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

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**ABSTRACT**

The present study proposes two straightforward, responsive, and inveterate spectrophotometric techniques for the detection of Amiloride hydrochloride (AMD). The methodologies are mainly relay on the generation 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 an enduring 1:1 stoichiometric complex were accomplished, which are yellow and pink colour correspondingly. The generated chromogens optical densities were measured at 476 and 508 nm accordingly. Beer’s law been tested in the range of 5-30 and 1-6 µg mL-1. The other analytical parameters like, Limit of Detection (LOD), Limit of Quantification (LOQ), accuracy and precisions were examined. The mentioned approaches were successfully adopted for the determination of the AMD in tablet formulation with high accuracy, better recoveries and acceptable relative standard deviation.

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

**INTRODUCTION**

Chemically Amiloride, 3,5-diamino-6-chloro-N-(diaminomethylene) pyrazine-2-carboxamide (Fig 1), which operates through blocking the epithelial sodium channel through decreasing sodium reabsorption in the distal convoluted tubules and collecting ducts in the kidneys [1]. This stimulates the elimination of salt and water from the body, but without depleting potassium. The medicine is typically used in combination with Thiazide (e.g. co-amilozide) or loop diuretics (e.g. co-amilofruse) (e.g. co-amilofruse). Due to its potassium-sparing qualities, hyperkalemia (high blood potassium levels) are sometimes recorded in patients on Amiloride. It is used in the therapy of hypertension and congestive heart failure. It is beneficial for the prevention of hypokalemia generated by hydrochlorothiazide especially during extended therapy. Amiloride is available on the market as combination medicine with furosemide such as Amifru tab, Amimide, Exna-k tab. It is also available in conjunction with Atenelol & Hydrochlorothiazide namely Beta-Biduret cap, BP-Loride tab, Hipres-D cap.

The inclusive literature study affirms that there are various methods available for the evaluation 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 assess the AMD in formulations and biological samples. However, these procedures are connected with sophistication, expertise, extraction, and more expensive than other methods. Additionally the procedures have been closely followed several of the widely known standards and approaches [21-25]. In the suggested approaches, we have employed spectrophotometer, which is a method of choice still in many pharmaceutical businesses for the regular analysis in poor nations. It serves to be one of the quickest, promising and most trustworthy strategies. The rationale of the current work is to develop and verify novel straightforward, receptive, swift, reliable and accurate spectrophotometric techniques for the measurement of AMD in its dose forms.



Fig 1: Amiloride structure

**EXPERIMENTAL**

**Equipment**

Double beam T90+ UV-Spectrophotometer (PG Instruments Ltd. USA) with quartz cells of 1cm thickness were utilized to obtain the absorbance data.

**Materials**

The reference standard Amiloride hydrochloride pure (99.5%) was offered by Shreeji Pharma International Pvt. Ltd. Gujarat, India. which is used for the analysis, (10 mg of reference standard in 10 mL methanol the apparent conc. is 1000 µg mL-1) the lesser conc. of this solution are created by suitable dilutions with methanol (100 and 50 µg mL-1). Biduret® pills (Biduret® -5) which has been developed and sold by Glaxo Smithkline Pharmaceuticals Ltd. were acquired from the medical shop, which are utilised as test samples. DDQ from Sigma Aldrich chemical (0.1% in methanol), PC from Sigma Aldrich chemicals (0.1% in acetonitrile), AR grade solvents methanol and acetonitrile were used, deionized water was used throughout the analysis.

**Investigative procedure:**

***Modus operandi A***

To a series of 10 mL volumetric flasks, varying fractions of working standard solution of AMD (100 µg mL-1) varying from 0.5-3.0 mL were transferred, one mL of (0.1%) DDQ was added and swirled physically to get homogenous solutions, heated on a steam bath 5 min at 60ºC, an orange-yellow coloured species was obtained, left the solution for 10 min to develop colour at ambient conditions 28±3ºC. The whole bulk of the solution was brought to 10 mL using methanol [25]. Optical density of the coloured species was then verified at 476 nm versus blank solution. A calibration chart was created by graphing abs. vs conc. of medication (µg mL-1), the regression equation was derived. From this generalization, the sample conc. was estimated.

***Modus operandi B***

Variable fractions of AMD working standard solution (50 g mL-1) varying from 0.2-1.2 mL were transferred to a set of 10 mL volumetric flaks. One mL of (0.1%) p-chloranilic acid solution was added and thoroughly blended, a pink coloured complex formed, and the flask was left for 10 minutes to complete the colour development at room temperature 28±3ºC. Acetonitrile was used to reduce the total volume to 10 mL. The optical density of the coloured species was verified at 508 nm against a blank solution. By graphing abs. vs drug conc. (μg mL-1) on a calibration chart, the regression equation was derived [25]. The sample concentrations were determined using this equation.

***Assay procedure for samples***

The content of AMD in tablet formulations was investigated. Ten [Biduret®-5] pills, each weighing 237 mg, are crushed to a fine powder. A portion of this grind, equal to 100 mg of AMD, was taken to a 100 mL standard flask and extracted with 15 mL of water by thorough physical shaking, filtered into a 100 mL volumetric flask through filter paper no.41, and brought to the mark with methanol (Method A) and acetonitrile (Method B). The obvious conc. of the solution was 1000 g mL-1; subordinate conc.s of this solution were created by stepwise dilutions with methanol/acetonitrile to match the conc. of both procedures' reference standards [25]. Following the typical approach, the amount of AMD contained in the tablets was estimated, and the conc. was computed using regression equations.

***Estimation of molar ratios***

In all procedures, the molar ratio of AMD: reagents, was determined using Job's approach. AMD : reagents were evaluated at equimolar doses of 2X10-3 M. A series of solutions in the ratios of [0:10, 1:9, 2:8...10:0] were produced, and the associated absorbances were measured. The absorbance measurements were plotted against the mole ratio in each reagent used in the two techniques.

**RESULT AND DISCUSSION**

***Assay development strategy***

To meet the needs, we chose a medication of therapeutic value in antihypertensive action and conducted this work to establish easy and accurate testing methodologies in clean and tablet dose forms. The active ingredient has a greater proclivity to give its non-bonding pair of electrons, which causes the charge transfer between the-acceptor reagent DDQ and the AMD; this is the foundation for the invention of method A.

The interaction of AMD and DDQ in method A results in the production of an orange-yellow colour charge transfer complex. The likely chemical route for the complex's creation was shown in (Fig. 2). The synthesised chromogen gave an absorbance maximum at 476 nm (Fig. 3). Intensities of chromogen grew as the conc. of AMD increased, becoming the foundation for the study.



**Fig. 2:** Mechanism of coloured species for method A

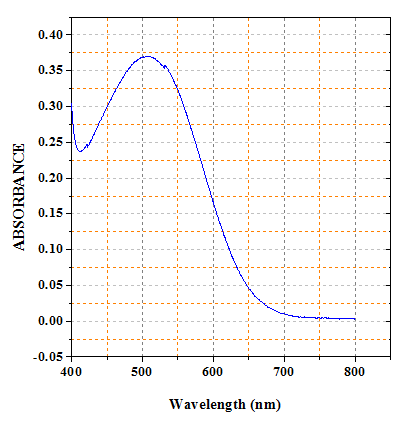
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**Fig. 3:** Lambda maximum for method A

The technique B was established on the charge transfer mechanism amongst the drug and the reagent p-chloranilic acid, which resulted in a stable pink-coloured complex (Fig. 4). The generated compound exhibits a maximum absorbance of 508 nm (Fig. 5). The colour intensities grew as the concentrations of AMD increased, becoming the foundation for the study.

The sections that follow detail the improvement parameters that impact the chemical reaction and the analytical efficiencies of the proposed tests.



**Fig. 4:** Mechanism of coloured species for method B

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

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

To achieve a robust technique, we tuned all of the required parameters based on their optical densities, the conc. of DDQ solution, the reaction time for the creation of chromogen and volume of DDQ solution, and also temperature effects on the formation of chromogen, listed in (**Table 1**). We tweaked all the essential settings based on the preceding findings to get reproducible outcomes.

**Table 1:** shows the ideal circumstances for the creation of coloured complexes.

|  |  |  |
| --- | --- | --- |
| Parameters | 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***

We tuned all of the essential parameters for a robust approach based on their optical densities the volume of p-chloranilic acid solution, conc. of p-chloranilic acid solution and the reaction time for the chromogen formation. Thermal dependence is also established in the (**Table 2**).

**Table 2**: shows the ideal circumstances for the creation of coloured complexes.

|  |  |  |
| --- | --- | --- |
| Parameters | 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 |

***The molar ratio and the reaction mechanism***

The mole ratio of AMD:DDQ was calculated using Job's method. Based on the Job's graphs, it was confirmed AMD:DDQ and AMD:PCA ratios come out to be both 1:1.

This establishes that each mole of each AMD reacted with one mole of DDQ and PCA (Fig. 6 & 7).

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**Fig. 6:** Drug to reagent ratio for method A

**Fig. 7:** Drug to reagent ratio for method B

**Validation of assay method A**

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

The projected assay was generated by graphing the optical density as a function of the respective AMD conc. under the aforementioned optimal reaction conditions. The least-squares approach was used to create the regression equation for the findings, and the Beer's law plot (11 points) was found to be linear in the conc. range of 5.0-30.0 μg mL-1.

The straight line equation was as follows: Y = 0.015x + 0.1334 (r = 0.9998), where 'Y,' 'x,' and 'r' are the optical density, AMD conc., and correlation coefficient, correspondingly. (Fig. 8).

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**Fig. 8:** Linear regression curve for method A

**Validation of assay method B**

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

Calibration graph for the determination of AMD using the intended assay was generated by graphing the optical density as a function of the respective AMD conc. under the aforementioned optimal reaction conditions. The least-squares approach was used to construct regression equation for the results. Beer's law plot (11 points) was found to be linear in the conc. 1.0-6.0 μg/ml range.

The straight line equation was: Y= 0.6675x+0.0158 (r = 0.99987), with 'Y', 'x', and 'r' representing optical density, AMD conc., and correlation coefficient, respectively (Fig. 9).

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**Fig. 9:** Linear regression curve for method B

According to International Conference on Harmonization (ICH) guidelines[25-27], the limits of detection (LOD) and quantification (LOQ) were calculated as follows: LOD = 3.3 S/b and LOQ = 10 S/b, 'S' is the standard deviation of blank absorbance values (n = 6) and b is the slope of the calibration line. The LOD and LOQ values were determined, 0.7660 and 2.553 µg mL-1, respectively. The suggested assay's quantitative parameters are presented in (Table 3).

**Table 3:** Parameters of analytical interest 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 |
| 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 |

**Precision and accuracy**

Recovery experiments for reference solution at various conc. proved their accuracy and precision.

**For (method A), an (intra-day) precision at three distinct concentrations (15, 17.5, and 20 g mL-1) was determined for five replicates of the AMD in pure form, as shown in (Table 4).** The standard analytical errors, relative standard deviations (%RSD), and recoveries achieved in the suggested method's intra-day investigation were judged to be satisfactory. As a result, the suggested approach is successful in determining AMD.

**Table 4:** Assessment of the suggested technique's accuracy and precision (method A) by intra-day test observed conc.of AMD (g mL-1)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Observed conc. of AMD (μg mL-1) | | | | |
| Conc.  of AMD (μg mL-1) | **Intra-day** | | | |
| **Average\*** | **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 suggested method's (Method A) accuracy was further tested by completing recovery tests on the pre-analyzed dose forms, which were then calculated using the recommended process (Table 5). Average recoveries were determined to be 99.4-100.4 percent, with relative standard deviations (percent RSD) ranging from 0.084 to 0.231%. According to the validation requirements for analytical procedures, this range is acceptable. This shows that the approach is repeatable.

Using the conventional addition procedure, no influence from typical tablet excipients was found. A known quantity of pure AMD was added for this reason.

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

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Qty. of  drug before  addition (μg) | Amount of  drug added (μg) | Theoretical  Amount  (μg) | Avg.  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 determinations of AMD in pure form at three distinct concentrations (1.5, 2.5, and 5.0 g mL-1) using short term (intra-day) precisions, as demonstrated in (Table 6). The standard analytical errors, relative standard deviations (percent RSD), and recoveries achieved in the suggested method's intra-day analysis were judged to be satisfactory. As a result, the suggested approach is successful in determining AMD.

The proposed method's (Method B) accuracy was further tested by completing recovery tests using the usual addition procedure. A determinate amount of pure AMD was added to the pre-analyzed dosage forms for this purpose, and the results were determined using the prescribed approach (Table 7). The average recoveries were determined to be 98.9101.3%, with relative standard deviations (% RSD) ranging from 0.11-0.14 %. According to the validation requirements for analytical procedures, this range is acceptable. This shows that the approach is repeatable. The usual tablet excipients did not cause any interference.

**Table 6:** Evaluation of intra-day test for the suggested technique's accuracy and precision (method B) by observed conc. of AMD (g mL-1)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Observed conc. of AMD (μg mL-1) | | | | |
| Conc.  of AMD (μg mL-1) | **Intra-day** | | | |
| **Avg\*** | **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:** Estimation of AMD in pharmaceutical dosage form by standard addition technique (method B)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Amount of  drug before  addition (μg) | Amount of  drug added (μg) | Expected  Amount  (μg) | The mean  Amount  recovered (μg) | Avg. % 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 |

***Ruggedness and Robustness of method A and B***

The analytical performance of each technique was investigated by assessing the impact of tiny variations in each method variable on its robustness. In these trials, one parameter was tweaked while the others remained the same, and the recovery percentages were calculated every time. Small adjustments in the technique variables were shown to have no compelling effect on the operations; recovery values ranged from 98.4 to 101.1%. This demonstrated the dependability of the proposed approaches in frequent use for AMD analysis in QC laboratories.

Further, ruggedness was examined by utilising the suggested approach of the AMD assay under similar working conditions, but with two separate instruments from two different laboratories and with varying elapsed time. Because the relative standard deviations (RSD) did not surpass 2%, the results obtained from lab-to-lab and day-to-day changes were repeatable.

**Suggested assay's application**

The suggested approach was used to analyse AMD in pharmaceutical dosage forms [Biduret®5 tablet formulations], findings were statistically compared with the reference methodolgy using student's t-values. According to the data compiled in, the estimated t-values were fewer than the tabulated values at the 95% confidence level for five degrees of freedom (Table 8). This signifies that the suggested technique is as exact and accurate as the reference method [5, 25].

At the 95% confidence level, no noteworthy differences were discovered in the calculated and theoretical values of both the suggested and reported assays using the t- and F-tests. This demonstrated that the planned and reported approaches provided comparable accuracy and precision in the analysis.

**Table 8:** Recovery and assay studies of AMD in tablet, two methods A and B

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | % Content±SDa | | | | |
| Method | **Tablet** | **Claimed**  **(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 includes the design and validation of two straightforward, precise, reliable, and cost-effective assay methods for assessing AMD in pure and formulated forms utilizing charge transfer complex spectrophotometry. The tests were carefully checked in line with the standards for analytical method validation, and the results were satisfactory. The acceptable recovery values verified the usefulness of the suggested approaches for the quality control of AMD. The following benefits are associated with the tests reported herein:

* The UV-visible spectrophotometer instrument, in contrast to the LC/MS and HPLC methods, is simple and affordable; then again, the superiority resides in simplicity and user friendliness. The approach might be considered, and it is superior to the ways previously published. Furthermore, typical additives and excipients do not interfere with the procedure.
* Reduction in the use of organic solvents in the charge transfer-based spectrophotometric measurement of AMD, hence decreasing analyst exposure to the hazardous effects of organic solvents.
* Escalates costs by 50-folds, which can be reflected in the price of completed dosage forms, lowering the costs of pharmaceuticals for patients.

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**CONFLICT OF INTEREST**

The content of this work has no conflict of interest as stated by the authors.

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