**Spectrophotometric assay methods for Amiloride: A charge-transfer reaction approach with DDQ and p-Chloranilic acid reagents**

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**Spectrophotometric assay for Amiloride drug by using charge-transfer reactions with DDQ and p-Chloranilic acid as analytical reagents**

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**ABSTRACT**

The present study accounts for two simple, sensitive, and validated spectrophotometric methods for the determination of Amiloride hydrochloride (AMD) in pure and formulated forms. The methods are mainly relay on the formation of charge transfer (CT) complex, with the reagents 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (Method A) and p-Chloranilic acid (PCA) (Method B), hence a stable and 1:1 stoichiometric complex were obtained, which are yellow and pink color respectively. The produced chromogens optical densities were measured at 476 and 508 nm respectively. The Beer’s law was tested in the range of 5-30 and 1-6 µg mL-1. The optical characteristics such as molar absorptivity (*ɛ*), Sandell’s sensitivity values were calculated. The other analytical parameters like, Limit of Detection (LOD), Limit of Quantification (LOQ), accuracy and precisions were evaluated. The proposed methods were successfully applied for the determination of the AMD in tablet formulation with high accuracy, better recoveries and permissible relative standard deviation.

**Keywords:** Amiloride hydrochloride, Charge transfer complex, DDQ, p-Chloranilic acid, Spectrophotometry, Validation

**INTRODUCTION**

Amiloride chemically, 3,5-diamino-6-chloro-N-(diaminomethylene) pyrazine-2-carboxamide (Fig 1) which works by directly blocking the epithelial sodium channel there by inhibiting sodium reabsorption in the distal convoluted tubules and collecting ducts in the kidneys [1]. This promotes the loss of sodium and water from the body, but without depleting potassium. The drug is often used in conjunction with Thiazide (e.g. co-amilozide) or loop diuretics (e.g. co-amilofruse). Due to its potassium-sparing capacities, hyperkalemia (high blood potassium levels) are occasionally observed in patients taking Amiloride.

It is used in the management of hypertension and congestive heart failure. It is useful for the prevention of hypokalemia induced by hydrochlorothiazide especially during prolonged treatment. **A**miloride is available in the market as combination drug with furosemide such as Amifru tab, Amimide, Exna-k tab. It is also available in combination with Atenelol & Hydrochlorothiazide namely Beta-Biduret cap, BP-Loride tab, Hipres-D cap.

The inclusive literature survey reveals that there are various methods available for the determination of amiloride, such as High Performance Liquid Chromatography (HPLC) [1-6], High Performance Thin Layer Chromatography [7], UV-spectrophotometric methods in which derivative methods have been illustrated [8-13], some of the method uses the chemometrics in conjunction with UV-spectrophotometer[14-16], also has a method uses stopped flow analysis in conjunction with UV [17], a method with Capillary Zone Electrophoresis [18], a micro sensor potentiometric method [19], a method uses spectrofluorimeter [20] to measure the AMD in formulations and biological samples. However, these methods are involved with sophistication, skills, extraction, and more expensive than proposed methods. Additionally the methods have been strictly followed some of the well known guidelines and methods [21-24]. In the proposed methods, we have used spectrophotometer, which is a method of choice still in many pharmaceutical industries for the routine analysis in developing countries. It serves to be one of the quickest, promising and most reliable techniques. The aim of the present study is to develop and validate new simple, sensitive, rapid, reliable and precise spectrophotometric methods for the analysis of AMD in its dosage forms.



Fig 1: Amiloride structure

**EXPERIMENTAL**

**Apparatus**

All spectrophotometric measurements were carried out using a dual beam T90+ UV-Spectrophotometer (PG Instruments Ltd. USA) with quartz cells of 1cm thickness. Officially, calibrated Pyrex glassware was used throughout this study.

**Materials**

The Amiloride hydrochloride pure (99.5%) was supplied by Shreeji Pharma International Pvt. Ltd. Gujarat, India. as a gift sample which is used as reference standard for the analysis, (10 mg of reference standard in 10 mL methanol the apparent concentration is 1000 µg mL-1) the lower concentrations of this solution are prepared by appropriate dilutions with methanol (100 and 50 µg mL-1). Biduret® tablets (Biduret® -5) which has been prepared and marketed by Glaxo Smithkline Pharmaceuticals Ltd. were purchased from the medical store, which are used as test samples. Analytical Reagent grade DDQ from Sigma Aldrich chemical (0.1% in methanol), Analytical Reagent grade PC from Sigma Aldrich chemicals (0.1% in acetonitrile), Analytical Reagent grade solvents methanol and acetonitrile were used, double distilled water was used throughout the experiment.

**General analytical procedure:**

***Method A***

To a set of 10 mL volumetric flasks, variable aliquots of working standard solution of AMD (100 µg mL-1) ranging from 0.5-3.0 mL were transferred, 1 mL of (0.1%) DDQ was added swirled mechanically to mix the solutions, heated the solution on a water bath up to 60ºC for 5 min. there was formation of orange yellow colored complex, left the solution for 10 min to develop color at room temperature 28±3ºC. The total volume brought to 10 mL with methanol. The absorbance of the colored species was then measured at 476 nm against blank solution. A calibration graph was drawn by plotting absorbance versus concentration of drug (µg mL-1), the regression equation was obtained. From this equation the sample concentrations were calculated.

***Method B***

To a set of 10 ml volumetric flaks, variable aliquots of working standard solution of AMD (50 µg mL-1) ranging from 0.2-1.2 mL were transferred, 1 mL of (0.1%) p-chloranilic acid solution was added and mixed thoroughly, there was a formation of pink colored complex, the flask were left for 10 min. to complete the color development at room temperature 28±3ºC. The total volume brought to 10 mL with acetonitrile. The absorbance of the colored species was then measured at 508 nm against blank solution. A calibration graph was drawn by plotting absorbance versus concentration of drug (µg mL-1), the regression equation was obtained. From this equation the sample concentrations were calculated.

***Assay procedure for samples***

Tablet formulations were studied for their content of AMD. 10 tablets [Biduret-5] were taken, the average weight of each tablet was 237 mg and triturated to a fine powder. A portion of this powder equivalent to 100 mg of AMD was transferred to a 100 mL volumetric flask and extracted with 15 mL water by rigorous mechanical shaking, filtered through Whatmann filter paper no.41 in to a 100 mL volumetric flask and the solution is made up to the mark with methanol (Method A) and acetonitrile (Method B). The apparent concentration of the solution was 1000 µg mL-1; the lower concentrations of this solution were prepared by step wise dilutions with methanol/acetonitrile to suit the concentrations of reference standard solutions of both methods. The amount of AMD present in the tablets were calculated by following the general procedure, the concentration was calculated by regression equations.

***Determination of molar ratios***

The Job’s continuous variation method has been employed to establish the molar ratio between the AMD and the reagents in the two methods. A equimolar concentrations of 2X10-3 M solution of AMD and the reagents has been tested. A series of solutions have been prepared in the ratios of [0:10, 1:9, 2:8…10:0], the corresponding absorbances were measured. Absorbance values versus the mole ratio in each reagent used in the two methods were plotted.

**RESULT AND DISCUSSION**

***Strategy for assay development***

The selected drug has a therapeutic importance in antihypertensive activity, in order to cater the demands; we have taken this study for the development of simple and accurate assay procedures in the pure and pharmaceutical dosage forms. The drug has a higher tendency to donate its non-bonding pair of electrons which induces the charge transfer reaction between the π-acceptor reagent DDQ and the AMD; this forms the basis for the development of the method A.

The method A was mainly based on the interaction of AMD and DDQ, this leads to the formation of orange yellow color charge transfer complex. The possible reaction pathway for the formation of the complex was shown in (**Fig. 2**). The complex formed shown an absorption maximum at 476 nm (**Fig. 3**). The color intensities were increased with increase in concentrations of AMD, which forms the basis for the analysis.



**Fig. 2:** Proposed pathway for the formation of coloured complex for method A

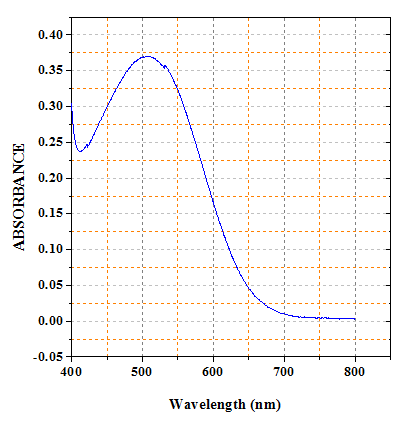
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**Fig. 3:** Absorption maximum for method A

The method B was based on the charge transfer reaction between the drug and the reagent p-chloranilic acid a stable pink colored complex was obtained (**Fig. 4**). The complex formed has a maximum absorption at 508 nm (**Fig. 5**). The color intensities were increased with increase in concentrations of AMD, which forms the basis for the analysis.

The following sections describe the establishment of the optimization factors that influence the chemical reaction and the analytical performance of the proposed assays.



**Fig. 4:** Proposed pathway for the formation of colored complex for method B

**Fig. 5:** Absorption maximum for method B

***Optimization studies for the method A***

To obtain a rugged method, we have optimized all the necessary parameters based on their optical characteristic absorptions, the concentration of DDQ solution, the volume of DDQ solution and the reaction time for the colored species generation. The temperature dependencies were also determined, and shown in (**Table 1**). From the above findings, we have optimized all the relevant parameters to obtain reproducible results.

**Table 1:** Optimum conditions for the formation of colored complex

|  |  |  |
| --- | --- | --- |
| Condition | Studied range | Optimum |
| DDQ conc. (%) | 0.02-1.0 | 0.1 |
| Vol. of 0.1% DDQ (ml) | 0.5-2.5 | 2.0 |
| Reaction time (min) | 0-30 | 10 |
| Temperature (ºC) | 28-75 | 60 |
| λmax (nm) | 400-600 | 476 |

***Optimization studies for the method B***

To establish all the optimum parameters for a rugged method we have optimized all the necessary parameters based on their optical characteristic absorptions, the concentration of p-chloranilic acid solution, Volume of p-chloranilic acid solution and the reaction time for the colored species generation. The temperature dependencies are also determined in the (**Table 2**).

|  |  |  |
| --- | --- | --- |
| Condition | Studied range | Optimum |
| PCA conc. (%) | 0.02-1.0 | 0.1 |
| Vol. of 0.1% PCA (ml) | 0.5-2.5 | 1.0 |
| Reaction time (min) | 0-30 | 10 |
| Temperature (ºC) | 28-75 | 60 |
| λmax (nm) | 400-600 | 508 |

**Table 2:** Optimum conditions for the formation of colored complex

***Molar ratio and mechanism of the reaction***

Job’s method of continuous variation was used for determining the molar ratio of AMD to DDQ. From the obtained Job’s plots, it was concluded that the AMD:DDQ and AMD:PCA ratios were 1:1.

This indicated that one mole for each AMD interacted with one mole of DDQand PCA was confirmed (**Fig. 6 & 7**).

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**Fig. 6:** Stoichiometric ratio for the complex formation in method A

**Fig. 7:** Stoichiometric ratio for the complex formation in method B

**Validation of the proposed assay method A**

***Calibration, range, and sensitivity***

Under the above mentioned optimum reaction conditions, the calibration curve for the analysis of AMD by the proposed assay was constructed by plotting the absorbances as a function of the corresponding AMD concentrations. The regression equation for the results was derived using the least-squares method, Beer’s law plot (11-points) was linear in the range of 5.0–30.0 μg mL-1.

The regression equation was: Y = 0.015x + 0.1334 (r = 0.9998); where, ‘Y’, ‘x’ and ‘r’ are the absorbance, concentration of AMD and correlation coefficient, respectively

(**Fig. 8**).

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**Fig. 8:** Beer’s Law calibration curve for method A

**Validation of the proposed assay method B**

***Calibration, range, and sensitivity***

Under the above mentioned optimum reaction conditions, the calibration curve for the analysis of AMD by the proposed assay was constructed by plotting the absorbances as a function of the corresponding AMD concentrations. The regression equation for the results was derived using the least-squares method Beer’s law plot (11-points) was linear in the range of 1.0–6.0 μg/ml.

The regression equation was: Y= 0.6675x+0.0158 (r = 0.99987); where, ‘Y’, ‘x’ and ‘r’ being the absorbance, concentration of AMD and correlation coefficient, respectively (**Fig. 9**).

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**Fig. 9:** Beer’s Law calibration curve for method B

The limits of detection (LOD) and quantification (LOQ) were calculated according to International Conference on Harmonization (ICH) guidelines using the formulas:

LOD = 3.3 *S/b* and LOQ = 10 *S/b*, where ‘*S’* is the standard deviation of blank absorbance values (*n* = 6) and *b* is the slope of the calibration line. The LOD and LOQ were found to be 0.7660 and 2.553 μg mL-1, respectively. The quantitative parameters of the proposed assay are given in (**Table 3**).

**Table 3:** Analytical parameters for the methods A and B

|  |  |  |
| --- | --- | --- |
| Parameters | Method A | Method B |
| λmax  (nm) | 476 | 508 |
| Linearity range (µg mL-1) | 5.0-30.0 | 1.0-6.0 |
| Apparent molar  absorptivity (L Mol-1 cm-1) | 0.331x104 | 0.204 x 104 |
|  |  | Contd… |
| Sandell’s sensitivity  (ng cm-2 0.001 abs units) | 6.9592 | 11.2500 |
| Linear regression | Y=0.03175x+0.19604 | Y= 0.6675x+0.0158 |
| Slope (b) | 0.03175 | 0.6675 |
| Intercept (a) | 0.19604 | 0.0158 |
| Correlation coefficient (r) | 0.99980 | 0.99987 |
| % RSD | 0.160 | 0.118 |
| % Range of errors: |  |  |
| a). 0.05 level | 0.4487±0.686x10-3 | 0.2472±0.372x10-3 |
| b). 0.01 level | 0.4487±0.0121x10-3 | 0.2472±0.653x10-3 |
| LOD (µg mL-1) | 0.7660 | 0.0140 |
| LOQ (µg mL-1) | 2.553 | 0.0470 |

***Accuracy and precision***

Accuracy and precision were established by recovery studies of standard solution at different concentrations.

***For (method A):*** Five replicates determination of the AMD in pure form at three different concentrations (15, 17.5 and 20 µg mL-1) by short term (intra-day) precisions as shown in (**Table 4)**. The standard analytical errors, relative standard deviations (%RSD) and recoveries obtained in the intra-day analysis of the proposed method were found to be acceptable. Thus the proposed method is effective in the determination of AMD.

**Table 4:** Evaluation of the accuracy and precision of the proposed method (method A) by intra-day assay observed concentration of AMD (μg mL-1)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Observed concentration of AMD (μg mL-1) | | | | |
| Concentration  of AMD (μg mL-1) | **Intra-day** | | | |
| **Mean\*** | **Error (%)** | **RSD (%)** | **Recovery (%)** |
| 15 | 15.24 | 15.2±0.0032 | 0.87 | 101.16 |
| 17.5 | 17.64 | 17.6±0.0034 | 1.09 | 100.8 |
| 20 | 19.84 | 19.8±0.0038 | 0.87 | 99.2 |

\* For five determinations.

The accuracy of the proposed method (Method A) was further checked by performing recovery experiments added to the pre-analyzed dosage forms and then determined by the recommended procedure. The results are as shown in (**Table 5**). The mean recoveries were calculated and found to be 99.4−100.4% and the relative standard deviations (%RSD) were in the range of 0.084-0.231%. This range is acceptable according to the guidelines for the validation of analytical methods. This indicates the reproducibility of the method.

No interference was observed from the common excipients of tablet, through standard addition technique. For this purpose, a known amount of pure AMD was added.

**Table 5:** Determination of AMD in pharmaceutical formulation by standard addition technique (method A)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Amount of  drug before  addition (μg) | Amount of  drug added (μg) | Theoretical  Amount  (μg) | The mean  Amount  recovered (μg) | Mean % of  Recovery  (n=5) | RSD% |
| 4 | 4 | 8 | 7.96 | 99.7 | 0.23 |
| 8 | 4 | 12 | 12.1 | 100.4 | 0.15 |
| 10 | 4 | 14 | 13.9 | 99.4 | 0.08 |

***For (method B):***Five replicate determination of AMD in pure form at three different concentrations (1.5, 2.5 and 5.0 μg mL-1) by short term (intra-day) precisions as shown in (**Table 6**). The standard analytical errors, relative standard deviations (%RSD) and recoveries obtained in the intra-day analysis of the proposed method were found to be acceptable. Thus the proposed method is effective in the determination of AMD.

The accuracy of the proposed method (Method B) was further checked by performing recovery experiments through standard addition technique. For this purpose, a known amount of pure AMD was added to the pre-analyzed dosage forms and then determined by the recommended procedure. The results are as shown in (**Table 7)**. The mean recoveries were calculated and found to be 98.9−101.3% and the relative standard deviations (%RSD) were in the range of 0.11-0.14%. This range is acceptable according to the guidelines for the validation of analytical methods. This indicates the reproducibility of the method. No interference was observed from the common excipients of tablet.

**Table 6:** Evaluation of the accuracy and precision of the proposed method (method B) by intra-day assay observed concentration of AMD (μg mL-1)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Observed concentration of AMD (μg mL-1) | | | | |
| Concentration  of AMD (μg mL-1) | **Intra-day** | | | |
| **Mean\*** | **Error (%)** | **RSD (%)** | **Recovery (%)** |
| 1.5 | 1.514 | 1.514±0.0032 | 0.87 | 101 |
| 2.5 | 2.502 | 2.502±0.0034 | 1.09 | 100.1 |
| 5.0 | 4.985 | 4.985±0.0038 | 0.87 | 99.7 |

\* For five determinations.

**Table 7:** Determination of AMD in pharmaceutical formulation by standard addition technique (method B)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Amount of  drug before  addition (μg) | Amount of  drug added (μg) | Theoretical  Amount  (μg) | The mean  Amount  recovered (μg) | Mean % of  Recovery  (n=5) | RSD% |
| 2 | 2 | 4 | 3.96 | 99.0 | 0.23 |
| 2 | 3 | 5 | 5.01 | 100.2 | 0.15 |
| 2 | 4 | 6 | 5.95 | 99.17 | 0.08 |

***Robustness and ruggedness of method A and B***

Robustness of each method has been examined by evaluating the influence of small variation in the each method variable on its analytical performance. In these experiments, one parameter was changed, whereas the others were kept unaltered, and the recovery percentages were calculated each time. It was found that small variations in the method variables did not significantly affect the procedures; recovery values were 98.4–101.1%. This indicated that, the reliability of the proposed methodologies during their routine application, for the analysis of AMD in quality control laboratories.

Ruggedness was also tested by applying the proposed methodology to the assay of AMD using the same operational conditions, but using two different instruments at two different laboratories and different elapsed time. Results obtained from lab-to-lab and day-to-day variations were reproducible, as the relative standard deviations (RSD) did not exceed 2%.

**Application of the proposed assay**

The proposed method applied to the analysis of the AMD in pharmaceutical dosage forms [Biduret®5 tablet formulations] and the results were statistically compared with reference method by calculating the student’s t-values. The evaluated t-values were less than the tabulated values at the 95% confidence level for five degrees of freedom, as revealed by the results complied in (**Table 8**). This suggests that the proposed method is accurate and precise as the reference method [5].

On the t- and F-tests, no significant differences were found between the calculated and theoretical values of both the proposed and the reported assays at 95% confidence level. This indicated a similar accuracy and precision in the analysis by the proposed and reported methods.

**Table 8:** Assay of AMD in the formulations by the two methods and the recovery

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | % Content±SDa | | | | |
| Method | **Formulation** | **Label claim**  **(mg)** | **Obtained**  **(mg)** | **%Recovery** | **Reported method\*** | **t-valueb** | **F-valueb** |
| A | Biduret®5 | 5 | 4.97 | 99.4±0.0006 | 95.35±0.455 | 1.104 | 1.327 |
| B | 5.05 | 101±0.0010 | 1.102 | 3.835 |

aMean values of five determinations±SD.

bThe tabulated values at 95% confidence limit are 2.78 and 6.39, respectively.

\*Reference method [5]

**CONCLUSIONS**

The present study describes the development and validation of two simple, sensitive, accurate and cost effective assay methods for the charge transfer complex spectrophotometric determination of AMD in pure and formulated forms. The assays were fully validated according to the guidelines for validation of analytical procedures and the results were satisfactory.

The applicability of the proposed methodologies for the quality control of AMD was confirmed as indicated by the acceptable recovery values. The assays described herein, have the following advantages:

* Unlike the LC/MS procedure and HPLC procedures, the UV-visible spectrophotometer instrument is simple and highly inexpensive; on the other hand, the superiority lies in simplicity and user friendliness. The method could be considered, better in comparison with the previously reported methods. Moreover the method is free from interferences by common additives and excipients.
* Reducing the consumption of organic solvents in the charge transfer based spectrophotometric analysis of AMD, accordingly limiting the exposures of the analysts to the toxic effects of organic solvents.
* Reduction in the analysis cost by 50-folds, which can be reflected in the price for the finished dosage forms, thus it can reduce the expenses for the medications to the patients.

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**CONFLICT OF INTEREST**

The authors declare that the content of this article has no conflict of interest.

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