

# COLORIMETRIC DETERMINATION OF CHLORAMPHENICOL AND METRONIDAZOLE IN PHARMACEUTICAL FORMULATIONS AFTER SCHIFF-BASE FORMATION WITH VANILLIN AND ANISALDEHYDE

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## ABSTRACT

The nitro groups in chloramphenicol and metronidazole drugs were reduced to their amino functions using zinc dust in concentrated hydrochloric acid. Vanillin and anisaldehyde were used to form Schiff-base adducts with chloramphenicol and metronidazole respectively. The chloramphenicol couple gave an absorption maximum ( $\lambda_m$ ) at 385 nm while that of metronidazole absorbed ( $\lambda_m$ ) at 400 nm. Both colours were stable over a period of 9h. Both derivatives obey Beer/Lambert law within the range of 1 mg/ml and 10 mg/ml that was investigated. The molar absorptivities were 2491 and 3151 l mol<sup>-1</sup> cm<sup>-1</sup> for chloramphenicol and metronidazole adducts respectively. Sandell's sensitivity values were 8.5 ng/ml for chloramphenicol and 22.5 ng/ml for metronidazole suggesting that the vanillin couple was more sensitive than the anisaldehyde adduct. The adduct formation was highly selective so quantitative recoveries indicated high precision although statistical comparison employing Student's t-test did not suggest any significant ( $p > 0.05$ ) difference between the proposed and the Pharmacopoeia method. This proposed method is more sensitive, accurate and simple to carry out.

**Key words:** Chloramphenicol and Metronidazole assay; Schiff-base adduct

## INTRODUCTION

Molecules that do not ordinarily absorb radiation in the visible region of the spectrum can be made to absorb by introducing chromagens that will facilitate electronic transitions to occur. The commonest functional groups in drug molecules that are readily derivatized are the nitro, amino, carbonyl and carboxyl groups. Drugs containing nitro groups like metronidazole, chloramphenicol, nitrofurantoin can be assayed very accurately after reduction because the reduction is quantitative. The subsequent reaction may be diazotization, nitrite titration, complexation or Schiff-base formation most of which absorb in the visible region.

The Pharmacopoeia protocol (British Pharmacopoeia, 1993) for the analysis of chloramphenicol in pure and dosage forms utilizes spectrophotometric method at the ultraviolet region ( $\lambda_m$  278 nm) while that of metronidazole employs non-aqueous titration. In spite of the Official protocol, a large number of colorimetric determinations have been presented in the literature (Taha et al., 1980; Nekoz et al. 1974; Mahrous and Abdul-Khalek, 1984; Morelli, 1987; Cieszynski and Kociemba, 1977) with considerable degree of accuracy.

Chloramphenicol when heated with alkaline solution first produces a yellow colour that intensifies on further heating. When the solution boils, a red precipitate of azo-benzoic

acid is formed. This reaction pathway has been used to establish the identity of chloramphenicol and quantitative determination of the drug (British Pharmacopoeia, 1993). By far the most employed protocol for the colorimetric determination of chloramphenicol employs nitro reduction followed by various derivatization reactions. For example, aminoanthraquinone dye was used (Krishna et al. 1980) for the determination of chloramphenicol. Similar treatment with benzyl chloride and ferric chloride produced a sensitivity factor of between 0.80  $\mu$ g/ml and 1.60  $\mu$ g/ml (Auterhoff and Beiuorth, 1980). Diazotization of reduced chloramphenicol and coupling with Brantton-Marshall reagent [N-(1-naphthyl)-ethylenediamine] dihydrochloride has produced a highly sensitive procedure, but suffers the disadvantage of being very expensive Nekoz et al. 1974; Wolfgang and Gertruida, 1980). Other reactions include the use of dimethylformamide in the presence of tetramethylammonium hydroxide (Sakar, 1976); cobaltinitrite method (Mahrous and Abdul-Khalek, 1984); the formation of blue complex with o-methoxyphenol in alkaline medium (Cieszynski and Kociemba, 1977).

Chloramphenicol has been analyzed in cerebral fluid and serum by modified enzymatic acetylation assay (Botha et al. 1981). Also a highly sensitive fluorimetric technique was developed for the assay of chloramphenicol in the presence of its metabolites (Clarenberg and Rao, 1977) with a sensitivity in the region of 20 ng/ml.

Many of these colorimetric methods have proved more sensitive than the Official and recommended procedure particularly in the presence of excipients and metabolites.

Pharmaceutical analysis of metronidazole has similarly been well reported in the literature. Technically, the colorimetric determinations of chloramphenicol based on the nitro-reduction are applicable to metronidazole drug. In addition to the examples illustrated above, polarography (Gratteri and Cruciani, 1999), first derivative spectrophotometry (Verga and Sola, 2001), difference spectrophotometry (Liu, 1997), ratio spectra derivative spectrophotometry (Erk and Altun, 2001) has been reported for the assay of metronidazole with accuracy comparable with the Official method. Also techniques like bivariate spectrophotometry (Lopez de Alba et al. 1997) and absorbance-subtraction method (Du, 1997) have been reported. The phenomenon of  $\pi$ -donor to  $\pi$ -acceptor interaction has been adapted to the quantitative analysis of metronidazole after nitro-reduction (Onah, 2002, in press).

Aldehydes and ketones have long been established as reacting with primary amines to form imines or Schiff-bases. These imines have been very useful in organic synthesis and in biochemical transformations. Schiff-bases have various absorbances depending upon the chromagen used and the condition of formation; such absorbances must have to be stabilized if intended for analytical purposes. In most situations, the chromagen must be present in excess since equilibrium condition depends upon its concentration and its absorbance does not interfere with that of the Schiff-base formed.

Vanillin is the most frequently used reducing agent in the Schiff-base formation so we decided to compare it with anisaldehyde in this investigation to illustrate the nature of chromagen on the absorbance maximum of the Schiff-base and the sensitivity to spectrophotometric measurement. The non-aqueous titration of metronidazole is severely limited by low sensitivity and poor sharpness at end-point; similarly the ultraviolet spectrophotometric method for chloramphenicol is relatively less selective particularly in the presence of metabolites and excipients. These limitations have necessitated the development of simpler, cheaper but accurate methods for the assay of these drugs particularly in the presence of contaminants.

## EXPERIMENTAL

### Materials and method

Chloramphenicol and metronidazole

powders were kindly donated by ECWA Pharmacy Jos, Nigeria. Vanillin powder (AnalaR grade, BDH, UK), zinc granules (Samok Combined Industries Jos, Nigeria), hydrochloric acid (AnalaR, BDH,); ethanol (AnalaR, BDH) were all used. All other reagents were of analytical grade.

### Nitro-reduction of chloramphenicol and metronidazole

100 mg of pure chloramphenicol and metronidazole powders were quantitatively transferred into 50 ml conical flasks and 25 ml of warm water added to each flask for dissolution. After cooling to room temperature, 10 ml of concentrated hydrochloric acid and 3.0 g of zinc granules were added to each drug and the mixture heated for 15 minutes. The reaction mixtures were allowed to return to room temperature, then filtered and the residues further washed with sufficient water (50 ml). The filtrate respectively were transferred into an 100 ml volumetric flasks and the volumes made up to mark.

### Coupling reaction and determination of absorption maxima

Stock solutions equivalent to 250  $\mu\text{g/ml}$  each of reduced chloramphenicol and metronidazole were transferred to 100 ml volumetric flasks, 10 ml of vanillin (0.1974 M) was added to the reduced chloramphenicol and 10 ml of anisaldehyde (0.2206 M) to the reduced metronidazole and the volumes made up to mark with 1 M hydrochloric acid. Blank solutions were similarly prepared. The solutions were thoroughly mixed and allowed to stand in the dark for 15 minutes before absorption maxima were determined using Spectronic 20 Spectrophotometer (Nottingham, UK). The stability of the colours were monitored at intervals of 1 h for 12 h.

### Determination of extinction coefficient

Serial volumes of 2, 4, 6, 8 and 10 ml each of the solutions of reduced chloramphenicol and metronidazole equivalent to 10  $\mu\text{g/ml}$  each were quantitatively transferred to separate 50 ml volumetric flasks (in quintuplets each). 10 ml of vanillin and anisaldehyde were added to chloramphenicol and metronidazole, respectively. Each of the volumes was made up to mark with hydrochloric acid. The contents were thoroughly mixed and absorbance measured after 15 minutes. The stability of each colour was again monitored for another 9 h at intervals of 1h. The average values of the absorbances were calculated and plotted against the concentrations.

The plots were regressed by the method of least squares (E.L. Bauer, 1971) and molar absorptivity determine from the values of the slopes. The Sandell's sensitivity was similarly calculated for each solution.

**Optimization of the analytical technique**

The optimization of this assay technique was established essentially by the method described earlier (Onah, 2002, in press). The sensitivity of the spectrophotometer was established by standard protocols, the limit of detection (LOD) and quantitation (LOQ) were determined. The concentrations of the analytes were randomly varied with their corresponding absorbances recorded. The relationships between concentrations and absorbances were plotted and regressed by the method of least squares (Bauer, 1971). Selectivity was established by *in situ* reduction of these drugs in their pharmaceutical formulations. Other optimizations parameters were carried out by the standard procedures.

**Calibration curves and their validation**

Serial volumes of each drug equivalent to 10, 20, 30, 40 and 50 µg/ml were taken and 10 ml of vanillin (0.1974 M), anisaldehyde (0.2206 M) were respectively added; absorbances were then measured after 15 minutes at their respective absorption maximum using matched cells.

Suitable blanks were similarly prepared. This procedure was repeated five times for each drug. The means of the absorbances corresponding to various concentrations were calculated and plotted graphically. Linearity was tested for by regression analysis (Bauer, 1971). Further validation was carried out by taking different concentrations of the derivatives and analyzing them for their absorbances. Again five replicate determinations were carried out. The mean absorbances were converted directly to their corresponding concentrations using the calibration curve prepared earlier; the percent recoveries were noted.

**Assay of chloramphenicol and metronidazole in their formulations**

Twenty capsules of chloramphenicol and 20 tablets of metronidazole were powdered. 100 mg of each powder was accurately weighed out and subjected to nitro-reduction as described earlier (section 2.3). Stock solutions of each drug equivalent to 250 µg/ml were prepared in standard volumetric flasks from which 5.0 ml was transferred accurately into other 50 ml volumetric flasks in quintuplets; 10 ml of 0.1974 M vanillin

and 10 ml of 0.2206 M anisaldehyde were added to chloramphenicol and metronidazole, respectively. Absorbances were determined at the respective absorption maximum using matched cells. The absorbances measured were converted directly to concentrations using the validated calibration curve. The procedure was repeated five times for each drug using different concentrations and corresponding recoveries calculated. Official methods were also used to assay the drugs for comparative purposes.

**RESULTS AND DISCUSSION**

The reduction of nitro groups of chloramphenicol and metronidazole was quantitative (Onah, 2002, in press). The Schiff-base adduct formed between vanillin and chloramphenicol and that between metronidazole and anisaldehyde were similarly quantitative. The mechanism of Schiff-base formation is electrophilic in nature. Evidence for the existence of these bases was confirmed by infra-red spectrophotometry which showed the characteristic absorption band. The adducts of chloramphenicol and metronidazole absorbed maximally ( $\lambda_m$ ) at 385 nm and 400 nm, respectively (Fig. 1). Because the colours were stable for 6h, we believe the colours were also stable throughout the 45 minutes duration of the analysis.

The limit of detection (LOD for chloramphenicol and metronidazole adducts were 0.15 µg/ml and 0.10 µg/ml respectively while that of quantitation (LOQ) were 0.52 µg/ml and 0.30 µg/ml. The reactions obeyed Beer/Lambert law between 1.0 mg/ml and 10 mg/ml that was investigated and fully validated for each drug substance. Linearity tests were carried out at different concentrations; the following equations satisfies the mathematical equation derived for each experiment:

$$A_{385} = 0.00844x_1 + 0.000239 \text{ (for chloramphenicol couple) and}$$

$$A_{400} = 0.0222x_2 + 0.00295 \text{ ( for metronidazole)}$$

where  $A_{380}$  and  $A_{400}$  represents absorbances at their absorption maximum,  $x_1$  and  $x_2$  were the respective concentrations in the media. Linear regression analysis carried out gave the correlation coefficient of 0.98 and 1.0 respectively, suggesting complete correlation between theoretical and experimental data. The errors of slopes and intercepts were  $3.8 \times 10^{-4}$  and  $1.67 \times 10^{-4}$ , for chloramphenicol and  $1.30 \times 10^{-3}$  and  $1.18 \times 10^{-4}$  for metronidazole. The molar absorptivities were  $2491 \text{ l mol}^{-1} \text{ cm}^{-1}$  for chloramphenicol and  $3151 \text{ l mol}^{-1} \text{ cm}^{-1}$  for metronidazole. The Sandell's sensitivity

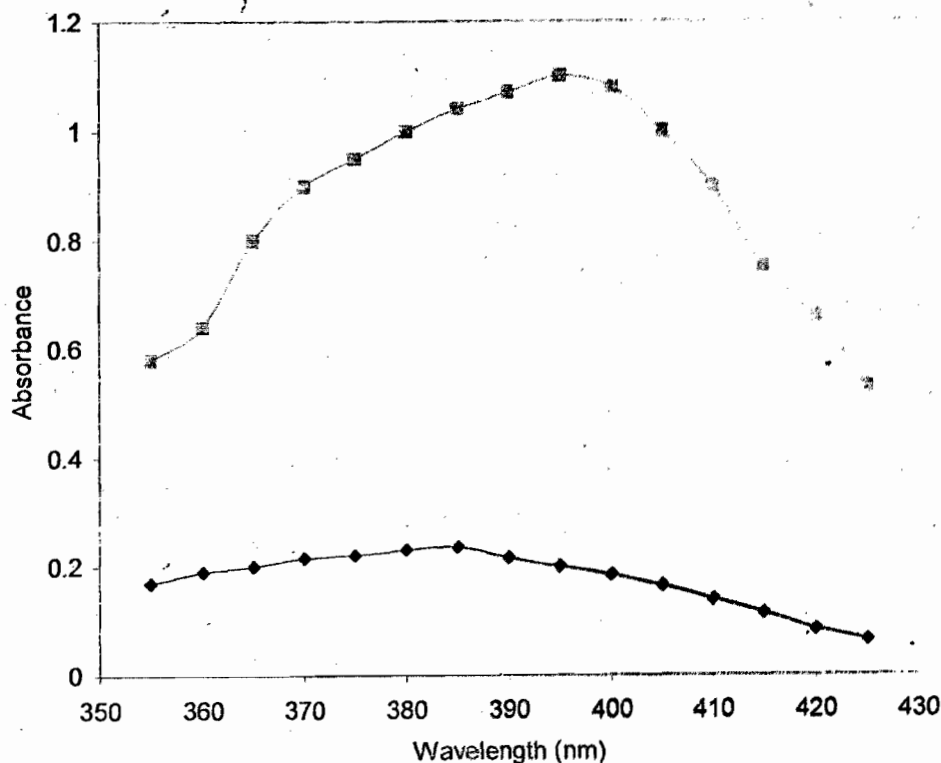


Fig.1 Determination of absorption maxima for the adducts of chloramphenicol (■) and metronidazole (◆)

coefficient similarly gave 8.5 ng/ml and 22.51 ng/ml for chloramphenicol and metronidazole respectively. These data reveal that vanillin adduct was more sensitive and most suitable for analytical investigation. The precision of the analytical technique (RSD) varied between 0.80 and 0.90 %. Confirmatory test of the selectivity of nitro reduction and coupling was carried out in the presence of excipients; the margin of error was between 3 and 4 % of the pure compound.

Table 1 illustrates the quantitative recoveries obtained experimentally. The relatively low standard deviation indicates high precision of the proposed technique. Statistical comparison of the proposed method with the Official methods at 95 % confidence limit (Student's t-test) did not show any significant difference. We therefore conclude that the analysis of Schiff-base adduct is sufficiently accurate for their application in quality assurance.

The reactivity of aromatic aldehydes and ketones with primary amines is acid catalyzed and so experimental conditions must be adjusted such that the colour formed is stable and reflects the actual concentration of the product (analyte) and not that of the chromagen. This condition is achieved by using excess chromagen so that a constant amount of the product is present. Although vanillin and anisaldehyde belong to the same family of aromatic aldehydes, we have

deliberately chosen them to compare their reactivity and colour sensitivity for colorimetric purposes. We observed that vanillin produced colour more rapidly than anisaldehyde. The Sandell's sensitivity test confirms this assertion. This can be explained by the electronic influence of the hydroxy group in vanillin.

The non aqueous method is the recommended technique for the analysis of metronidazole. This technique is entirely empirical because the nature of the indicator determines the sensitivity of colour transition at the end-point. The presumption that perchloric acid ionizes completely during the titration cannot be ascertained with absolute certainty. The accuracy and reproducibility of non-aqueous titration largely depend upon the skill and experience of the researcher. Spectrophotometric method of analysis by comparison is economical, sensitive and accurate although in the ultraviolet region selectivity and specificity may pose a serious problem particularly in the presence of degradation products and metabolites.

This investigation demonstrates that Schiff-base derivative of chloramphenicol and metronidazole can be used to assay these drugs either as industrial raw materials or in their pharmaceutical formulations. The suitability of the technique for stability-indicating assay is not immediately obvious since the nitro-group itself is

**Table 1: Recovery experiments by the proposed and Official methods:**  
 Chloramphenicol: Molar absorptivity  $\epsilon = 2491 \text{ l mol}^{-1} \text{ cm}^{-1}$

Proposed method					Official method	
Sample no	Pure Drug % n= 5	RSD %	Tablet Formulation n= 5	RSD %	Tablet formulation n= 5	RSD %
1	99.60	0.98	97.60	0.95	96.50	1.30
2	98.67	1.05	97.22	1.09	96.50	1.01
3	99.8	1.14	98.34	1.18	97.80	0.97
4	98.86	1.21	97.39	1.18	95.90	1.25
5	99.44	1.28	98.52	1.24	96.7	0.94
mean	99.27	1.13	97.81	1.13	96.68	1.09

Metronidazole: Molar absorptivity  $\epsilon = 3151 \text{ l mol}^{-1} \text{ cm}^{-1}$

Proposed Method				Official method		
Sample no	Pure drug n= 5	RSD %	Tablet formulation	RSD %	Tablet formulation	RSD %
1	98.72	0.75	96.76	1.07	95.17	1.30
2	99.50	1.03	96.02	1.00	94.10	1.51
3	99.15	1.10	96.18	1.14	94.75	1.45
4	98.98	1.05	96.52	1.09	95.35	1.55
5	98.50	1.25	96.04	1.02	95.51	1.46
Mean	98.97	1.20	96.30	1.06	94.98	1.45

not affected when the drugs deteriorate on storage.

## CONCLUSION

The technique of analyzing chloramphenicol and metronidazole through the formation of Schiff-bases represents an accurate, sensitive and easy method that can be used for the quality assurance of these drugs in their pharmaceutical formulations. Vanillin adduct is more sensitive to measurements than the anisaldehyde couple as demonstrated by the Sandell's sensitivity values. Non-aqueous titration method of analysis of metronidazole recommended by Pharmacopoeia is not as sensitive as the proposed method principally because of the inherent limitations of the method. Similarly, spectrophotometric assay of chloramphenicol in the UV region is not very

selective and specific particularly in the presence of degradation products, contaminants and/or excipients. This proposed method when validated by other laboratories could be used to compliment the recommended technique.

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