ORIGINAL PAPER

Nig. J. Pharm. Res. 2015, 1, pp. 147-153
ISSN 0189-8434

# Development of a visible spectrophotometric method for the assay of methyldopa following oxidative coupling with N-(1-naphthyl) ethylenediamine dihydrochloride

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

## Abstract

**Background**: Methyldopa is a centrally acting hypertensive drug that remains one of the choice treatment options in the management of hypertension in pregnancy and during surgical anaesthesia. Although a number of methods of analysis are available, most employ sophisticated techniques and strict experimental conditions.
**Objective**: To develop and validate a simple visible spectrophotometric analysis for the quantitative determination of methyldopa in bulk and dosage forms.
**Method**: The method was based on the oxidative coupling reaction between methyldopa and N-(1-naphthyl) ethylenediamine (Bratton-Marshall's reagent) to generate a blue chromogen. Reactions variables critical to optimal response were established. Various analytical and validation parameters including repeatability, reproducibility and selectivity were also determined.
**Results**: The calibration graph was linear between 30 and 80 µg/ml at 580 nm with a correlation coefficient of 0.9996. The apparent molar absorptivity was $1.52 \times 10^3$ L $mol^{-1}$ $cm^{-1}$ while the limits of detection and quantification were 9.3 and 31 µg/ml respectively. The method was accurate and precise with recovery in the range of 99.26-101.30 % and intra- and inter-day precision (%RSD) at three different concentrations less than 1.0%. When applied to the analysis of dosage form, there was no statistical difference between the new method and the official method. There was no interference from commonly used excipients.
**Conclusion**: The method is rapid, simple and cost-effective. It can serve as a reliable and affordable assay method for the routine analysis of methyldopa in bulk and dosage forms

**Keywords**: Methyldopa, Oxidative coupling, N-(1-naphthyl) ethylenediamine dihydrochloride

## INTRODUCTION

Methyldopa, chemically (2S)-2-amino-3-(3, 4-dihydroxyphenyl)-2-methylpropanoic acid, is a centrally acting antihypertensive that acts via an active metabolite. Methyldopa is metabolized by the L-aromatic amino acid decarboxylase in adrenergic neurons first to α-methyldopamine and then to α-methylnorepinephrine that is preferentially released instead of the endogenous neurotransmitter norepinephrine (Martindale, 2009).
Although some of its attendant side effects like pseudo tolerance limit its use, methyldopa remains one of the choice treatment options in the management of hypertension in pregnancy and during surgical anaesthesia (Martindale, 2009; Hoffman, 2005).
Several methods have been reported for the analysis of methyldopa in bulk, dosage forms, in combination with other drugs as well as in biological fluids. These methodsinclude liquid chromatography (Emara et al., 2015; Sahithi et al., 2013), titrimetry (Walash et al., 1982) and electroanalytical method (Hussein et al., 2013). A number of spectrophotometric methods have also been reported with many of these based on the readily oxidisable nature of methyldopa and its subsequent coupling with suitable chromogenic substances. The formation of highly coloured adducts have allowed the determination of methyldopa in the visible region and the elimination of the effects of interfering substances in the sample matrix. Some of the agents reported include potassium periodate and para-phenylenediamine for colour enhancement (Al-Da'amy & Al-Moswi, 2014), ferric chloride and nitroso R salt (Al Abachi & Hadi, 2013) potassium periodate and o-tolidine (Al-Da'amy & Al-Moswi, 2013), 2-furoic acid hydrazine (Al-Abachi et al., 2009), molybdate (Ribeiro et al., 2005), hydrogen peroxide

and p-chloranil (Gotardo et al., 2008), ammonium cerium nitrate and 3-methyl-2-benzothiazolinon hydrazine hydrochloride MBTH (Ashour & Alboushi, 2009), ferric ions and 1,10-phenanthroline (Al-Ghabsha et al., 2007) and 2,6-dichloroquinone-4-chlorimide (Gadkariem et al., 2009).

Other reported spectrophotometric methods involve the use of methyldopa as a coupling agent for diazotized 4-amino acetophenone (AbdulSattar, 2014) and use of simultaneous equations (Sawant & Mhaske, 2014).

Many of the methods described however, suffer the drawbacks of requiring strict control of reaction pH using strongly acidic or alkaline medium, narrow linear concentration ranges, use of non-aqueous medium and buffer systems.

In this report, a simple, fast and cost-effective visible spectrophotometric method that is based on the oxidative coupling of methyldopa with N-(1-naphthyl) ethylenediamine dihydrochloride (Bratton- Marshall's reagent) is presented.

## MATERIALS AND METHODS

### Materials and reagents

All solvents, salts and acids used in this study are of analytical grade including 60% v/v ferric chloride solution, N-(1-naphthyl) ethylenediamine dihydrochloride, hydrochloric acid (BDH, UK) and methanol (Sigma-Aldrich, Germany). The two brands of methyldopa tablets were purchased from retail outlets in Ibadan, Nigeria. Distilled water was used for all preparations and dilutions.

### Instrumentation

Analytical balance, precoated aluminum plate, Digital colorimeter (6051 Jenway, UK) equipped with a 1cm matched quartz cell, magnetic stirrer (Gallenkamp), ultraviolet lamp 254/364nm (PW Allen and Co, London), vortex mixer (Griffin and George Ltd), thermostated controlled water bath (Uniscope), test tubes and test tube rack, thermometer, stop watch, UV-Visible spectrophotometer (spectrumlab 752s)

### Preparation of standard solutions

A 4.8 x $10^{-3}$ M solution of the analyte was prepared by dissolving 10mg of anhydrous methyldopa crystals in 10 ml methanol. An equimolar concentration of the coupling agent was prepared by dissolving 0.029g of N-(1-naphthyl) ethylenediamine dihydrochloride in sufficient distilled water to give 25ml final solution. The ferric chloride solution (0.32%w/v) was prepared by diluting 0.13mls of 60%w/v ferric chloride stock solution with sufficient distilled water to produce 25ml final solution.

### Derivatisation reaction

A 0.5ml aliquot of methyldopa stock solution was transferred into a test tube and 0.5 ml ferric chloride solution was added and then vortex mixed for 2 minutes. To the brownish-yellow product, 0.5 ml N-(1-naphthyl) ethylenediamine dihydrochloride solution was added and the mixture was vortex mixed for 10 secs and then incubated at 50℃ for 5 mins. Thereafter, the reaction was terminated by cooling in an ice bath and making the volume up to 5 ml with methanol. The absorbance readings were taken against a reagent blank.

### Spot test

Samples for spot test were prepared by mixing methyldopa stock solution and ferric chloride solution as described. A 0.5 ml aliquot of N-(1-naphthyl) ethylenediamine dihydrochloride solution was then added and the colour of the mixture was noted after incubation for 5 to 20 mins at 30℃. The procedure was repeated with the samples incubated at 70℃ for 5 mins and 20 mins. Each determination was carried out in duplicate.

### Thin layer chromatography

Thin layer chromatography analysis was carried out using precoated aluminum TLC plate. Samples of methyldopa, N-(1-naphthyl) ethylenediamine dihydrochloride and the adduct were spotted and the plates developed using ethylacetate:methanol (5:5) v/v, butanol:glacial acetic acid:water (5:2.5:2.5) v/v and ethanol:ammonia:water (3:2:1.5) v/v.

### Optimization of reaction conditions

The analytical wavelength was selected by the inspection of the overlaid spectra of the drug, ferric chloride, N-(1-naphthyl) ethylenediamine dihydrochloride and the coupling product.

For the development of maximum colour intensity, the time and concentration of reagents required for oxidation, as well as oxidative coupling temperature were optimized.

The effect of the volume of ferric chloride needed for partial oxidation of methyldopa was studied by adding 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0ml of ferric chloride solution to different test tubes each containing 0.5mls of the drug solution and vortex mixed for 2 mins. A 0.5 ml aliquot of N-(1-naphthyl) ethylenediamine dihydrochloride solution was then added to each test tube and the reaction allowed to proceed for 5 minutes at $50^0$C after which the reaction was stopped by placing in an ice bath and then made up to 5 ml volume with methanol. The reaction was carried out in duplicate and the absorbance values determined at 580 nm against a reagent blank.

The effect of acid concentration on the oxidation process was studied by varying the concentration of hydrochloric used in preparing ferric chloride from 0-1 M.

The optimization of the coupling temperature and time was carried out by the method of steepest ascent (Karnes & March, 1993). This was carried out at temperatures of 30, 50, 60, 70 and 80 °C after incubation times of 5 and 10 mins.

Into ten test tubes each containing 0.5 mL of methyldopa stock solution, 0.5 ml of the ferric chloride solution was added and the mixture vortex mixed for 2 mins. A 0.5 ml aliquot of N-(1-naphthyl) ethylenediamine dihydrochloride reagent was then added and the mixture vortex mixed for 10 secs. At the end of the various reaction times and temperatures, the reaction was stopped by cooling in ice and dilution to 5 mL with methanol. The absorbance reading of each of the mixtures was taken at 580 nm against a reagent blank. Optimization of the time required for coupling to take place at the selected temperature (50 °C) was done at 0, 2, 5, 10, 20 and 30 minutes. The reaction was stopped and made up to 5 mL at these times with methanol. The absorbance readings were taken at 580 nm against a reagent blank. All of these determinations were done in duplicate.

Stoichiometric ratio determination using Job's method of continuous variation (Rose, 1964)

Aliquots of 0.5mls ferric chloride solution was added to different test tubes containing varying amounts of the drug stock solution 0, 0.25, 0.33, 0.5, 0.67, 0.75 and 1.0ml. Each test tube was vortex mixed for 2 mins and its volume made up to 1.5 ml with the coupling agent. The mixture was vortex mixed for about 10 seconds and the reaction maintained at $50^0$C for 5 minutes. The reaction was then terminated by rapid cooling in an ice bath and the volume made up to 5 ml with methanol. The absorbance values were taken at 580 nm against a reagent blank. Each determination was carried out in duplicates.

**Validation**

The calibration line was generated for oxidised methyldopa reacted with the coupling agent in the range of 30-80 µg/ml in methanol.

The validity of the proposed method for the assay of methyldopa was examined by determining the model recoveries and repeatability of the method on three successive days as stipulated by the USP (USP, 1999). The intra- and inter-day accuracy and precision of the proposed method were assessed from the results of replicate analyses on the pure drug solution. The mean values and relative standard deviation values for replicate analysis at three different concentration levels were calculated. The limit of detection (LOD) and limit of quantification (LOQ) were also determined.

**Method select**ivity

The selectivity of the proposed method was studied by determining the recovery of known amounts (equivalent to 55 µg/ml) of the drug from sample matrices that contain commonly used tablet excipients including starch, talc, magnesium stearate, lactose, gelatin and a mixture of all.

**Application to assay of dosage form**

The proposed method was employed on two different brands of methyldopa available in the market. An accurately weighed amount of the powdered tablets that is equivalent to 10 mg of methyldopa was dissolved in 10 ml methanol and then filtered. To 275µl of the filterate, equivalent to 55 µg/ml of the drug, 0.5 ml of ferric chloride solution was added and vortex mixed for 2 mins. Thereafter, 0.5 ml of N-(1-naphthyl) ethylenediamine dihydrochloride was added and the mixture vortex mixed for another 10 secs before incubating at $50^0$C for 5 mins. The reaction was then stopped by cooling in an ice bath and the volume made up to 5 ml with methanol. The absorbance reading was taken at 580 nm against a reagent blank.

The official method of analysis was also carried out on the dosage forms (B.P. 2009). Six replicate determinations were carried out for each brand using both methods.

**RESULTS AND DISCUSSION**

Evidence of coupling

Oxidative coupling yielded a blue adduct at both 5 mins and 20 mins incubation time. The adduct was stable at elevated temperatures of $70^{\circ}C$ and for up to 2 hours when monitored by absorbance values. The TLC analysis, in the three mobile phases, revealed the formation of a single-component adduct with $R_f$ values that were distinct from those of methyldopa and the coupling agent Table 1.

**Table 1**: **The Rf values for the TLC analysis**

| Mobile phase | Methyldopa | Bratton-Marshall's reagent | adduct |
|---|---|---|---|
| Ethylacetate:methanol (5:5) | 0.13 | 0.2 | 0.30 |
| Butanol:glacial acetic acid:water (5:2.5:2.5) | 0.53 | 0.60 | 0.70 |
| Ethanol:ammonia:water (3:2:1.5) | 0.83 | 0.79 | 0.56 |

**Selection of analytical wavelength**

The overlaid spectra of methyldopa, ferric chloride, N-(1-naphthyl) ethylenediamine dihydrochloride and the adduct is shown in Fig. 1

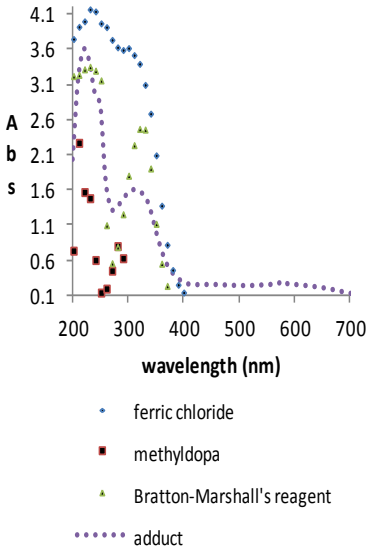


**Figure 1: Overlaid spectra of methyldopa, Bratton-Marshall's reagent, ferric chloride and adduct**

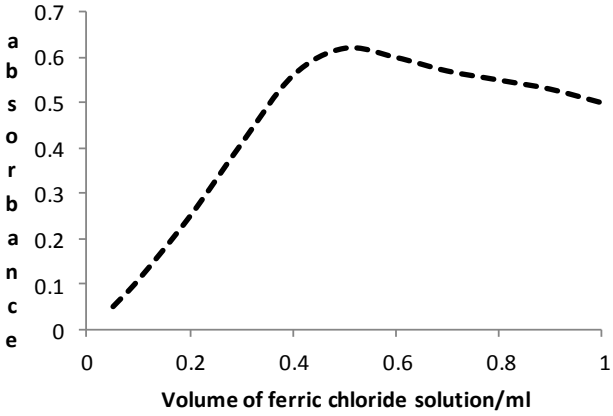


**Figure 2: Optimization of volume of ferric chloride solution**

**Selection of coupling temperature and time**

For both incubation times of 5 mins and 20 mins, the absorbance gradually increased with temperature and then plateaued at 50 ℃ as shown in Fig.3. The optimum coupling temperature and time was therefore established at 50 ℃ and 5 mins respectively as shown in Fig. 4.

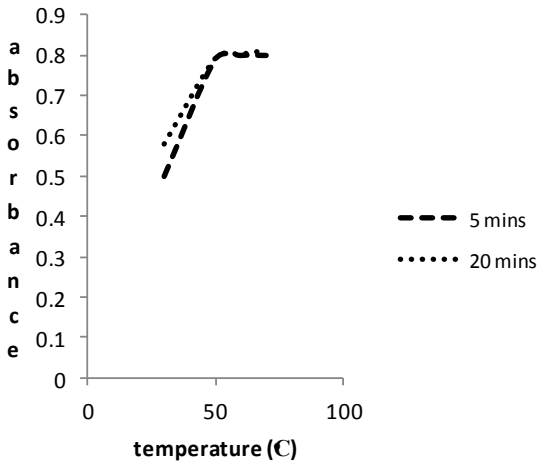


**Figure 3: Optimization of coupling temperature**

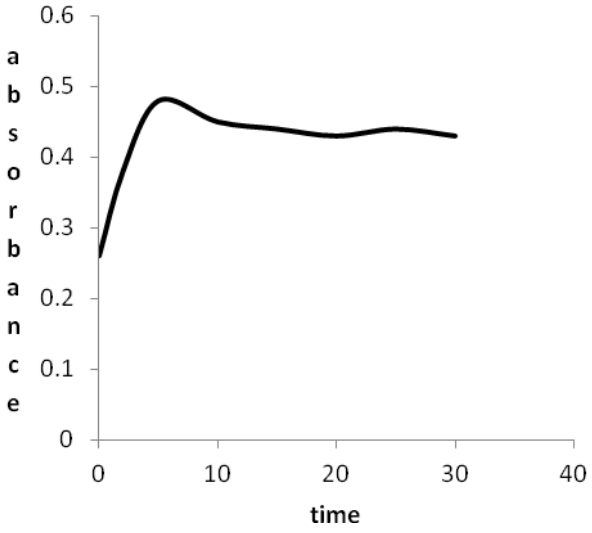


**Figure 4: Optimization of coupling time**

**Stoichiometric ratio determination and reaction mechanism**

Optimal response was observed when the oxidised methyldopa combined with Bratton-Marshall's reagent in 1:1 ratio as shown in Fig. 5.

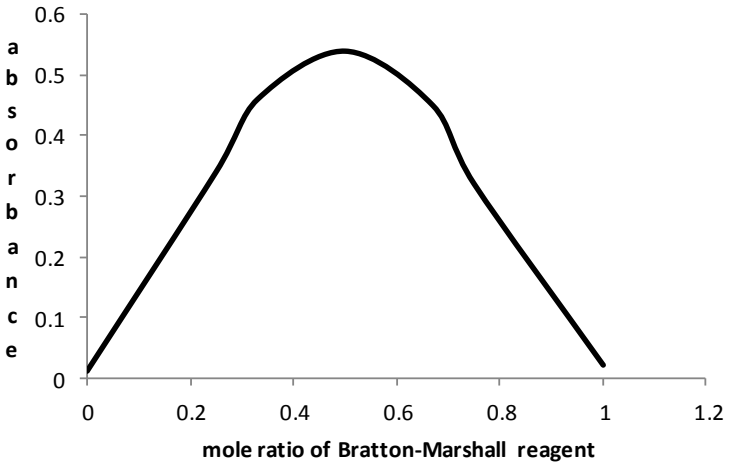


**Figure 5: Stoichiometric ratio of methyldopa and Bratton-Marshall'sreagent**

This further supported the TLC analysis that showed the formation of a single-component adduct. Ferric chloride partially oxidised one of the OH groups of methyldopa by dehydrogenation followed by one electron loss to give a radical. The single electron on the oxygen can delocalise within the ring and since OH groups are ortho-para directing, an electron deficient centre could be created on the carbon para to the oxidised OH group. The excess electron on the primary aliphatic amine of N-(1-naphthyl)ethylenediamine serving as a nucleophile attacked this electron deficient centre to form the adduct. The dye product has a probable structure as shown in Fig. 6

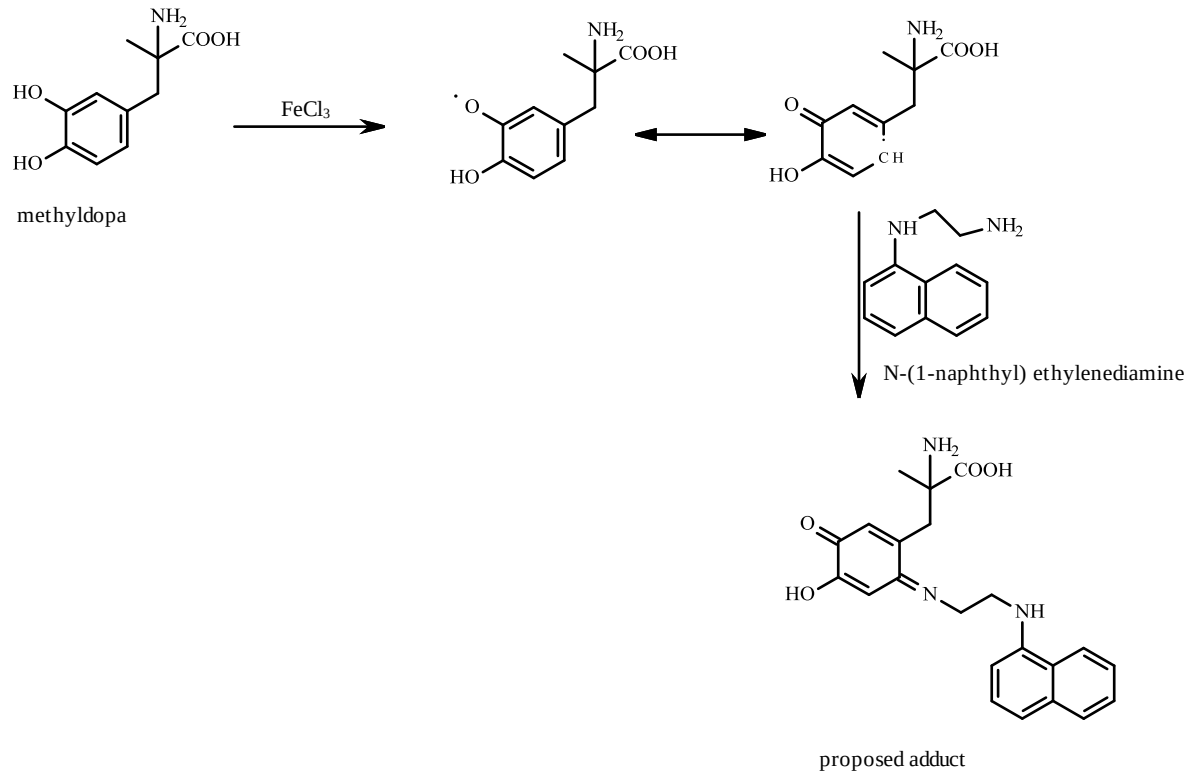


**Figure 6: Proposed coupling mechanism between oxidised methyldopa and N-(1-naphthyl) ethylenediamine**

**Validation of the proposed method**

A linear relationship was observed between absorbance at 580 nm and concentration of methyldopa in the range of 30-80 µg/ml. The limit of detection (LOD) and limit of quantification (LOQ) were determined as 3.3σ/S and 10σ/S respectively where, σ is the standard deviation of the absorbance of blank (n = 6) and S is the slope of the calibration line (ICH, 2011).The various analytical parameters are presented in table 2.

**Table 2**: **Analytical and validation parameters for proposed method**

| Performance Parameter | Value |
|---|---|
| Beer's Law Limit (µg/ml) | 30- 80 |
| Limit of detection (µg/ml) | 9.3 |
| Limit of quantification (µg/ml) | 31 |
| Molar absorptivity (L $mol^{-1}$ $cm^{-1}$) | $1.52x10^{3}$ |
| Slope ± SD | $0.0064±3.8x10^{-4}$ |
| Intercept ± SD | 0.0359±0.006 |
| Correlation coefficient | 0.9996 |
| Coefficient of determination | 0.9993 |

The intra- and inter day results are shown in table 3. The intra-day results showed that the new method having a percentage recovery of 99.26-101.25 % and a maximum percentage relative error of 1.17 % was accurate. The method also showed good repeatability as the percentage RSD did not exceed 0.2 %.

For the inter-day results, the percentage recovery and percentage relative error of the new method were 100.4-101.3 % and 1.3% respectively indicating high accuracy. Similarly, the percentage relative standard deviation was less than 1.0 % indicating good reproducibility.

**Table 3: Accuracy and precision of new method**

| | Intra-day (n=4) | | | Inter-day (n=12) | | |
|---|---|---|---|---|---|---|
| Amount added (µg/ml) | Mean recovery (%) | % RSD | % Relative error | Mean recovery (%) | %RSD | % Relative error |
| 30 | 100.57±0.01 | 0.03 | 0.56 | 100.40±0.01 | 0.03 | 0.52 |
| 50 | 99.26±0.20 | 0.2 | 0.78 | 101.11±0.02 | 0.04 | 1.24 |
| 70 | 101.25±0.01 | 0.01 | 1.17 | 101.30±0.02 | 0.03 | 1.30 |

**Accuracy and precision**

**Method selectivity**

The selectivity of the proposed method to quantify methyldopa in the presence of commonly employed pharmaceutical aids was established. The results showed that the method has a recovery between 95.0 to 104.1%

**Application to dosage form analysis**

The proposed method was applied to two brands of methyldopa with label claims of 250 mg active ingredient per tablet. The results obtained were statistically compared with those obtained using the official method (B.P. 2009) as shown in table 4. The F test was used to estimate the difference in variance between the two methods, while student's t test was used to compare the mean recovery, with 95% confidence intervals.

**Table 4: Comparative dosage form analysis using the new method and official method**

| | New Method | | Official method | | Statistics | | |
|---|---|---|---|---|---|---|---|
| Drug Formulation (0.25g strength) | Amount found (g) | % RSD | Amount found (g) | % RSD | *Mean recovery (%) | F test | t test |
| Brand 1 | 0.29 | 4.33 | 0.29 | 2.31 | 100.01 | 0.41 | 0.99 |
| Brand 2 | 0.28 | 1.46 | 0.28 | 1.24 | 101.30 | 0.73 | 0.1 |

**ratio of the results obtained with the new method to that of the official method*

No significant differences exist between the accuracy and precision of the two methods. The proposed method can therefore serve as a reliable alternative in the routine analysis of methyldopa in bulk and dosage forms.

The prescribed method was simple and affordable and did not require sophisticated and complex procedures. The test was also rapid and did not involve the use of buffers or extensive extraction procedures.

## CONCLUSION

A novel visible spectrophotometric method for the analysis of methyldopa has been developed and validated. The method can serve as a reliable and affordable assay method for the routine analysis of methyldopa in bulk and dosage forms.

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Conflict of Interest: None declared
Received: 30 April, 2015
Accepted: 4 July, 2015