# COLRIMETRIC DETERMINATION OF TETRACYCLINE HYDROCHLORIDE WITH 4-NITROBENZENEDIAZONIUM HYDROGEN SULPHATE

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## ABSTRACT

NIGERIATetracycline hydrochloride was coupled with 4-nitrobenzenediazonium hydrogen sulphate in aqueous medium. Freshly prepared cold saturated solution of sodium acetate facilitated the coupling reaction at room temperature thus obviating the high temperature required for coupling in the method of Kakemi, Arita, Sezaki and Nadal ( 1 ). The azo product in 0.1M sodium hydroxide solution had a $_{max}$ of 425 nm and an $E_{max}$ of 27,800 L-mol$^{1}$ cm$^{1}$. Beer's law was obeyed at this $_{max}$ in the range of 0 15 g/ml of tetracycline hydrochloride. The developed method was applied to the determination of tetracycline hydrochloride in the presence of capsule excipients and in capsule dosage form.

**KEYWORDS:** Colorimetric, tetracycline hydrochloride, couple, 4-nitrobenzenediazonium hydrogen sulphate.Compounds containing the phenolic group undergo certain characteristic reactions such as complexation with ferric ion, bromination with bromide-bromate solution in acidic medium and coupling with diazotised primary aromatic amines. These reactions have formed the basis for the development of many analytical methods for such compounds. Colorimetric methods are common in analysis because of their simplicity, accuracy and sensitivity without resort to expansive instrumentation. Amongst the above- named analytical reactions for phenolic compounds, the coupling reaction with diazotised primary aromatic amines is the most sensitive. This is as a result of the extensive conjugation in the azo product which enables it to strongly absorb visible light energy thus making it possible for trace quantities of phenolic compounds to be determined. Variations in the structure of the primary aromatic amine used for forming the diazonium salt, the coupling medium and the final medium in which the azo product is measured, can have profound effects on the absorption characteristics of the azo product. The reaction conditions for such coupling have been established. Generally, the primary aromatic amine is diazotised in acidic medium and coupled with the phenolic compound in mildly alkaline medium ( 2 ).

Many methods have been developed for the determination of the bacteriostatic antibiotic, 4-(dimethylamino)-1, 4, 4a, 5, 5a, 6, 11, 12a octahydro-3, 6, 10, 12 12a-pentahydroxyl-6-methyl-1, 11, -dioxo-2-naphthacene-carboxamide (tetracycline) and its hydrochloride. These include titrimetric ( 3-5 ); densitometric (6 ); polarographic ( 7,8 ); spectrophotometric ( 9- 15 ); fluorimetric ( 16- 21 ); liquid chromatographic(22- 32); high performance liquid chromatographic ( 33-34 ); chemiluminescence ( 35 ) and fourier transform medium infra-red and near infra-red spectroscopic ( 36 ) methods. Tetracycline has also been assayed by microbiological method (37, 38 ).The colorimetric method of Kakemi et al. ( 1 ) involves the coupling of tetracycline with diazotised 4-nitroaniline. However, the experimental conditions for the reaction involved coupling at 65C for 45 minutes. Generally, diazotisation and coupling reactions take place at reduced temperature of below 15C and occasionally at room temperature because of the instability of diazonium salts. Also, some diazonium salts have been reported to explode violently when heated ( 39 ). More importantly, for routine analytical purposes, reactions that are fast under ambient conditions would be preferred. With this background, the coupling of tetracycline hydrochloride with diazotised 4-nitroaniline was investigated to establish fast analytical conditions at room temperature.

## EXPERIMENTAL

### Apparatus

Double beam Pye Unicam SP1800 ultra-violet/visible spectrophotometer with model AR55 linear recorder (Pye Unicam, England) and 1-cm quartz cells were used to obtain all spectral measurements.

### Reagents

The following reagents were of analytical or pharmaceutical grade and were used as obtained. 4-nitroaniline, sodium nitrite,, 95% ethanol, acetone and magnesium stearate were obtained from Merck, Darmstadt, West Germany; sodium acetate trihydrate, -lactose monohydrate and soluble starch were from BDH Chemicals Ltd., Poole, UK. Sulphamic acid was from Fluka A.G., Buchs; sodium hydroxide pellets from Halewood Chemicals Ltd., Middlesex, UK; glacial acetic acid from Haig Laboratory Chemical Corporation, Middlesex, UK; sulphuric acid 98% (˚/.) from May and Baker Ltd. Daggenheim, UK and tetracycline hydrochloride from Afro-Arab Techni-Chemicals, Lagos, Nigeria. Reference standard tetracycline hydrochloride was obtained from Council of Europe Pharmacopoeia, Strasbourg, France, Brands of tetracycline hydrochloride capsule were purchased from local pharmacy shops. Distilled water was used for preparing all the solutions and all the reactions were carried out at room temperature except when specified.

### Procedure

Stock 4-nitroaniline solution was

prepared by suspending 3g in 30 ml distilled water in a 100ml beaker. 30ml concentrated sulphuric acid was slowly added to it with stirring. After cooling, the red coloured solution was transferred into a 100ml volumetric flask with the aid of distilled water and made up to mark withdistilled water.

### Diazotisation

To 95ml of distilled water in a 100ml volumetric flask was added 1ml of the 4-nitroaniline stock solution followed by addition of 1ml 10% (%) of sodium nitrite. After allowing 10 minutes, 1ml of 10% (%) of sulphamic acid was added and after another 5 minutes, the solution was made up to mark with distilled water.

### Coupling

Appropriate volumes (0.5 2.5ml) of freshly prepared stock solution of tetracycline hydrochloride (120μg/ml) were taken in 20 ml volumetric flasks. Distilled water was added to bring total volume of each flask to 2.5ml and 1.0ml of freshly prepared cold saturated solution of sodium acetate was added to each flask followed by addition of 1.0ml of the freshly prepared diazonium salt. The flasks were left at room temperature for 30 minutes and thereafter brought to mark with 0.1 M NaOH. A blank was similarly prepared by using distilled water in place of the tetracycline hydrochloride stock solution.

### Excipients and Dosage Form

For the determination of tetracycline hydrochloride in the presence of excipients, appropriate amounts of drug and excipient were weighed, mixed and dissolved/suspended in distilled water. For the dosage form, the contents of twenty capsules were weighed, mixed and appropriate weight of powder containing a known amount of tetracycline hydrochloride was dissolved/suspended in water. From the separate solutions, stock solutions of tetracycline hydrochloride containing 120μg/ml were prepared. Thereafter the procedure for coupling was followed.

## RESULTS AND DISCUSSION

Phenolic compounds generally couple with diazotised primary aromatic amines at the para-position and when this position is not free, coupling occurs at the ortho position ( 40 ). In tetracycline, one of the ortho and one of the meta positions to the hydroxyl group of the phenolic moiety are substituted. Moreover, the substituted ortho position carries a conjugated carbonyl and since coupling is an electrophilic substitution in which the diazonium salt is the attacking electrophile, this would lead to deactivation of the aromatic ring hence decreased reactivity to electrophilic reagents. This is why the coupling reaction in the method of Kakemi et al. ( 1 ) was carried out at an elevated temperature to help accelerate the reaction. It was also observed that at room temperature, the coupling reaction between tetracycline hydrochloride and 4-nitrobenzenediazonium hydrogen sulphate proceeded slowly in water. Thus it became necessary to look for alternative ways of accelerating this reaction without resort to high temperature.

For diazotisation at room temperature, it was observed that the same results were obtained when 5, 30, 60 and 120 minutes were allowed for the reaction. In conjunction with the instantaneous discharge of the yellow colour of 4-nitroaniline solution upon the addition of sodium nitrite, diazotisation occurred very quickly and was complete within 5 minutes. The destruction of excess sodium nitrite by sulphamic acid was observed to give more stable absorbance readings of the azo product.

The effect of cold saturated solution of sodium acetate, 0.1M sodium hydroxide and distilled water on the progress of the coupling reaction at room temperature is shown in Fig. 1. In distilled water, 0.1M sodium hydroxide and 0.2ml of saturated sodium acetate, the coupling reaction was quite slow at room temperature as it did not reach completion even after one hour. However, with 1ml of cold saturated solution of sodium acetate, the reaction was accelerated and within 30 minutes, it had reached completion as the reading of the azo product became constant.

It was however observed that the absorbance readings of the azo product when coupling was carried out in presence of 1ml 0.1M sodium hydroxide and 0.2ml of saturated sodium acetate were higher than those obtained when reaction was carried out in 1ml saturated sodium acetate. The effect of varying amounts of cold saturated solution of sodium acetate on the coupling reaction was therefore investigated. Fig. 2 shows that as the amount of sodium acetate increased, the absorbance of the azo product decreased. This is due to the effect of the medium on the absorptivity of the azo product. Thus, the amount of sodium acetate has effect both on the rate of the coupling reaction and on the absorptivity of the azo product.

Table 1 shows the stability of the azo product in the presence of varying amounts of saturated sodium acetate. It shows that the product when coupling was carried out in the presence of 1ml sodium acetate was more stable. In 24 hours variation in reading was only 0.6%. However, Beer's law was obeyed when coupling was carried out for 30 minutes in the presence of 0.2 ml of sodium acetate and readings taken within one hour. The regression equation for the results in this medium is $A = 0.001 + 0.072C$, $E_{max}$ = 34,500 L $mol^{-1}$ $cm^{-1}$. r = 0.9998 where A and C represent absorbance and concentration (μg/ml) respectively. The azo product obtained for coupling in 0.1M sodium hydroxide was however very unstable as the colour faded quite quickly. Sensitivity considerations would favour coupling in low amounts of sodium acetate or dilute sodium hydroxide, however coupling in the presence of 1ml cold saturated solution of sodium acetate was adopted because of the completeness of the

coupling reaction and stability considerations.

It was observed that optimum result was attained when the amount of diazonium salt was equal to or greater than 3.5 times the amount of the drug. Also, when higher amounts of the diazonium salt were used, the blank became more coloured. Thus in the procedure adopted, the ratio of the diazonium salt to drug was not less than 4 even for the highest drug concentrations.

The effect of alcohol and acetone which have been reported to repress the development of colour in certain coupling reactions ( 41 ), was investigated. While both of them decreased the absorption of the azo product, the decrease by acetone was more than that by ethanol. Also, in the presence of acetone, the blank was coloured green and this colouration increased as the amount of acetone increased.

The effect of the diluting medium on the absorption characteristics of the azo product is shown in Fig. 3. Under the conditions of the experiment, the optimum diluting medium is 0.1M NaOH. It was however observed that using more concentrated solutions of sodium hydroxide as diluting solvent led to decrease in the 425 nm peak and the subsequent appearance of another peak at 570 nm. The intensity of the 570 nm peak increased with increase in the concentration of the diluting sodium hydroxide solution while the 425 nm peak decreased simultaneously. When the diluting medium was 2.0M NaOH, the azo product was coloured violet but the colour faded very quickly. Thus, this 570 nm peak in such a medium cannot be used for quantitative purposes. It was also observed that increased acidity of the medium during coupling led to decrease in the absorption of the azo product.

Fig. 4 shows the effect of temperature on the coupling reaction. It was observed that as temperature increased, the colouration of the blank increased significantly. This may be due to increased hydrolysis of the diazonium salt and the accelerated reaction between the 4-nitrophenol produced with the remaining diazonium salt. This increased colouration of the blank, decreased amount of diazonium salt left for coupling with tetracycline hydrochloride and the increased rate of the coupling reaction between tetracycline hydrochloride and the 4-nitrobenzenediazonium salt as temperature increased can explain the observed temperature profile.

The above observations have culminated in the optimum experimental conditions as stated in the procedure above. When the procedure was applied to standard tetracycline hydrochloride solutions, Beer's law was obeyed as 425 nm from 0 $15_{\mu}$ g/ml. The equation of the straight line is A = 0.002 + 0.058C where A is absorbance and C is the concentration of the drug in µg/ml. The correlation coefficient is 0.9999. The method was validated by applying it in the determination of tetracycline hydrochloride in the presence of common excipients and finally used to determine tetracycline hydrochloride in capsule dosage form. The results are shown in Table II. The excipients do not interfere in the determination of tetracycline hydrochloride using this method. F-test was used to compare the results obtained using the method developed here with that by Kakemi et al. ( 1 ) for the determination of tetracycline hydrochloride in capsule dosage form. The F-ratio obtained (0.57) is less than the tabulated $F_{1, 18, 0.5} = 4.41$. Therefore there is statistically no significant difference between the results obtained by both methods at 95% confidence level. Comparing the absorption characteristics, $\lambda_{max}$ values are 425 nm and 435 nm while $E_{max}$ values are 27,800 and 13,500 L mol$^{-1}$ cm$^{-1}$ respectively by the method developed here and by their method. Thus the method developed here is equally as accurate but more sensitive than their method. More importantly, the reaction for the method developed here is at room temperature and occurs quite quickly. The method developed here can be quite valuable for routine and trace determinations of tetracycline hydrochloride.

**Table I: Stability of the azo product as a function of the amount of saturated sodium acetate**

| Volume of saturated Sodium acetate (ml) | Absorbance at 425 nm* | | | |
|---|---|---|---|---|
| | 1 hour | 2 hours | 4 hours | 24 hours |
| 0.01 | 0.721 | 0.708 | 0.696 | 0.685 |
| 0.20 | 0.740 | 0.735 | 0.725 | 0.716 |
| 1.00 | 0.506 | 0.507 | 0.504 | 0.503 |

* Concentration of tetracycline hydrochloride in each case is 8.856 µg/ml.

**Table II: Determination of tetracycline hydrochloride in the presence of excipients and in capsule dosage form**

| Excipient | Mean Recovery | Standard Error of Mean (n = 5) | |
|---|---|---|---|
| Starch | 99.23 | 0.95 | |
| Lactose | 98.25 | 0.76 | |
| Magnesium Stearate | 98.59 | 0.81 | |
| Brands | | | |
| A | 98.20 | 0.58 | 97.65 1.08[a] |
| B | 98.80 | 1.30 | 98.65 1.37[a] |

[a] Results obtained using the method of Kakemi et al (1).

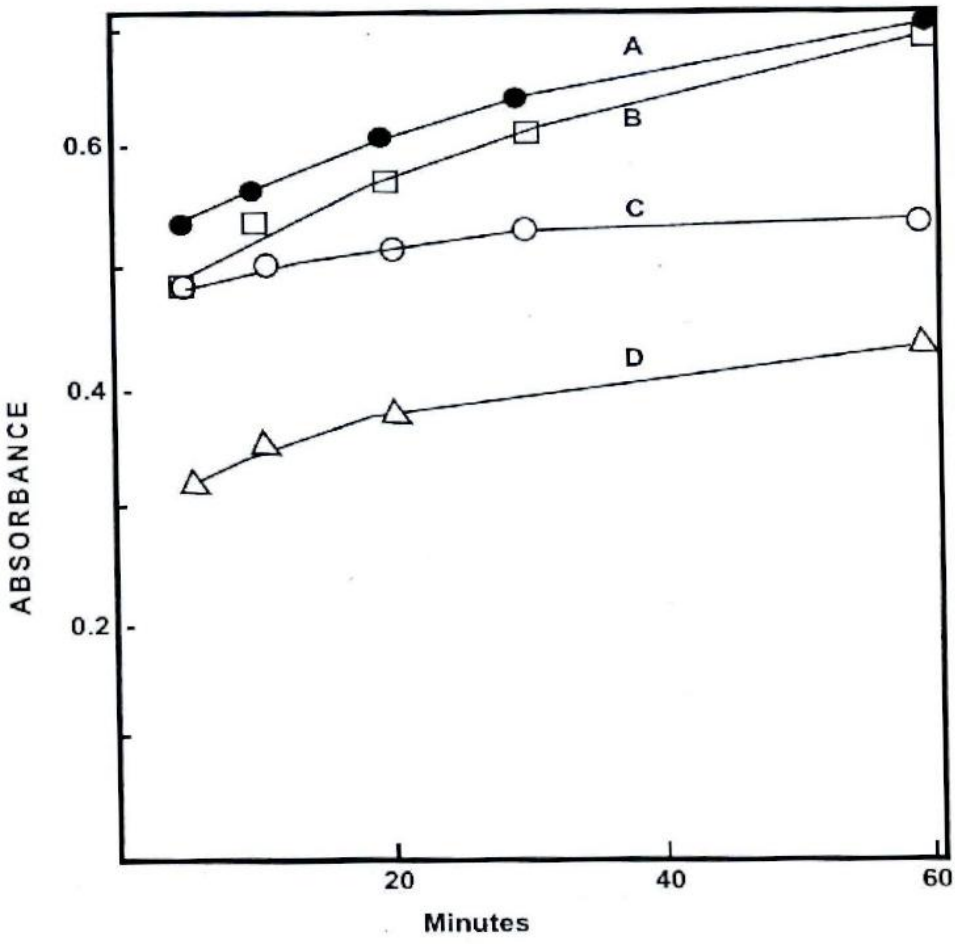


FIG. 1. Progress of the coupling reaction under different conditions. A coupling in 0.2ml saturated sodium acetate; B coupling in 1ml 0.1M sodium hydroxide; C coupling in 1ml saturated sodium acetate and D coupling in 1ml distilled water. Concentration of tetracycline hydrochloride 8.856μg/ml.

Fig 1

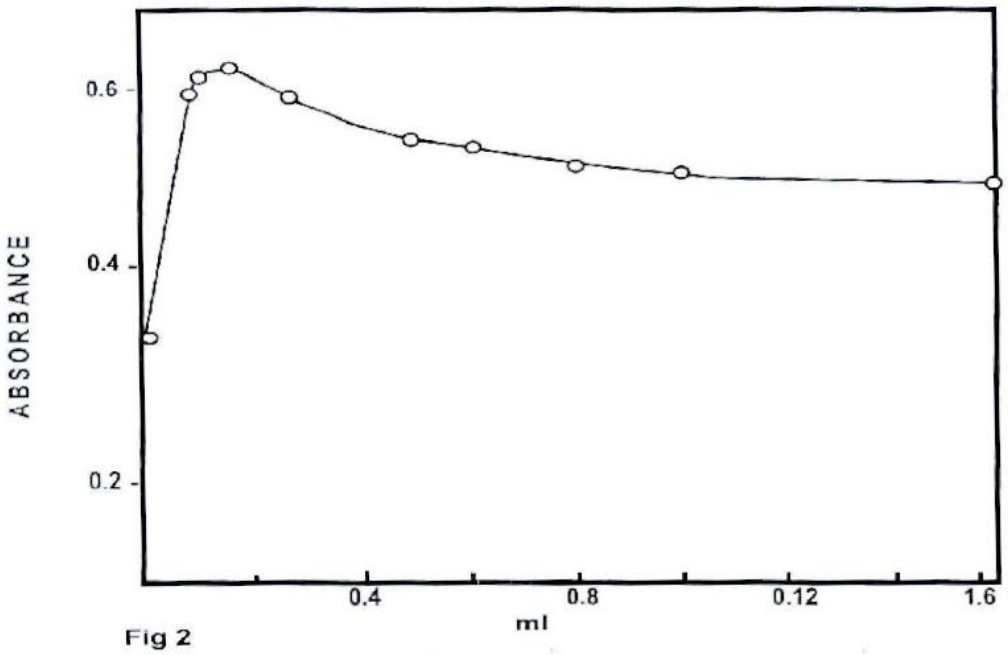


Fig 2

FIG. 2. Absorption of the azo product as a function of the volume of saturated sodium acetate in the coupling medium. Coupling time is 30 minutes in all cases and concentration of tetracycline hydrochloride is 8.856µg/ml.

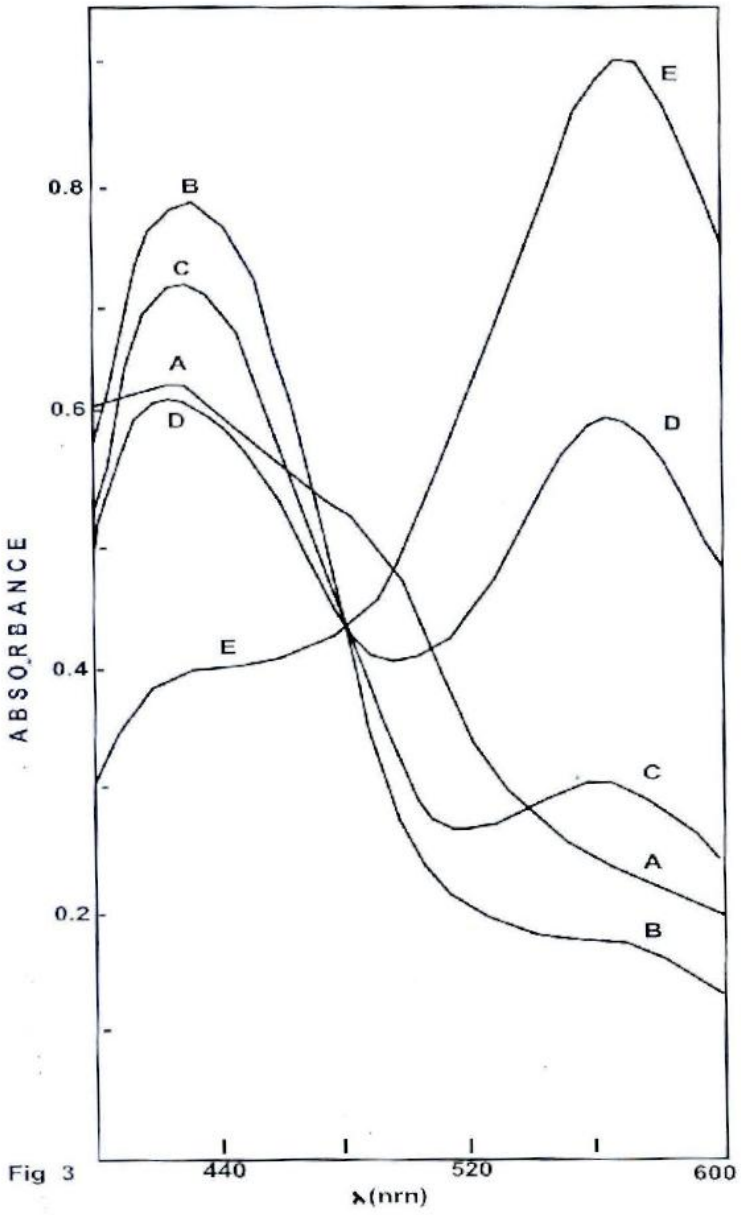


Fig 3

FIG. 3. Absorption of the azo product as a function of the concentration of the diluting sodium hydroxide solution. A 0.05M; B 0.1M; C 0.2M; D 1.0M and E 2.0M. Concentration of tetracycline hydrochloride is 13.60 µg/ml.

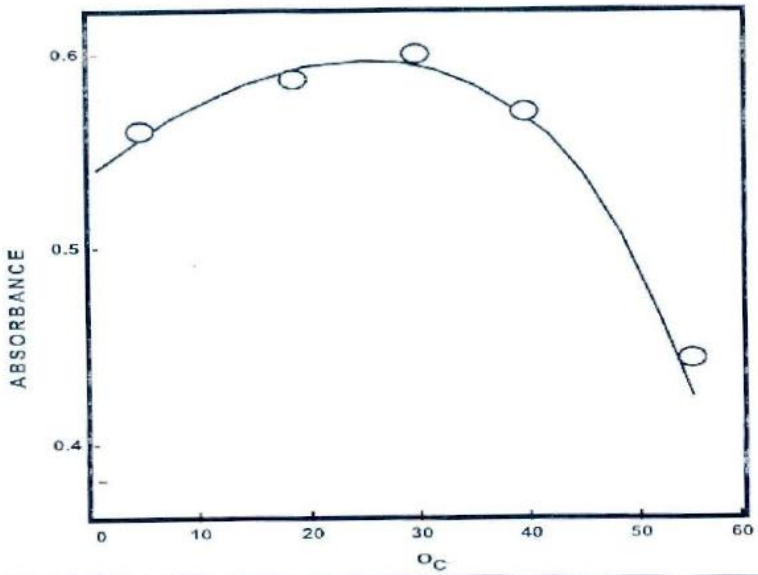


FIG. 4. Effect of temperature on the coupling reaction. Coupling time is 30 minutes and diluting medium is 0.1M NaOH. Concentration of tetracycline hydrochloride is 10.63 µg/ml.

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