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ORIGINAL ARTICLE

Novel spectrophotometric determinations of some cephalosporins following azo dye formation with *p*-dimethylaminobenzaldehyde

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p-Dimethylaminobenzaldehyde;
Diazo coupling reaction;
Spectrophotometric analysis

Abstract A new simple, accurate and cost-effective spectrophotometric method has been developed for the analysis of some cephalosporins (ceftriaxone, ceftazidime, cefixime, cefotaxime and cefuroxime) in bulk samples and pharmaceutical dosage forms. The reaction involves a two-step process of diazotization of the cephalosporins with acidified $NaNO_2$ at 0–5 °C and coupling with acidified *p*-dimethylaminobenzaldehyde (DMAB). Optimal temperature and time for coupling were established at 50 and 60 °C with coupling time ranging from 15 to 20 min. All the cephalosporins gave azo adducts with DMAB that absorbed light optimally at 400–430 nm. The reaction with DMAB occurred at a stoichiometric ratio of 1:1. Optimization of DMAB concentration revealed the superiority of using 0.3% DMAB in 0.0625 M H_2SO_4 with the best diluting solvent being methanol. Beer's law was obeyed at concentrations ranging from 5 to 60 μg/mL with correlation coefficients >0.9980 in all cases. Overall recoveries were of the order of 95–103% with errors generally less than 6%. The method was successfully applied to the determination of the cephalosporins in dosage forms and it was found to be equivalent accuracy to the official (USP and BP) HPLC assay procedures for these drugs. There was no interference from commonly adopted excipients.

The method could find application as a rapid and cost-effective alternative for the quality control of these cephalosporins, especially in preliminary studies.

1. Introduction

Cephalosporins are a class of β-lactam antibiotics discovered in the 1950's and are produced by various species of the mold *Cephalosporium* and from semi-synthetic processes. Cephalosporin-C which is the prototype of the cephalosporins was isolated in 1956 by Norton and Abraham from *Cephalosporium acremorium*, and its total synthesis was accomplished by Woodward and co-workers in 1966. Cephalosporins are broad spectrum antibiotics, with high potency against Gram-negative

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organisms. They are more resistant to inactivation by β-lactamases, particularly those produced by Gram-positive bacteria. They also have relatively low serum protein binding potential and almost all are excreted through the renal route. The cephalosporins are generally used in the treatment of upper respiratory and urinary tract infections. They are also generally useful for the rare patient who is sensitive to penicillin although sensitivity to cephalosporins is also sometimes found (Delgado and Remers, 2004; Dollery, 1999). The basic nucleus of the cephalosporins is 7-amino-cephalosporanic acid (7-ACA), which comprises a dihydrothiazine nucleus and a β-lactam ring. Cephalosporins are traditionally divided into first, second, third and fourth generation agents, based roughly on the time of their discovery and their antimicrobial properties i.e. spectrum of activity.

The official methods for the analysis of the cephalosporins are HPLC techniques in the reversed phase mode (BP, 2009; USP, 2009). Various other alternative methods have been reported for the analyzes of the cephalosporins. The methods include spectrophotometric (Abdel-Hamid, 1998; Kelani et al., 1998; El-Walily et al., 2000a,b; Agbaba et al., 1997; Yang et al., 1998), spectrofluorometric (Aly et al., 1996; Yang et al., 1999; Hefnawy et al., 1999; El-Walily et al., 1999), chemiluminescence (Li and Lu, 2006; Yao et al., 2003; Thongpoon et al., 2006), chromatographic (Shinde and Shabadi, 1997, 1998; Misztal, 1998; LaCourse and Dasenbrock, 1999) and electrochemical methods (Özkan et al., 2000; Rodrigues et al., 1999; El-Maali et al., 1993; Ferreira et al., 1999). There are many methods which have been reported based on the hydrolysis of cephalosporins to the residual 7-ACA for the assay of cephalosporins in both bulk samples and pharmaceutical dosage forms. These methods include use of derivatization of the α-aminoacyl functions (El-Obeid et al., 1999), flow injection analysis following formation of dyes (Metwally et al., 2001), iodine and wool fast blue (Sastry et al., 1998), alkaline hydrolysis (Helaleh and Abu-Nameh, 1998), flow injection (Al-Momani, 2004), vanadophosphoric acid (Amin and Shama, 2000) and more recently 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (Rageh et al., 2010). While each of these reported methods has advantages, majority of them are extensively time-consuming and utilizes reagents which are expensive or may in some cases present with certain stability problems.

Recently, we demonstrated the ability of *p*-dimethylaminobenzaldehyde (DMAB) to form azo dyes with diazotizable functional groups and we successfully adopted the method for the spectrophotometric determination of metronidazole and tinidazole with accuracies comparable to official methods (Adegoke and Umoh, 2009). Generic brands of cephalosporins circulating in the drug market have become too overwhelming in recent times that a simple, rapid and equally accurate method will be required for their quality assessment. In this report, we developed simple approaches for the spectrophotometric determination of five cephalosporins possessing amino groups that can be diazotized and coupled to acidic solution of DMAB in methanol with a view to providing relatively simple and easily adoptable technique for their analysis with the use of cheap reagents. The drug market has witnessed the proliferation of many multisource generic brands of cephalosporins in the last decade. The official method for their assay is HPLC which is beyond the reach of the third world economies. A simple spectrophotometric method with readily available reagent will aid in the quality control of these anti-bacterials.

2. Experimental

2.1. Reagents and materials

Cefuroxime (CEFU), cefixime (CEFM), ceftazidime (CEZD), ceftriaxone (CEFT) and cefotaxime (CEFX) chemical reference substances. Methanol, ethyl acetate, acetone, sulfuric acid, sodium nitrite, distilled water, ethanol, lactose, starch, magnesium stearate, talc, gelatin, *p*-dimethylaminobenzaldehyde (DMAB), hydrochloric acid (all analytical reagent grade from BDH, Poole, England).

Drug samples consisted of cefuroxime injection, cefuroxime tablets, cefixime tablets, ceftazidime injection, ceftriaxone injection, cefotaxime injection.

2.2. Equipment

Analytical balance (Mettler AE 160, Mettler PC 400), thermostated water bath (Buchi, Switzerland), vortex mixer (Griffins and George Ltd., UK), UV–vis spectrophotometer (Lambda 25 UV/VIS Spectrometer, Perkin Elmer Inc., Singapore), HPLC equipment (Cecil adept system 4-programmable wavelength detector CE 4200, dual piston pump CE 4100, chromatographic system manager CE 4900, auto sampler CE 4800).

2.3. Preparation of stock solutions

2.3.1. Preparation of DMAB solution

A 0.3% solution of *p*-dimethylaminobenzaldehyde (DMAB) was prepared by dissolving 0.3 g of the crystals in 100 mL of 0.0625 M sulfuric acid to give a molarity of 0.02013 M.

2.3.2. Preparation of drug stock solution

Equimolar concentrations (0.02013 M) of the five cephalosporins were prepared by dissolving 11.14 mg (CEFT), 8.54 mg (CEFU), 10.99 mg (CEFD), 9.16 mg (CEFX) and 9.12 mg (CEFM) in 10 mL of methanol to obtain the respective stock solutions.

2.3.3. Preparation of sodium nitrite solution

A 10% solution of sodium nitrite was prepared by dissolving 1 g of sodium nitrite crystal, (activated at 105 °C for 1 h in the oven) in 10 mL of distilled water.

2.3.4. Preparation of diazotized drugs

A 0.3 mL aliquot of the 10% of solution of sodium nitrite was added to a mixture of 0.3 mL of the drug stock solution and 0.12 mL of HCl solution was then added and the mixture cooled. The diazotization was carried out with stirring for 20 min. The reaction was stopped by the addition of 13 mL of ice-cold water and the mixture was allowed to stand in ice for 15 min before use. All the drug samples are diazotized in similar manner.

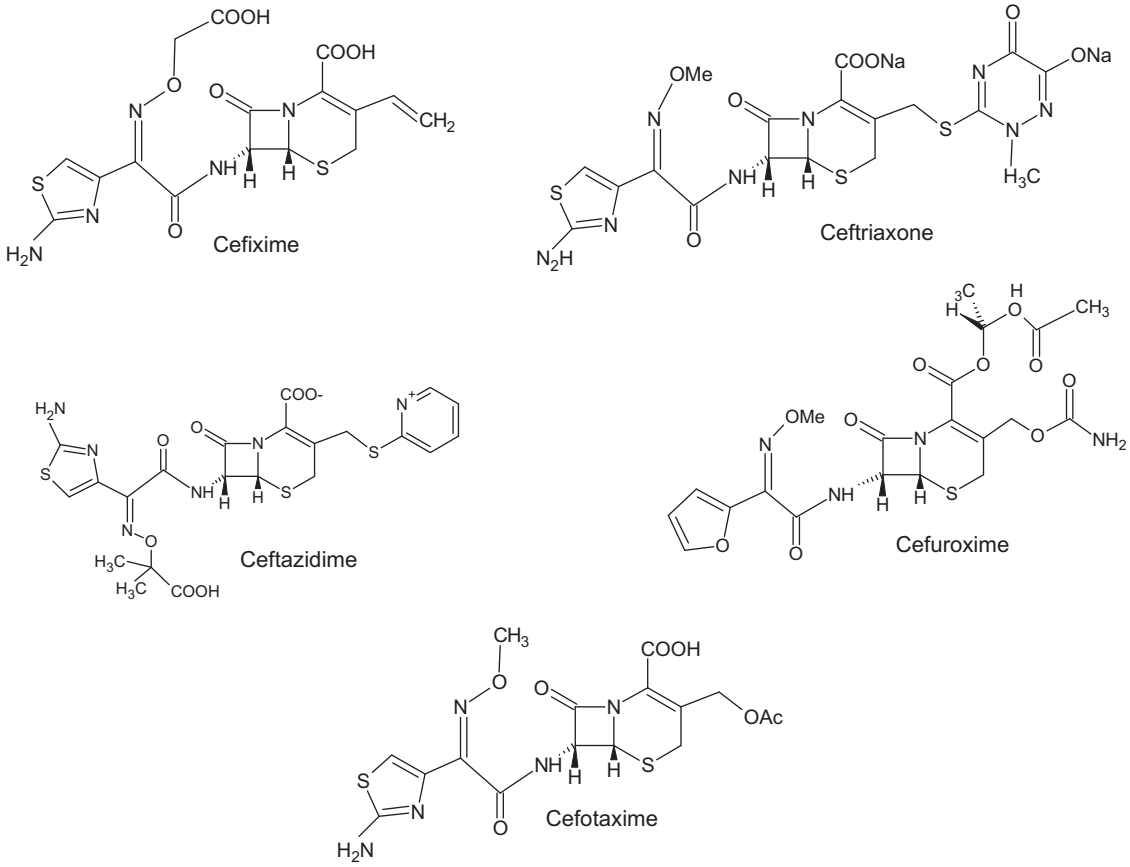


Figure 1 Structures of the cephalosporins studied.

2.4. Selection of analytical wavelength

A 0.5 mL aliquot of DMAB solution was pipetted into a test tube and 0.5 mL of the diazotized drug solution was added. The immediate yellow colored complex formed was allowed to stay at room temperature 30 and 70 °C for 5 min. The solution was made up to 5 mL with methanol. The absorption of the azo adduct at different wavelength ranging from 200 to 700 nm was recorded by scanning on a UV–vis spectrophotometer against methanol as solvent blank. The wavelength with the highest absorption was noted. This procedure was carried out with the different diazotized drug solutions.

2.5. Optimization studies

2.5.1. Optimization of reaction temperature and time

The method of steepest ascent was adopted for this assessment (Karnes and March, 1993). Aliquots of the diazotized drug solution (0.5 mL) was added to 0.5 mL DMAB solution in test tubes and the reaction mixtures were mixed in a vortex mixer followed by incubation at 30, 50, 60 and 80 °C for 5 and 20 min. Each determination was done in duplicate. The absorbance readings of the complex were taken at their respective wavelength maxima after making them up to 5 mL with methanol. The optimal temperature was taken as the temperature corresponding to the maximal absorbance of the samples.

For the optimization of reaction time, aliquots of the diazotized drug solution (0.5 mL) was added to 0.5 mL DMAB solution in test tubes and the reaction mixtures were mixed in a vortex mixer followed by incubation at 50 °C (CEFT and CEFX adducts) and 60 °C (CEZD, CEFM and CEFU adducts). The reaction was terminated by making up to 5 mL with methanol at 5, 10, 15, 20, 25 and 30 min and cooling in ice-bath. Each determination was done in duplicate. The absorbance readings of the complex were taken at their respective wavelength maxima and the optimal reaction time was taken as the time corresponding to the maximal absorbance of the samples.

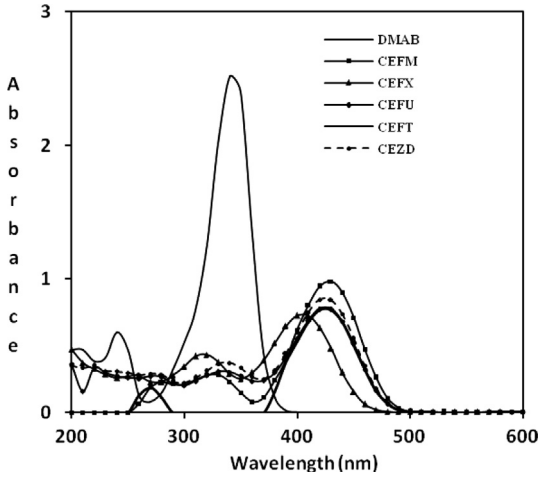
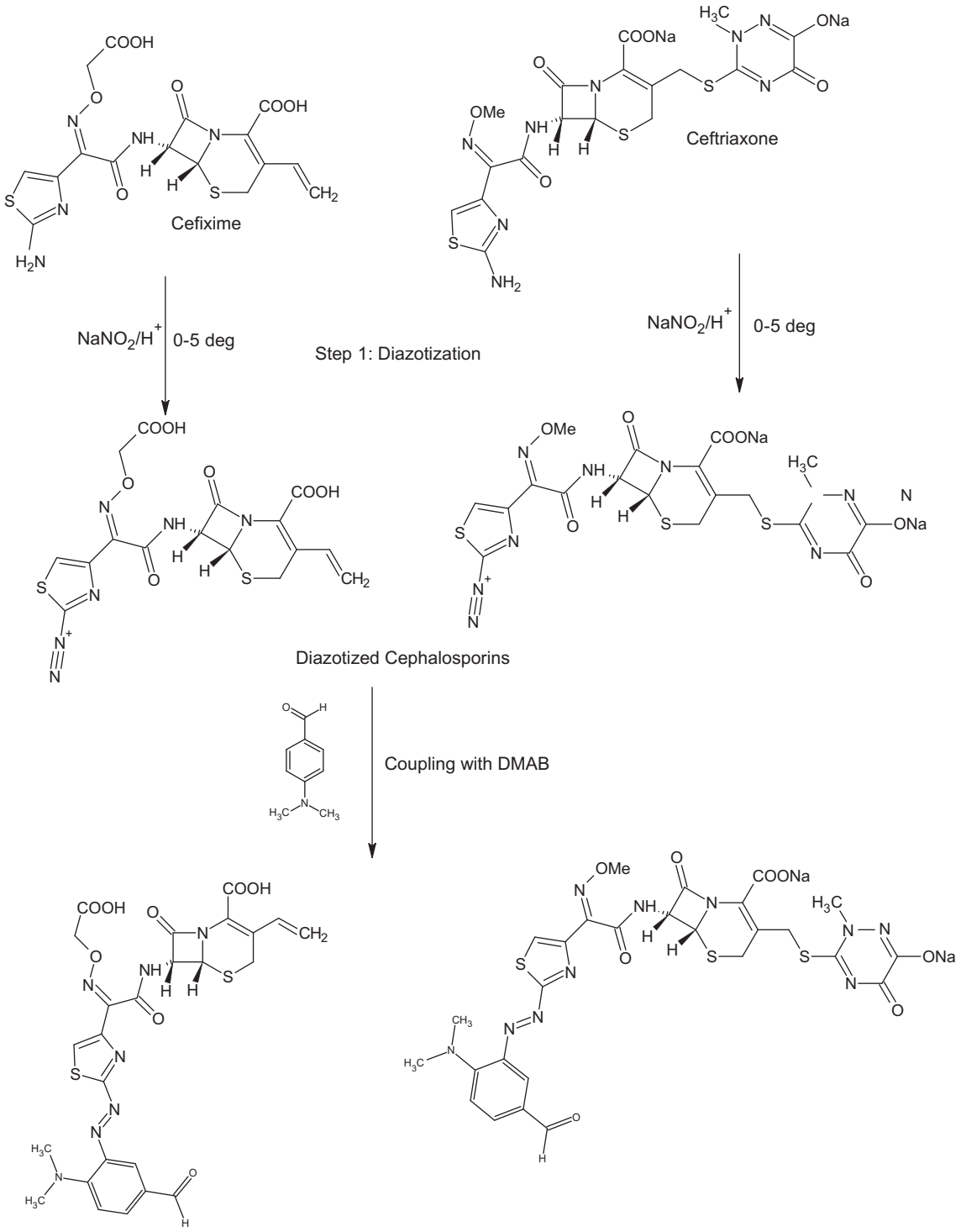


Figure 2 Overlaid absorption spectra of azo adducts formed between the cephalosporins and DMAB.



Scheme 1 Proposed coupling patterns for the azo dye formation.

2.5.2. Other optimization studies

The effect of varying the concentration of the acid used in the preparation of the DMAB solution on the azo adduct formation was studied by preparing DMAB reagent solution with 0.00156, 0.0312, 0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0 M sulfuric acid. In each case, the absorbance reading of each reaction mixture containing 0.5 mL DMAB and 0.5 mL diazotized drug solution incubated at optimized temperature and time was taken. Optimal acid concentration was taken as one which gave the highest absorbance reading.

In order to determine the effect of the solvent used in stopping the coupling reaction, water, ethanol, propan-1-ol, propan-2-ol and methanol were each used as the diluting sol-

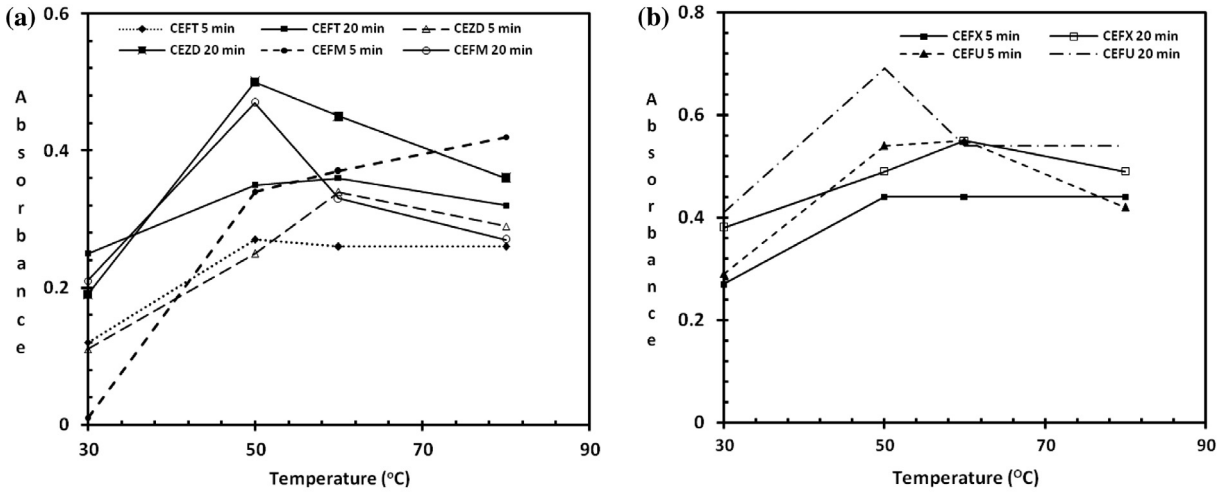


Figure 3 Optimization of coupling reaction temperature; (a) for ceftriaxone, ceftazidime and cefixime. (b) For cefotaxime and cefuroxime.

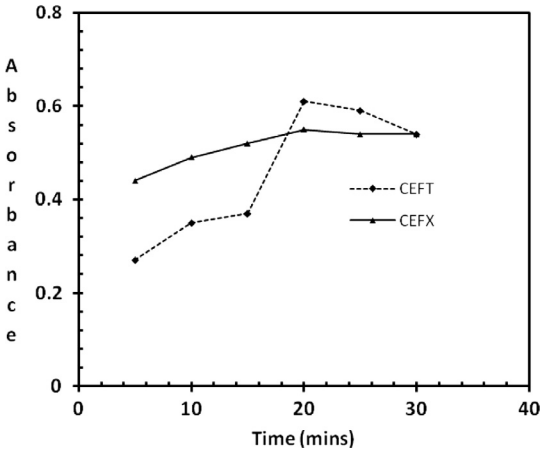


Figure 4 Optimization of coupling reaction time at 50 °C for ceftriaxone and cefotaxime.

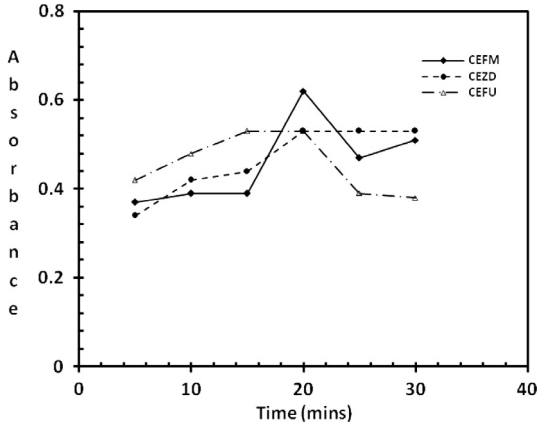


Figure 5 Optimisation of coupling reaction time at 60 °C for ceftazidime, cefixime and cefuroxime.

vent. The different solvents were used to make up the reaction mixture (0.5 mL DMAB and 0.5 mL diazotized drug solution) to volume after incubation at the optimal temperature and time). In each case, the absorbance reading of the resulting mixture was taken at their respective wavelength maxima.

The effect of varying the concentration of DMAB was carried out using reagent concentrations of 0.1%, 0.2%, 0.3%, 0.5%, 1.0%, 2.0% and 3.0% in 0.0625 M H_2SO_4. After mixing 0.5 mL of each reagent concentration with 0.5 mL of the diazotized drug solution and incubating at specific temperature and time, the reaction mixture was made up to 5 mL with methanol. The absorbance readings of the complex formed were determined at their respective wavelength maxima.

2.5.3. Stoichiometric ratio determination

Job's method of continuous variation (Rose, 1964) was adopted for this study. Equimolar solutions (0.02013 M) of DMAB and diazotized drug solution were prepared in their respective solvents. Into ten different test tubes, 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.75, 0.8 and 1 mL of DMAB was added, respectively. The solutions were made up to 1.0 mL with the diazotized drug solution. The mixtures were incubated at different temperature and time made up to 5 mL with methanol and the absorbance readings were determined.

2.6. Validation studies

The calibration curves were prepared using concentrations of the diazotized drugs ranging from 0 to 60 μg/mL, depending on the cephalosporin. 0.5 mL of DMAB was added to each of these test tubes. These test tubes were incubated at different temperature and time (specific for the different drugs used), after which respective volumes of methanol were added to make up to 5 mL reaction mixture. The absorbance readings of each of the mixture were then recorded at their respective wavelength maxima. The average absorbance reading was obtained from the determinations, and used to generate the calibration curves. Calibration curves were prepared on three successive days. The particular range for each diazotized drug

Table 1 Analytical and validation parameters for the analysis of the cephalosporins.

Parameter	CEFT	CEZD	CEFM	CEFX	CEFU
Color at room temperature	Deep yellow	Golden yellow	Yellow	Yellow	Golden yellow
Color at 70 °C (5 min)	Deep orange	Deep orange	Deep orange	Deep orange	Deep orange
λ_{max} (nm)	420	420	430	400	420
Limit of detection (μg mL^{-1})	5.093	1.901	6.152	1.775	1.751
Limit of quantitation (μg mL^{-1})	15.44	5.76	18.64	5.38	5.84
Molar absorptivity (mol^{-1} cm^2)	$(9.25 \pm 0.14) \times 10^4$	$(1.033 \pm 0.088) \times 10^3$	$(9.91 \pm 0.53) \times 10^2$	$(8.488 \pm 0.209) \times 10^2$	$(1.143 \pm 0.001) \times 10^4$

Table 2 Regression analysis for the calibration data.[a]

Parameter	CEFT	CEZD	CEFM	CEFX	CEFU
Beer's law range (μg mL^{-1})	25–60	5–35	30–55	20–45	10–40
Slope	0.006348	0.01701	0.005256	0.01822	0.01679
95% Confidence interval for slope	5.62×10^{-4}	5.36×10^{-4}	1.273×10^{-3}	4.35×10^{-3}	2.20×10^{-3}
Intercept	0.3822	0.0623	0.475	0.0327	0.0826
95% Confidence interval for intercept	2.51×10^{-2}	9.5×10^{-3}	5.36×10^{-2}	5.51×10^{-3}	7.92×10^{-4}
Correlation coefficient (r)	0.9991	0.9993	0.9982	0.9988	0.9995
Coefficient of determination (r^2)	0.9982	0.9986	0.9964	0.9976	0.9990

[a] $y = bx + c$; where y is the absorbance, x is the concentration.

Table 3 Intra-day accuracy and precision of the new method.

Drug	Amount taken (μg mL^{-1})	Amount found (μg mL^{-1})	Recovery[a] (%)	RSD[b] (%)	Relative error (%)
CEFT	25.0	25.26	101.04	2.69	1.03
	40.0	40.03	100.08	1.65	0.08
	50.0	49.87	99.74	0.68	−0.26
CEZD	10.0	10.15	101.98	2.4	1.94
	20.0	20.64	103.18	1.19	3.08
	30.0	30.04	100.13	0.81	0.13
CEFM	30.0	29.97	99.9	1.58	−0.1
	40.0	40.43	101.08	0.85	1.06
	55.0	55.66	101.2	0.85	1.19
CEFX	25.0	25.20	100.78	0.913	0.77
	35.0	35.56	101.6	0.79	1.57
	45.0	45.03	100.07	0.30	0.07
CEFU	15.0	15.41	102.71	0.84	2.66
	25.0	26.50	106.0	0.57	5.66
	35.0	34.55	98.71	0.38	−1.29

[a] Average of six determinations.
[b] RSD, relative standard deviation.

was arrived at by carrying out linear regression analysis of the results obtained from linearity of response. The range that gave the best slope, least intercept and highest correlation coefficient was adopted for each cephalosporin.

Accuracy and repeatability of the new methods were carried out in 3 successive days as stipulated by the USP (USP, 2000). Other validation parameters were assessed using the ICH guidelines (ICH Topic Q2, 2010). The limit of detection (LOD) and limit of quantitation (LOQ) were obtained from the ICH guidelines using the expressions:

$$\text{LOD} = \frac{3.3\sigma}{s} \quad \text{and} \quad \text{LQD} = \frac{10\sigma}{s}$$

where σ is the standard deviation of the blank signals and s is the slope of the calibration graph.

2.7. Dosage form analysis

2.7.1. New method

Various dosage forms, mainly tablets and powder for injection, were sourced from retail outlets and assessed for their content of the cephalosporins using the new DMAB method and the official HPLC assay procedures for each cephalosporin. In each instance, stock solutions were made containing the equivalent of each cephalosporin to give a molarity of 0.02013 M and then diazotization of the dosage form was carried out as done for the pure cephalosporins. For ceftazidime, the following brands were studied – Orzid® Injection and Betazidim® Injection 0.1 mL (equivalent to 20 μg/mL of ceftazidime) of the diazotized drug solution were reacted with 0.5 mL DMAB

Table 4 Inter-day accuracy and precision of the new method.

Drug	Amount taken (μg mL^{-1})	Amount found (μg mL^{-1})	Recovery[a] (%)	RSD[b] (%)	Relative error (%)
CEFT	25.0	25.46	101.84	2.55	1.81
	40.0	40.82	102.05	2.25	2.01
	50.0	49.68	99.36	0.79	−0.64
CEZD	10.0	10.15	101.49	2.21	1.47
	20.0	20.78	103.89	1.03	3.74
	30.0	20.94	99.81	1.01	−0.19
CEFM	30.0	30.36	101.20	2.34	1.19
	40.0	40.59	101.48	1.11	1.15
	55.0	55.73	101.33	0.84	1.31
CEFX	25.0	25.44	101.76	1.26	1.73
	35.0	35.53	101.51	1.07	1.49
	45.0	45.03	100.67	0.31	0.07
CEFU	15.0	15.51	103.37	1.23	3.26
	25.0	26.60	106.40	0.79	6.02
	35.0	35.90	102.57	2.7	2.51

[a] Average of twelve determinations.
[b] RSD, relative standard deviation.

and incubated at 60°C for 20 min. The solutions were made up to 5 mL with methanol and their absorbance values recorded.

For cefuroxime, the following brands were studied – Axacef® 500 mg tablets, Zocef® 750 mg, Roxicef® 750 mg injections. One hundred and twenty five microliter (equivalent to 25 μg/mL cefuroxime) of the diazotized drug solutions reacted with 0.5 mL DMAB and incubated at 60 °C for 20 min.

For ceftriaxone, the following brands were studied – Duocef® 1 g, Rophex® 1 g and Ebecef® 1 g injections. 0.2 mL (equivalent to 40 μg/mL ceftriaxone) of the diazotized drug solutions were reacted with 0.5 mL DMAB and incubated at 50 °C for 20 min. The solutions were made up to 5 mL with methanol and their absorbances recorded.

For cefixime, two tablet brands were studied. 0.2 mL (equivalent to 40 μg/mL cefixime) of the diazotized drug solution were reacted with 0.5 mL DMAB and incubated at 60 °C for 20 min. The solutions were made up to 5 mL with methanol and their absorbances recorded.

For cefotaxime, the following brands were studied – Nitaxim® 1 g and Taxim® 500 mg injections. 0.175 mL (equivalent to 35 μg/mL cefotaxime) of the diazotized drug solutions were reacted with 0.5 mL DMAB and incubated at 50 °C for 20 min. The solutions were made up to 5 mL with methanol and their absorbances recorded. The analyzes above were carried out in six replicates.

2.7.2. Official method

The analyzes of the different brands of cephalosporins were carried out by high pressure liquid chromatography as stated in the BP (2009) for cefotaxime, ceftriaxone and cefuroxime while the HPLC method stated in USP 32/NF 27 (2009) was adopted for cefixime and ceftazidime.

3. Results and discussion

The structures of the five cephalosporins studied in this work are presented in Fig. 1. All the five cephalosporins upon diazotization formed immediate golden yellow color with DMAB in methanol. The colors of the azo adducts became intensified with elevated temperature. The formation of azo dye of diazotized molecules with DMAB was first reported by Adegoke and Umoh (2009). It was observed that when the reaction is carried out in methanol (as opposed to water), the internal mesomeric effect of the carbonyl group is repressed and thus giving room for the activating directing influence of the dialkylamino substituent on DMAB to be optimal. The end result is the formation of a highly colored product which can be used for the colorimetric assessment of pharmaceuticals. The method was successfully applied to the nitroimidazoles (metronidazole and tinidazole). The present assessment of cephalosporins is borne out of the desire to develop relatively simple, cheaper and easily adoptable method for the quality assessment of these important antibacterial agents.

3.1. Selection of analytical wavelength

All the cephalosporins studied gave clearly defined absorption maxima in the visible region. Fig. 2 shows the absorption spectra of the azo adducts formed between the cephalosporins and DMAB overlaid on the spectrum of DMAB. For CEFM, CEFU and CEZD, there were two clear-cut absorption maxima consisting of one high-energy band at around 320 nm and a low-energy band at greater than 400 nm. The high-energy band may be as a result of the chromophoric tagging with DMAB which leaves a residual peak of DMAB while the new bands at >400 nm represents the bands due to the new azo adducts formed between the cephalosporins and DMAB. CEFT, on the other hand, has its high-energy band at close to 270 nm. The new visible region maxima ranged from 400 (CEFX), 420 (CEFT, CEZD and CEFU) to 430 nm (CEFM). Since diazotization is a form of derivatization methodology, the major absorption maxima produced by the diazotized cephalosporins were around 230 nm. Thus, the primary aim of generating colored adducts that can be determined colorimetrically seems to have been accomplished with the formation of new compounds having absorption maxima in the visible region. The closeness of the absorption maxima of all the cephalosporin-DMAB adducts attests to the similarity in structures of the cephalospo-

Table 5 Comparative dosage form analysis using new method and the official HPLC methods.

Drug formulations	New method[a]		Official method[a]		Mean recovery ± SD[b] (%)	Error (%)	Statistics (*p*-values) [c]	
	Concentration found (μg mL^{-1})	RSD (%)	Concentration found (μg mL^{-1})	RSD (%)			*F*-test	*t*-test
Ceftriaxone brands								
Duocef injection	39.82	1.05	39.82	0.02	100.00 ± 1.08	0	0.004	0.48
Rophex injection	40.002	0.79	39.97	0.06	100.09 ± 0.73	0.08	0.002	0.86
Ebecef injection	39.94	0.68	40.06	0.05	99.70 ± 0.64	0.30	0.002	0.28
Ceftazidime brands								
Orzid injection	19.97	0.95	19.82	0.101	100.99 ± 0.95	0.76	0.004	0.13
Betazidim injection	22.64	4.33	23.52	0.094	96.61 ± 1.56	3.74	0.007	0.30
Cefixime brands								
Brand A (tablet)	31.14	2.51	30.28	5.38	103.86 ± 0.96	2.84	0.005	0.31
Brand B (tablet)	29.19	1.85	30.89	0.13	95.39 ± 0.91	5.50	0.10	0.34
Cefotaxime brands								
Taxim-500 injection	35.51	1.29	35.46	1.07	99.24 ± 1.32	0.14	0.22	0.58
Nitaxim injection	33.87	0.86	34.11	1.30	99.36 ± 0.93	0.70	0.21	0.79
Cefuroxime brands								
Axacef tablet	25.54	0.67	25.82	0.39	98.92 ± 0.72	1.08	0.37	0.06
Zocef injection	24.41	2.01	23.86	0.17	103.48 ± 1.12	2.31	0.001	0.15
Roxicef injection	25.33	1.07	25.66	0.02	98.71 ± 1.06	1.29	0.004	0.12

[a] Mean value, $n = 6$. Content of cephalosporins stated by BP ranges from 90% to 110% (cefotaxime), 92–108% (ceftriaxone) and 90–105% (cefuroxime injection, tablet is 92.5–105%) while USP content for cefixime and ceftazidime is 95–102%.
[b] % Recovery calculated as a ratio of the new method to the HPLC method.
[c] Statistical analyzes done between the results obtained from the proposed method and the BP official HPLC method.

rins with CEFM-DMAB azo adduct giving the highest absorptivity and λ_{max} of the five adducts. Thus, the cephalosporins were determined in the colorimetric region far removed from the non-specific absorption in the UV region. This holds promise for ready and easy determination of these important antibiotics.

The reactions leading to the formation of the new azo adducts between the cephalosporins and DMAB are illustrated with cefixime and ceftriaxone in Scheme 1. The reaction involves a two-step process, in which the cephalosporins were diazotized with acidified $NaNO_2$ (at 0–5 °C) and then coupling with acidified DMAB solution with methanol providing the best reaction medium. A cursory look at the adducts produced shows that the DMAB molecule is well staggered from the residual cephalosporin molecules and this may account for the extension of conjugation leading to new absorption maxima in the visible region. However, the conjugation appears to be limited to the heterocyclic rings alone as the conjugation is broken beyond these rings. The predicted stereochemistry of the azo adducts is of *E*-type (*trans* geometric isomers) which leads to a staggered conformation around the diazenyl linkage; hence accounting for the ease of formation of the azo adducts.

3.2. Optimization studies

Several critical parameters that could affect the propensity and the rate of the diazo coupling reaction between diazotized cephalosporins and DMAB were studied and optimized. These parameters include temperature, time, acid concentration, DMAB concentration, solvent for dilution after coupling as well as the determination of mole ratio of DMAB with respect to the diazotized compounds.

The results obtained for the optimization of temperature required for coupling are presented in Fig. 3a (CEFT, CEZD and CEFM) and b (CEFX and CEFU). For all the five cephalosporins, elevated temperature was required for optimal reaction to occur. This is understandable as the cephalosporins do not possess sufficient activated skeletons for the reaction to go on readily at room temperature. For CEFT and CEZD, the absorbance readings increased gradually from 30 °C and peaked at 50 °C. However, the values obtained for 5 min were lower than that obtained at 20 min implying that a longer time at 50 °C will be required for effective coupling. For CEFM, the value rises with increased temperature and 60 °C gave the optimal absorbance. For CEFT and CEZD, at temperatures beyond 50 °C, the absorbance values produced decreased. This might be due to thermal decomposition of the azo adducts aided by the acidic medium in which the reaction is occurring. Thus thermal hydrolytic cleavage of the azo adducts may be occurring at temperatures higher than 50 °C. Fig. 3b shows the optimization of coupling reaction temperature for CEFM and CEFU. For CEFX, the optimum temperature occurred at 60 °C and thereafter a decline was observed at 80 °C. Twenty minutes reaction time generally gave higher values than at 5 min. For CEFU, 50 °C was found optimal for the diazo coupling reaction with 20 min reaction time giving higher absorptivity compared to 5 min. Thus for the optimization of coupling reaction temperature, 50 °C was optimal for the reaction between DMAB and CEFT as well as for CEFX. The optimal temperature for CEZD, CEFM and CEFU adducts is 60 °C.

Based on the results of the optimization of coupling temperature, the time required for the optimal coupling to take place was investigated at the optimum temperature. The results of the assessment are presented in Fig. 4 (50 °C reaction temperature

for CEFT and CEFX) and Fig. 5 (60 °C reaction temperature for CEFM, CEZD and CEFU). For CEFT (Fig. 4), the absorbance of the azo adduct increased steadily from 5 to 20 min and thereafter declined beyond 20 min. Thus, 20 min was selected as the optimum coupling time at 50 °C. For CEFX, a not too clearly defined rise in absorbance was observed across the time intervals. However, 20 min still appears optimal for the coupling reaction and was thereafter selected. For the other three adducts (Fig. 5), optimization of coupling reaction time gave 15 min (CEFU) and 20 min (CEFM and CEZD).

The effects of varying the concentration of H_2SO_4 used in preparing the DMAB solution were investigated. For the DMAB-cephalosporin adducts, optimal difference in absorptivity was found at acid concentration of 0.0625 M. Thereafter absorbance readings declined to the lowest value at 0.5 M H_2SO_4 and attained a near plateau. Since the reaction is taking place at elevated temperatures, the decrease in absorbance with increasing acid concentration must be due to the hydrolytic cleavage of the reaction product by the protons. Thus the acid concentration for preparing DMAB was kept at 0.0625 M H_2SO_4.

The diluting solvents investigated were water, methanol, ethanol, propan-1-ol and propan-2-ol. In water, very low absorbance values were obtained while increasing values occurred in the order ethanol < propan-2-ol < propan-1-ol < methanol. Thus highest absorptivity was found with the use of methanol as diluting solvent for the five adducts of the cephalosporins. The low absorbance values obtained in water can be explained based on its ability to aid the breakdown of the azo adducts in the presence of an acid and high temperature. The alcohols will obviously make the azo adducts more stable by creating a less polar medium which will prevent such hydrolytic decomposition from taking place while at the same time mopping up excess proton from the medium by acting as a Lewis base.

The effect of DMAB concentration on the formation of azo adducts between DMAB and diazotized cephalosporins was studied by varying the DMAB concentration form 0.1% to 3.0% in 0.0625 M H_2SO_4. The DMAB had some problems dissolving optimally in 0.0625 M at concentrations beyond 0.5 M, hence colloidal solutions were obtained which scattered light and this led to increasing absorbance values as the concentration of DMAB increases. DMAB concentration between 0.1% and 0.3% gave uniform solutions with high absorbances; although 0.3% DMAB concentration did not give the highest absorbance, it was utilized as the optimum concentration for the reaction as it appears to be a limiting DMAB concentration beyond colloidal particles, in which extensively scattered light are observed and subsequently leading to high absorbance values.

3.3. Stoichiometric ratio determination

Using the optimized conditions, the absorbances of the azo adducts formed by the different diazotized drugs with DMAB were found to vary with the stoichiometric ratio of the diazotized drug and DMAB. The mole ratio of 1:1 was found to give the highest absorbance value, and therefore selected as the stoichiometric ratio for subsequent determinations, and the absorbance values decreased thereafter at lower and higher molar ratios of DMAB. This result confirms the presence and availability of only one primary aromatic amino group for diazotization and coupling on the cephalosporin structures.

3.4. Validation studies

Using the optimized conditions developed in this work, calibration curves were prepared for each of the azo adducts of the cephalosporins with DMAB on each of three successive days. Various linear regression equations were obtained for the five cephalosporins. The calibration range was from as low as 5 μg/mL (CEZD) to the highest concentration of 60 μg/mL (CEFT). The lower the calibration range, the higher the slope and the lower the intercept. In all the five instances, the correlation coefficient was higher than 0.9980. The various analytical and validation parameters are summarized in Table 1 while Table 2 presents the regression analyzes for the five adducts.

The LODs and LOQs ranged from 1.901 to 6.1525 μg/mL and 5.76 to 18.645 μg/mL, respectively. The higher the upper limit of the calibration range, the higher the LOD and LOQ. The molar absorptivities were also relatively high for all the five adducts of cephalosporins.

Accuracy and precision of the new methods were assessed on each of three successive days using the pooled calibration data according to the USP guidelines. Table 3 presents the intra-day accuracy and precision while the inter-day data are presented in Table 4. For the intra-day accuracy, errors ranged from 0.08% to 5.66% with recoveries in the range of 98.7–102.71%. When assessed between the three days, the errors were in the range 0.07–6.02% and the recoveries in the range 99.36–106.40%. There was no interference from commonly utilized excipients such as starch, talc, magnesium stearate and lactose. However, higher values were obtained for sample preparations containing gelatin. This is due to the breakdown of gelatin by acid in the presence of water and high temperature to generate amino acids and possibly some lower peptides which will form Schiff bases with DMAB and leading to high absorbance values.

3.5. Dosage form analyzes

The comparative analysis is presented in Table 5. All the brands assessed passed the tests except cefixime tablets that failed the quantitative determination by both the DMAB and HPLC methods. The results point to the need for constant monitoring of the drug market.

The mean recoveries were determined as a ratio of the results obtained from the new method and that of the official methods. The recoveries ranged from 95.39% to 103.86% with errors in the range 0–5.5%. The recoveries for the HPLC methods were generally higher than respective DMAB methods for each of the cephalosporins. This is understandable since HPLC is a separation technique. This is also reflected in the precision and this observation can explain why the statistical analysis yielded probability values for the *F*-ratio tests that were generally lower than 0.05. However, in all cases, the DMAB method was of equivalent accuracy as judged by the *p*-values for the Students' test which were generally higher than 0.05.

Some clearly observable advantages for the new DMAB method for the determination of cephalosporins in bulk samples and pharmaceutical dosage forms are simplicity, speed of analysis, low cost and the method is a two-step approach that utilizes minimal reagents without recourse to extraction into organic solvents that can compromise accuracy. It is also a departure from the need to hydrolyze the cephalosporins as it is common with most visible colorimetric determination of cephalosporins previously reported.

4. Conclusions

The proposed DMAB method has advantages over other previously reported visible spectrophotometric method with respect to its sensitivity, which permits the determination of very low concentration and simplicity of the procedures. Furthermore, all the analytical reagents are inexpensive, have excellent shelf life and are available in any analytical laboratory. The proposed method can be applied in quality control laboratories for the routine analysis of the investigated drugs in raw materials and pharmaceutical formulation and as an in-process quality control method in pharmaceutical industries, especially for preliminary studies.

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