ORIGINAL ARTICLE

RAPID COLORIMETRIC ASSAY OF DICLOFENAC SODIUM TABLETS USING 4-CARBOXYL-2,6-DINITROBENZENE DIAZONIUM ION (CDNBD)

OLAKUNLE S. IDOWU,* OLAJIRE A. ADEGOKE, BOLAJI A. ODERINU AND AJIBOLA A. OLANIYI

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria

ABSTRACT

This study describes a novel simple, rapid and sensitive colorimetric assay method for diclofenac sodium tablets. The method is based on a simple aromatic ring derivatization technique using newly developed 4-carboxyl-2, 6-dinitrobenzenediazonium ion (CDNBD) as chromogenic derivatizing reagent with subsequent formation of an azo dye. The diazo coupling reaction was carried out between CDNBD and diclofenac. Optimization studies for time and temperature was conducted using the method of steepest ascent. The UV absorption spectrum was recorded and the stoichiometric ratio for the drug and reagent was done by continuous variation method. Optimal calibration range was fixed (1-way ANOVA) and then the method was applied to dosage form analysis. Comparison of dosage form analysis was done with the BP HPLC method.

The diazo coupling reaction is very fast and optimization studies established an optimal reaction immediately after mixing the reaction mixture in a vortex mixer for 10 sec. A new absorption maximum (λ_{max}) at 470nm was selected as analytical wavelength. The assays were linear over 1.35 -10.8µg/ml of diclofenac and the reaction required a 2:1 reagent/drug stoichiometric ratio. The new method has a low limit of detection of 0.27 µg/ml, and was reproducible over a three-day assessment of precision (RSD 2.31%). The method has been successfully applied to the assay of diclofenac sodium slow-release tablets and found to be of equivalent accuracy ($p>0.05$) with the official (B.P 1998) HPLC method.

The new method has distinct advantages of speed, simplicity, sensitivity, and more affordable instrumentation and could find application as a rapid analytical method for diclofenac sodium tablets.

Keywords: Diclofenac assay, colorimetry, 4-carboxyl-2, 6-dinitrobenzenediazonium ion (CDNBD), diazo coupling reaction.

**Corresponding author*: e-mail: olakunleid@yahoo.com

INTRODUCTION

Diclofenac sodium, 2-[2,6-dichlorophenyl)-amino] benzene acetic acid monosodium salt, is a nonsteroidal anti-inflammatory drug with potent activity and outstanding tolerability in the treatment of rheumatic disease (Reynolds, 1990). It is also used as an analgesic and antipyretic. This phenylacetic acid derivative acts as an inhibitor of hyaluronidase, prostaglandins synthesis and platelet aggregation. Other commonly used salts of diclofenac are those of potassium and diethylammonium salt. Diclofenac is presented as tablets (enteric coated, controlled release), creams and injectables.

Several methods have been reported for the determination of diclofenac. The tablets and injection have been assessed by UV-VIS spectrophotometry (Vaidya and Prasad, 1995; Mo and Chen, 1988) and by non-aqueous titration using tetra-*n*-butylammonium hydroxide (Cakirer *et al.*, 1999), spectrofluorimetry using europium (III) as a fluorescent probe (Carreria *et al*, 1995), gas and liquid chromatography in biological fluids (De-Jong *et al*, 1989; Geiger *et al*, 1975; Riegel and Ellis, 1994; Abdel-Hamid *et al*, 2001; Bakkali *et al*, 1999), flow extraction spectrophotometric method (Perez-Ruiz *et al*, 1997) and Raman spectroscopy (Davies *et al*, 1990). Methods based upon formation of ion-pairs and their extraction in organic solvents using methylene blue (Agrawal and Shivramchandra, 1991; Botello and Perez-Caballero, 1995) and methylene violet (Sastry *et al* 1989) have been used to determine diclofenac in pure form and tablets by molecular spectroscopy. The BP 1998 method for the tablet is a reversed phase HPLC method (BP 1998).

In our previous work, we recently described the accurate and precise determination of mefenamic acid capsules (Idowu *et al.*, 2002) and propranolol tablets (Idowu *et al.*, 2004) by colorimetry. These assay procedures employed a derivatization procedure (aromatic ring derivatization technique), using the newly developed (Idowu, 1998; Idowu and Olaniyi, 2001) 4-carboxyl-2, 6-dinitrobenzene-diazonium ion (CDNBD) as a chromogenic derivatizing reagent**.** The unique combination of high technology (microprocessor and computer interfacing capabilities) and low-cost afforded by modern digital colorimeters and the applicability of this reagent to a wide range of drugs (Idowu and Olaniyi, 2001), led to this resurgence of interest in colorimetric assay of pharmaceuticals. This holds a promise of better affordability for routine in-process quality control, especially in poor resource economies.

In this paper, we report a novel colorimetric assay of diclofenac in slow-release tablets, using the dinitrobenzene

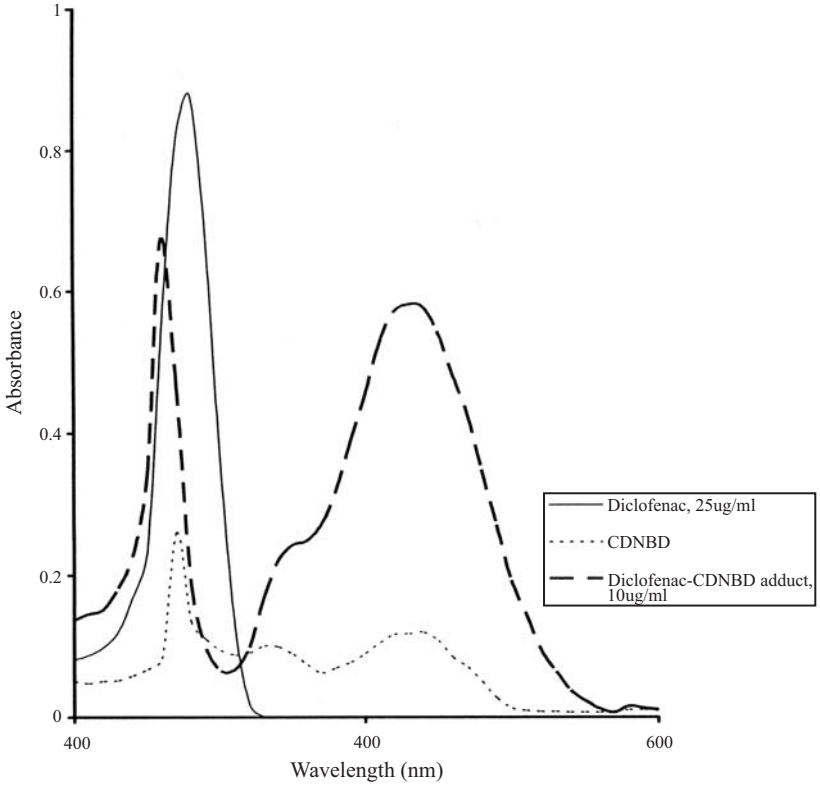


Figure 1: Overlaid absorption spectra of blank reagent (……), diclofenac (___) and diclofenac –CDNBD adduct (_ _ _ _)

diazonium ion, CDNBD, as a derivatizing reagent *via* diazo coupling reaction.

MATERIAL AND METHODS

Chemicals and reagents

Abitren (Teva-Pharmaceutical), Grofenac (Dr. Grossmann AG), Diclogesic (Dar Al Dawa, Jordan), Voltaren (Norvatis), diclofenac crystals (isolated and purified in our laboratory, mp 158-160^{o}C), methanol, ethyl acetate, glacial acetic acid, concentrated sulfuric acid, sodium nitrite (all are analytical reagents from BDH, UK) were used in the assay procedures.

Equipment

UV/visible spectrophotometer (Unicam Aurora, Helio Scan software v 1.1), digital colorimeter (6051, Jenway, UK), analytical balance (Mettler, H80, UK), ultrasonic bath (Langford, UK), vortex mixer (Griffins & George Ltd., UK).

Colorimetry

The solution of CDNBD in concentrated sulfuric acid was prepared as previously reported (Idowu *et al*, 2005). Diclofenac stock solution was made by dissolving 2.7 mg diclofenac crystals in 10ml glacial acetic acid solution.

In order to determine a suitable analytical wavelength for the reaction product, two critical response parameters (temperature and reaction time) were optimized using the method of steepest ascent (Miller and Miller, 1993a). Aliquot of diclofenac stock solution (100µl) was added to the reagent solution (500µl) in a test tube and the reaction mixture was mixed in a vortex mixer for 10 sec followed by incubation in turn at 30^{o}C and 50^{o}C for 5 minutes and 20 minutes, respectively. Similar experiments were performed at 60^{o}C and 80^{o}C. Each determination was done in duplicate. The reaction was terminated by addition of ice-cold water (5ml) to the reaction mixture and kept in ice-bath. Aqueous solution was extracted with ethyl acetate (10ml) and kept in a vial wrapped with aluminum foil. A blank reagent solution was prepared in a similar way but replacing the stock solution with glacial acetic acid. The absorption spectrum of the reaction mixture extract was determined against the absorption spectrum of the blank reagent extract, using the uv/visible spectrophotometer and the optimal absorption wavelength, 470 nm, was selected for sample determination.

Optimization of reaction time was done by adding aliquots of diclofenac stock solution (250µl) to the reagent solution (500µl) in seven tubes. The effect of varied time allowed for coupling to take place at 30^{o}C between diclofenac and CDNBD was studied at 0, 2, 5, 10,15, 20 and 30 minutes. The ethyl acetate extracts of the reaction mixture was prepared as usual after each reaction time and the absorbance was measured at 470 nm on the digital colorimeter. The procedure was repeated with replicate samples and optimal reaction time was then determined as the time corresponding to the maximal absorption of the samples.

Stoichiometric ratio of drug-reagent adduct formation

Equimolar solutions (9.18x 10^{-4}M) of the reagent and the diclofenac stock solution were prepared using the procedures described above. In six different test tubes, 0, 0.25, 0.5, 0.67, 0.75, and 1.0ml of the reagent solution were added respectively. Each tube was then made up to 1.0ml with the drug stock solution. A series of blank determination were carried out in which the volume of the drug stock solution was replaced with glacial acetic acid. The mixture was mixed in a vortex mixer for 10sec. Extraction into ethyl acetate, after terminating the coupling reaction with ice-cold water, was carried out afterwards as usual. The absorbance was measured at 470 nm against the blank and the absorbance values obtained were plotted against the mole fraction of the reagent solution. Each determination was carried out in duplicate.

Stability of the azo adduct in ethyl acetate

Test solutions of diclofenac containing 5.4µg/ml were prepared in four sample vials. Two of the vials were wrapped with aluminum foil, while the other two were left unwrapped. Both sets were kept on the laboratory bench. The absorbance reading of the extracts at 470 nm were taken at 30minutes interval for a period of three hours.

Assay of dosage forms

The amount of powdered tablets containing 2.7mg of diclofenac were weighed and dissolved in glacial acetic acid (6ml) and mixed for 5 min. The solution was filtered through a cotton wool plug into a 10ml volumetric flask. The volume was then made up with fresh glacial acetic acid, rinsing the filter aid in the process. An aliquot of the drug stock solution (100 µl) was added to the reagent solution (500µl). The mixture was mixed in a vortex mixer for 10sec and afterwards extraction into ethyl acetate was done as usual. The absorbance was measured at 470 nm. The content of diclofenac in each tablet was determined by interpolation from calibration lines. The sample analysis was repeated using the B.P 1998 HPLC procedure. Four brands of diclofenac sodium tablets (slow release tablets) were used in the assays.

The student's t-test was used in comparing the assay results from both the BP and the colorimetric assay reported here. A 2-tailed probability value less than or equal to 0.05 (95% confidence interval) was considered to be significant.

Assessment of method selectivity

Two approaches were adopted for investigating the method selectivity.

a *Tablets stored at elevated temperature*
Powdered samples of the four brands and the reference substance were kept at 100°C in the oven for 5 hours. Afterwards, methanolic extract of the tablets and the reference substance were analysed for possible degradation products. Three separate chromatographic systems were employed.

b *Spiking of reference samples into placebo*
An aliquot of the stock solution containing 5.4 µg/ml of diclofenac was spiked into tablet excipients containing each of lactose, corn starch, magnesium stearate, gelatin and a mixture of the four excipients.

The procedure for coupling, extraction and sample determination were carried out as above.

Validation of methods
Calibration lines using standard solutions of 0, 1.35, 2.7, 5.4, 8.1and 10.8 µg/ml diclofenac were carried out using the optimal analytical conditions as described above. Linear regression analysis (using Microsoft Excel) was used to calculate the slope, intercept and the correlation coefficient (r^2) of each calibration line. The slope of the calibration lines were compared by one way ANOVA using GraphPad Prism Version 4.01 for Windows (www.graphpad.com).

The precision and accuracy of the new method for diclofenac were determined using USP procedure on a three-day assessment (USP 2000). The limit of detection was computed as previously described as the analyte concentration giving a signal equal to the blank signal plus three standard deviations of the blank (Miller and Miller, 1993b). The limit of quantitation was computed as 3 x LOD.

RESULTS

The absorption spectrum of diclofenac in ethyl acetate showed absorption maximum at 280 nm (figure 1). The ethyl acetate extract of the adduct is deep orange in colour. The spectrum of the adduct shows an optimal absorbance at 470nm. The blank reagent exhibits absorption maxima at 260 nm, 340 nm and a broad peak around 430 nm (figure 1). Absorbance of the adduct was highest at 30°C and 5 min reaction time and lower at higher temperatures and longer reaction time (figure 2). Further optimization of reaction time at 30°C shows that the absorbance was at a peak value immediately after mixing the reaction mixture and decreased steadily within 5 minutes reaction time (figure 3). Maximum absorbance of the adduct was obtained at a mole fraction of 0.67 for the reagent solution (figure 4) and the absorbance was found to decrease at a lower or higher mole fractions. Ethyl acetate extract solution of the azo adduct formed by the coupling reaction was found to be stable to light in the laboratory environment over a period of 3h (figure 5), especially with wrapped samples.

Table 1: One way analysis of variance (ANOVA) of the slope of calibration lines for diclofenac assay by CDNBD method

Calibration Range (µg/mL)	b± S_b*	R^2
0-10.8 (n=6)	0.0401±0.00330	0.9964
0-9.45 (n=6)	0.0413±0.00113	0.9966
0-8.1 (n=5)	0.0412±0.00212	0.9944

* P<0.05 is taken as significant

ANOVA Statistics:

	SS	df	MS
Treatment (between columns)	0.00000573	2	0.00000286
Residual (within columns)	0.0000897	15	0.00000598

P value = 0.6285

The slope is not significantly different, (P>0.05) for the three calibration ranges considered. Calibration line with the widest dynamic range (0-10.8µg/mL) was adopted for routine analysis. The result of the ANOVA is presented in table 1. Beer-Lambert's law was obeyed for this concentration range of 0-10.8 µg/ml and had linear regression equation of y = 0.04012x + 0.09601 with r^2 of 0.9964. The 95% confidence limit for the slope and intercept are 0.04012 ± 0.00330 and 0.09601± 0.020 respectively. The limit of detection was determined as 0.27µg/ml, with LOQ of 0.81µg/ml. The overall recovery from the three-day assessment of accuracy and repeatability

Table 2: Three-day assessment of accuracy and precision of the new method of assay of Diclofenac

Concentration (µg/ml)	Day 1		Day 2		Day 3	
	Mean± S.D*	RSD%	Mean± S.D*	RSD%	Mean ± S.D*	RSD%
2.7	98.32±2.67	2.72	101.78±2.30	2.26	101.78 ±2.30	2.26
5.4	101.09±2.66	2.63	102.81±1.16	1.13	102.23±1.34	1.31
8.1	103.93±1.47	1.41	105.47±0.77	0.73	105.08±1.54	1.47

* n=8 for each analyte size, Regression equation: y = 0.04012 x + 0.09601(r^2 = 0.9964)

is102.50 ± 2.18% and the coefficient of variation is 2.13% (table 2). Repeatability of extraction was found to be better for the higher analyte size (8.1µg/ml) when compared to other analyte sizes. Comparative analysis of the four brands of diclofenac slow release tablets by the official HPLC (BP 1998) method and the new colorimetric method is presented in table 3. Both methods were found to be of equivalent accuracy ($p>0.05$).

Table 4 shows the results of the TLC analysis of the tablet brands at elevated temperature.

DISCUSSION

Diclofenac was found to couple readily with the reagent solution to give an instant blood-red colour in the acidic medium. The instant colour formation indicates the formation of an azo dye (scheme 1). The ethyl acetate extract is however orange in colour. The absorption spectrum of the adduct in ethyl acetate shows a bathochromic shift, relative to the underivatized pure drug and a pronounced hyperchromic shift relative to the blank reagent (figure 1). The peak at 470nm was selected as the analytical wavelength, because at this wavelength, the difference in absorptivity between the blank reagent and the adduct is maximal. Absorbance of the adduct was found to decrease sharply from 30^{o}C to 80^{o}C at 5 and 20 minutes incubation periods (figure 2). Thus, further investigation was carried out at 30^{o}C. Optimal reaction was however found to have been achieved immediately after mixing the reaction mixture in a vortex mixer for 10 seconds. Thus, further incubation of the reaction mixture was found unnecessary. Incubation for longer periods beyond 5 minutes led to a sharp decrease in absorbance (figure 3). This suggests the azo adduct formed is relatively unstable in the acidic coupling medium. In sharp contrast, the adduct has very good stability in ethyl acetate solution over a period of 3h (figure 4). This finding dictated the routine extraction of the adduct into ethyl acetate before measurement of absorbance reading. The molecular behavior of the azo adducts in acidic and neutral media was thus adapted to achieve a rapid assay method. Diazo coupling in alkaline medium was not applicable due to the high reactivity of the diazonium ion. Nucleophiles like hydroxyl ion (OH^-) preferably attack the cation before aromatic ring such that alkaline medium or aqueous medium could not support diazo coupling reaction with CDNBD.

The plot of absorbance against the mole fraction of the reagent exhibited a change of slope with a maximum absorbance at a mole fraction of 0.67 and decreasing absorbance as the value of the mole fraction deviate from 0.67. This signifies that the drug and reagent react optimally in a 1:2 stoichiometric ratio under the optimal conditions routinely employed for the coupling reaction. It is commonly interpreted in complex formation reaction that, for two interacting species at a constant total concentration, the complex (adduct) is at its greatest concentration at a point where the two species are combined in the ratio in which they occur in the complex (Martin *et al.*, 1983). Our result therefore suggests adduct with two moles of the reagent to one of the drug. However, for diazo coupling reaction, it is well known that a higher concentration of the diazonium ion will hasten the rate of coupling reaction (Herbst and Hunger, 1997). Secondly, the strong electron withdrawing groups on CDNBD would deactivate the aromatic rings in diclofenac such that electrophilic substitution of a second mole of CDNBD should be discouraged after an initial electrophilic attack. The ring bearing two mildly activating chlorine atoms in addition to the amino bridge should be more activated than the second ring bearing a deactivating carboxyl group. The free *para* position to the amino group is therefore a more likely point of attack to favor the proposed 1:1 CDNBD-diclofenac azo adduct (scheme 1), while a 2:1 reagent/drug stoichiometry is required for a very fast optimal reaction obtained. This explanation is corroborated by a similar pattern obtained for the diazo coupling of naproxen with CDNBD. A 2:1 stoichiometric ratio was obtained as described here (unpublished data). Yet unequivocal spectroscopic characterization of the isolated azo adduct formed shows a 1:1 mole ratio in the molecular structure (Adegoke, 2005).

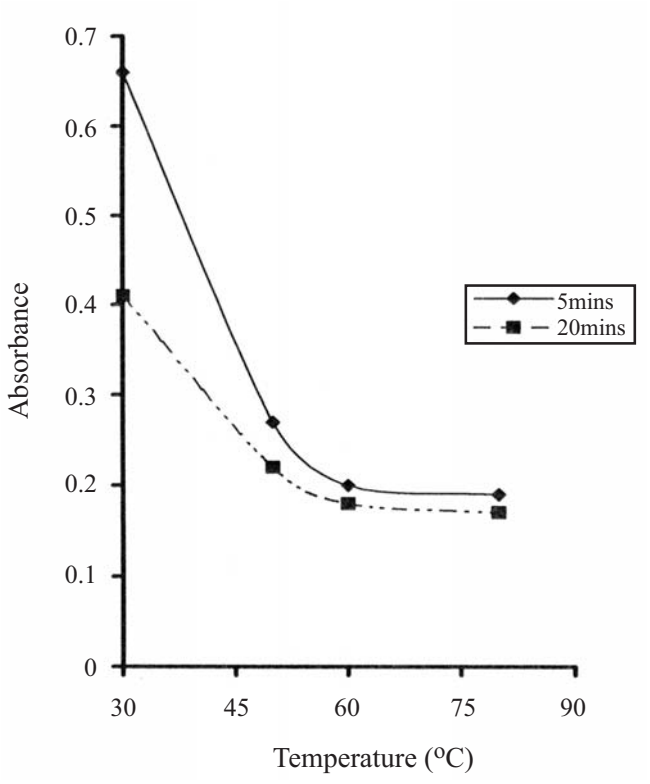


Figure 2: Optimization of coupling reaction temperature.

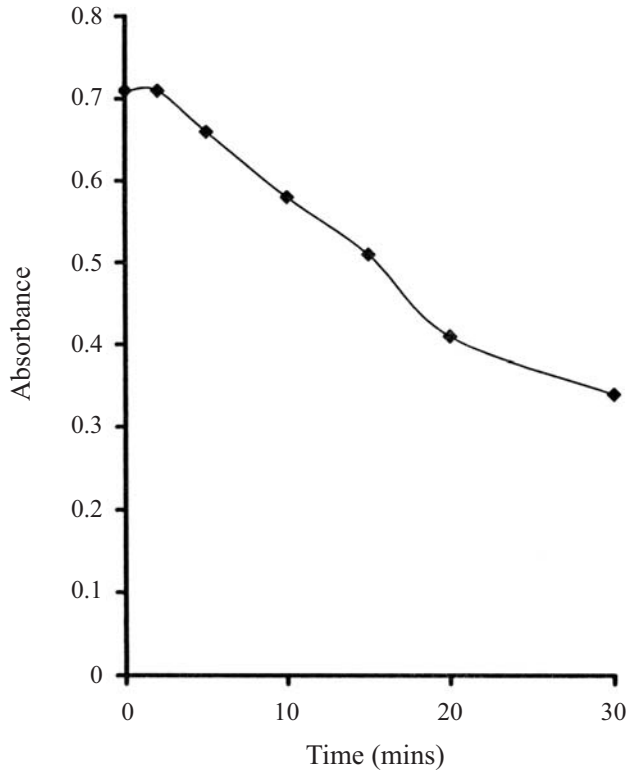


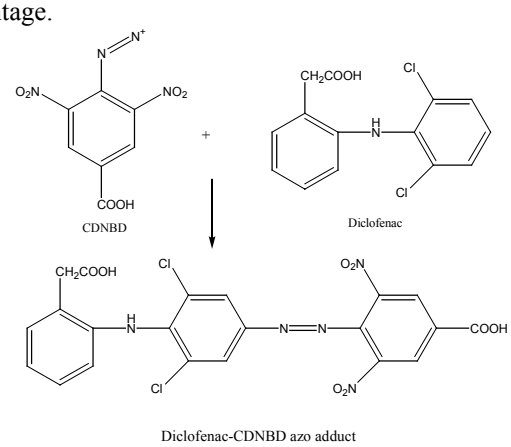
Figure 3: Optimization of coupling reaction time at 30°C.

Table 3: Assay of diclofenac sodium tablets by official method and the new method

Tablet Brand	Mean ± S.D*	
	B.P	CDNBD
Abitren	84.85 ± 0.58	85.18 ± 1.72
Grofenac	86.09 ± 0.83	85.74 ± 1.83
Diclogesic	87.34 ± 0.59	84.64 ± 2.49
Voltaren	100.17 ± 1.17	101.17 ± 1.85

n = 6, Official range = 92.5-107.5%, $p<0.05$ is taken as significant.
CDNBD = New colorimetric assay, B.P. = Official HPLC method

The colorimetric method of analysis for diclofenac sodium tablets using CDNBD as described in this work has some advantages over the official HPLC (BP 1998) method. The method is based on a very fast coupling reaction such that a single determination is completed in less than 5 minutes. Therefore, useful time of analysis is gained with the possibility of handling large sample throughput. The method could also be readily automated. Diclofenac has a peak with large absorptivity at 276nm, which could afford sensitive quantitative determination. This will however require a UV/visible spectrophotometer. The main attraction that warrants derivatization and perhaps a profound advantage of the developed method is that colorimetric determination was made possible with more affordable instrumentation (digital colorimeter), especially for routine analysis. The new method also employs simple reagent preparation compared to the use of potassium ferricyanide by Vaidya and Prasad (1995) and simple methodology compared to the ion-association complex formation with methylene violet, as previously reported by Sastry *et al* (1989). The extensive dilution stages, which could compromise accuracy and precision, in the method proposed for the analysis of diclofenac by Mo and Chen (1988) also places the new colorimetric procedure described in this work at a better advantage.



Scheme 1: Coupling reaction between CDNBD and Diclofenac.

Selectivity is the main advantage of the official HPLC method, being based on a separatory technique. In order to

Table 4: Thin layer chromatographic analysis of diclofenac tablets and diclofenac/CDNBD reaction mixture on silica gel GF_{254}.

	Tablet brands					Reaction mixture	
Mobile phase	DLC	D_1	D_2	D_3	D_4	Adduct	CDNBD
EtOAC: MeOH (9:1)	0.93	0.93	0.93	0.93	0.93	0.59	0.76
$CHCl_3$: MeOH (8:2)	0.79	0.79	0.79	0.79	0.79	0.62	0.88
MeOH: Strong NH_3 (100:1.5)*	0.76	0.76	0.76	0.76	0.76	0.55	0.60

*Plate was coated with 0.1M methanolic KOH before spotting samples on it visualization: UV-254nm and Iodine vapour.
DLC diclofenac reference substance; D_1=Abitren, D_2=Grofenac, D_3=Diclogesic, D_4= Voltaren

ascertain the specificity of the method described here, thin layer chromatographic analysis of the tablets stored at room temperature and elevated temperature was done to detect the presence of any thermal degradation products. The presence of any degradation product should invalidate the accuracy of the method described here, since such compounds may interfere with the coupling reaction. On the other hand, the absence of any degradation product in any batch of tablets intended for assay would guarantee that the colorimetric method is specific for diclofenac. As presented in Table IV, all the brands of the tablets were stable at elevated temperature of 100^{o}C for a period of 5 hours. Absence of any degradation product was observed in all the three chromatographic conditions adopted. In the other approach for method selectivity, the accuracy obtained for diclofenac was 98.77% and the following were obtained in the presence of the excipients; gelatin (96.47%), magnesium stearate (98.77%), starch (98.77), lactose (96.47%) a mixture of excipients (96.47%). The closeness of the values suggests lack of interference from commonly utilized excipients hence confirms selectivity of the CDNBD assay method for diclofenac.

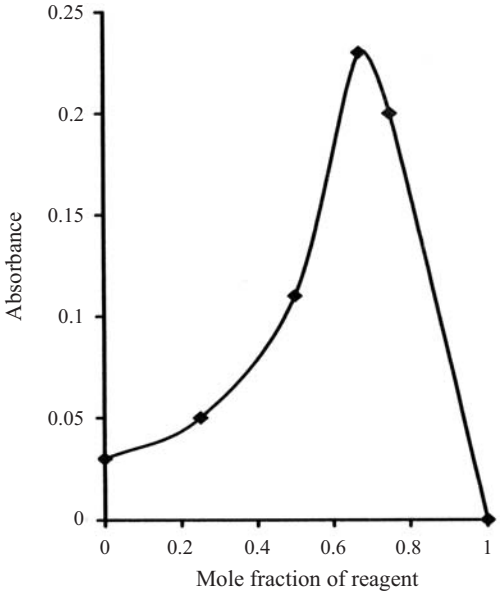


Figure 4: Stoichiometric ratio of Diclofenac-CDNBD azo adduct formation.

Only the innovator product passed the assay of active ingredient test by both the HPLC method (BP) and the new CDNBD method described in this work. This suggests that generic substitution in clinical practice should be done with caution.

In conclusion, the new method is of equivalent accuracy with the official (B.P) HPLC assay method and shares the typical advantages of instrumental methods with the official method. The simplicity of technique, speed of reaction and utilization of more affordable instrumentation (digital colorimeter) however places the new method at a better advantage than previously reported methods. The method described here could find application in the in-process quality control of diclofenac tablets.

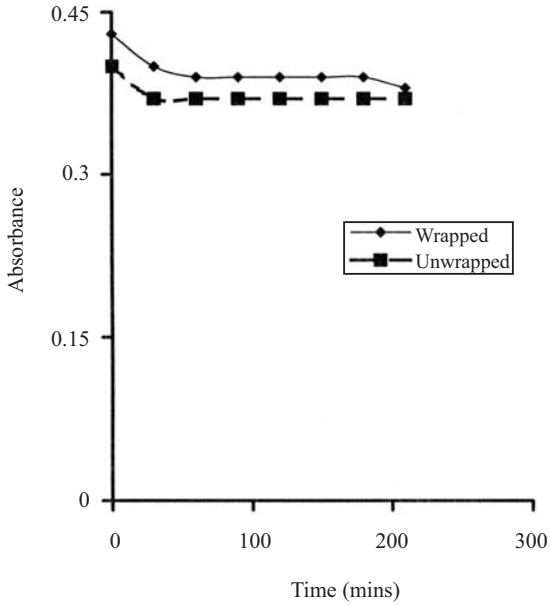


Figure 5: Stability of CDNBD-Diclofenac azo adduct in ethyl acetate

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