Development of a Validated Colorimetric Assay Method for Estimation of Amikacin Sulphate in Bulk and Injectable Dosage Form

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Abstract: Amikacin sulphate is devoid of any chromophore and/or conjugated system prerequisite for UV and florescent light detection (FLD). Hence, there is a need of simple and reliable methods for introducing chromophore in the structure of amikacin sulphate for its determination using UV and FLD. Therefore, the present study describes the development and validation of a simple, economical and fast colorimetric method for estimation of the drug. The analyte and aqueous ninhydrin solution upon heating for 2-5 min produced the Ruhemann purple colored drug-derivative which was detected at two wavelengths, 400 nm and 567 nm. Beer's law was obeyed over the concentration ranges from 0.417 mg/ml to 2.500 mg/ml. The method was found to be reliable (95.07 – 100.52 % recovery at 400 nm and 96.04 – 99.89 % at 567 nm), repeatable - intraday accuracy - (95.07 – 100.52 % at 400 nm and 96.04 – 99.84 % at 567 nm) and reproducible -inter day accuracy - (95.25– 99.91 % at 400 nm 96.52 – 99.89 % at 567 nm) with relative standard deviation less than 5 %. The results of the present study indicate that the method is easy to perform, specific and sensitive, and suitable to be used for the determination of amikacin sulphate in bulk and injectable dosage forms using less expensive/laborious derivatization.

Keywords: Amikacin sulphate, Colorimetric detection, Derivatization, Ninhydrin reagent, Validation.

Introduction

Amikacin sulphate (AS) - a broad spectrum semisynthetic aminoglycoside antibiotic, developed by strategic chemical alteration of kanamycin A - contains four primary and one secondary amino groups (Fig. 1), which are positively charged at neutral pH [1, 2]. The molecule of AS has inherent structural insufficiencies i.e. absence of chromophore or conjugated system to give a reliable UV/florescence signal(s). Therefore, quantification of the drug by direct UV-Visible or florescent light detectors is difficult or impossible [3]. The literature review indicated the detection of the drug at 212 nm [4], but this region was not suitable for quantification purposes. Fortunately, the drug contains such functional groups that can be derivatized chemically to produce detector-oriented response [5].

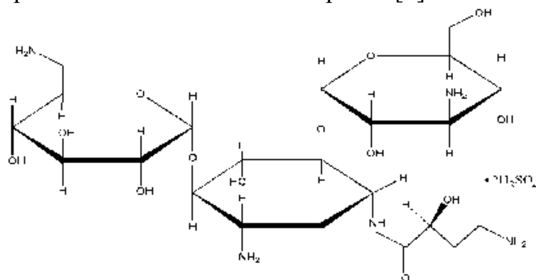

Fig. 1: Chemical structure of amikacin sulphate (AS).

Handsome literature documented the methods for the quantification of the drug alone and in various matrices with and without derivatization. Though, spectrophotometric methods are preferred due to their simplicity, economy and reasonable sensitivity, a very little data is available for spectrophotometric determination of AS in pharmaceutical formulation. Confino and Bontchev [6] developed a method based on Rimini test for determination of amikacin, kanamycin, tobramycin and neomycin. Various assay methods for determination of AS alone and in the presence of other aminoglycosides involved Hantzsch reaction for drug derivatization [7-9]. Al-Sabha [10] developed a method for quantification of amikacin based on charge transfer complex formation. Another method introduced for quantification of amikacin, kanamycin, streptomycin and neomycin described the use of cyanoacetamide as derivatizing agent for their UV measurement [11]. In a recent study, a spectrofluorimetric method for determination of AS and related compounds through their condensation with ninhydrin and phenyl acetaldehyde was developed [12]. Only few reports were found regarding the use of ninhydrin reagent alone for derivatization of the drug. According to the best of our literature survey, just one report described the use of ninhydrin test solution alone for

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spectrophotometric quantification of amikacin sulphate in injectable dosage and heat and pH condition of derivatization [13].

Our extensive literature review indicated that most of the derivatization processes introduced till date was not suitable for quality control and analysis due to time factor, laborious derivatization, expensive reagents and stability of the complex under mild reaction conditions [14, 15]. To overcome these issues, other instrumental techniques used included pulsed electrochemical detection on gold electrode [16, 17], mass spectrometric detection [2], evaporative light scattering detection [18], post-column ligand displacement reaction with fluorescence detection [19] and chemiluminescence detection [20]. Nonetheless, all such techniques need special and expensive instrumentation; therefore there is a need of developing simple methods that can be employed in less developed or equipped laboratories. Keeping all the aforementioned factors in view, the present study aimed to develop a simple colorimetric method for the determination of amikacin sulphate.

Experimental

Chemicals, solvents and other supplies

Analytical grade AS was gifted by M/S Global Pharmaceuticals, Islamabad, Pakistan. The commercial samples of AS injections (Grasil-250mg/2ml, Batch No. 09M, and Grasil- 500mg/2ml, Batch No. 022N, M/S SAMI Pharmaceuticals (Pvt.) Ltd. Karachi, Pakistan) were purchased from local pharmacies. Sterile water for injection (USP) having batch number W13013, manufactured by M/S Bosch Pharmaceuticals, 209/23, K.I.A. Karachi, Pakistan, was also purchased from the local market. Ninhydrin reagent (Simpsons (UK) Ltd., Unit 6b, Lodgeway, Severnbridge, Industrial Estate, Caldicot, Monmouthshire, NP 26 5Pt.) and analytical grade acetone, Lot No. 911103 298, Product code No. 100034 Q were procured from the local market.

Instruments

UV/Visible analysis was performed using a double beam UV/Visible spectrophotometer (Shimadzu Corporation, Kayoto, Japan), Serial No. A10844403603 LP, CAT 206-24401-93, Model UV-2550, connected to Fujitech computer and equipped with UV Probe 2.21 operating software. The absorption spectra of the standard and test solution were recorded in 1 cm quartz cells over the whole UV/Visible range (800 nm to 200 nm). A sonicator (Model, DSA50-SK1-1.8L, made in Germany was used for mixing and cleaning purposes.

Preparation of Ninhydrin Test Solution

Ninhydrin test solution was prepared by adding 0.2 g ninhydrin in 94 ml distilled water and 6 ml acetone. The resulting solution was sonicated till the formation of a clear solution and stored in a tightly capped container, protected from light and heat.

Preparation of Standard Solutions

Standard Stock Solution: Standard stock solution of AS without derivatization having concentrations 125 mg/ml was prepared in water for injection. To introduce chromophore in the AS, 200 µl from the abovementioned standard stock solution (125 mg/ml) was added in a test tube containing 2 ml of ninhydrin solution. The tube was heated until the development of Ruhemann purple color. Then the contents were transferred to a 10 ml volumetric flask and made up the volume with distilled water. The derivatized standard stock solution of the drug was having concentration 2.500 mg/ml.

Working Standard Solution: A range (0.417 mg/ml to 2.500 mg/ml) of working standard solution of derivatized amikacin sulphate (dAS) was prepared by diluting the stock solution (2.500 mg/ml) with distilled water.

Optimization of Variables in Color Development

Various variables, heating time for effective derivatization, heating type (direct on flame and water bath), drug-reagent ratio, reagent composition and storage temperature for the derivatized drug, involving in the development of Ruhemann purple color were optimized.

Determination of λ max

The standard stock solutions of AS and dAS having concentration 125 mg/ml and 2.500 mg/ml respectively, were scanned in UV/Visible region (800-200 nm) using distilled water and ninhydrin reagent solution as blanks. The ninhydrin solution was also scanned similarly for comparison and estimation of interference in estimation of AS, if any.

Method Development and Validation

Assessment of Linearity: Five working standard solutions in a concentration range 0.417 mg/ml to 2.500 mg/ml were analyzed in triplicate at

400 and 567 nm. The linearity was evaluated by visual inspection of the plot of concentration versus absorbance. The correlation of the data points was assessed by finding the correlation coefficient (R^2).

Beer-Lambert Range: The Beer-Lambert range of the assay method was derived from the linearity studies, carried out as mentioned earlier using standard solutions in a concentration range (0.083 mg/ml to 2.500 mg/ml).

Sensitivity: Limit of detection (LOD) and limit of quantification (LOQ): Five standards solutions having a concentration from 0.417 mg/ml to 2.500 mg/ml were analyzed in quintuplicate and the data were used to construct five standard curves. LOD at a signal to noise (S/N) ratio 3:1 and LOQ at S/N ratio 10:1 were determined statistically.

Recovery, intraday and inter-day accuracy and precision: Three working standard solutions (0.417, 1.250 and 2.500 mg/ml) were used to determine recovery, intra- and inter-day accuracy and precision. For recovery, the standard solutions were analyzed in triplicates and their concentrations were determined from the calibration curve. The percentage recovery was determined by comparing the obtained values to the true values.

In order to assess the quality of the assay method, accuracy and precision estimates i.e. repeatability and reproducibility were carried out at three concentration levels of the standard solution. For intra- and inter- day accuracy and precision, each of the three standard solutions was analyzed 6 times in a single day and once daily for six consecutive days, respectively. The amounts were determined from the calibration curves constructed on each day.

Specificity: Determination of the drug in commercial samples: Specificity is the ability of the assay method to estimate unequivocally the compound of interest in the presence of other components e.g. excipients, impurities, degradants and matrix, etc. For evaluating the specificity of the method, commercial injections of AS were used. Commercial injection (200 µl) was treated like the standard stock solution stated above. The working sample solution was prepared by diluting the stock solution (2.500 mg/ml) appropriately so that the concentration fell within the range of the calibration curve. Then five standard solutions and two sample solutions were analyzed in triplicate at 400 and 567 nm, and amount of the drug was quantified from the standard curve, using the linear regression equation.

Robustness: The effect of small deliberate changes in the detection wavelength on the recovery of the drug was investigated to find the robustness of the method.

Stability of complex: The stability of the derivatized standard and sample solution was evaluated by keeping them in screw capped test tubes, protected from light, at room temperature, in the refrigerator and, freezer. Recovery of these solutions was compared with the freshly prepared solution.

Results and Discussions

λ max of the drug

The scan of AS solution at 800-200 nm did not give any peak (Fig. 2). In very high concentrations, however, the drug without derivatization gave a peak at 210 nm. But, this wavelength is not suitable for quantification because many solvents absorb at such low wavelength. On the other hand, after derivatization with ninhydrin reagent, the drug gave two peaks, one at 400 nm and the second at 567 nm (Fig. 3). To the best of our knowledge, we did not find any report regarding the absorption behavior of the AS at 400 nm. However, only one study by Feng-ming *et al*. [13] report the maximum absorption of ninhydrin derivative of the drug at 568 nm, which is consistent with that reported in this study.

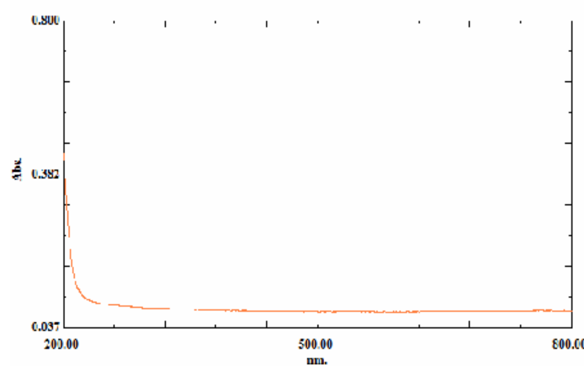

Fig. 2: UV-Visible scan of aqueous solution of amikacin sulphate (AS).

As mentioned earlier, the scans were taken by using distilled water and ninhydrin reagent as blank; we found no difference in the spectra of AS using both of the blanks. For evaluating any interference by reagent itself in the estimation of the drug, the ninhydrin solution (without analyte) was also scanned in the UV/Visible range (800-200 nm). The scan of ninhydrin (Fig. 4) shows absorption at

254 nm and 231 nm, which indicates that the reagent does not interfere with the estimation of drug at 400 nm and 567 nm.

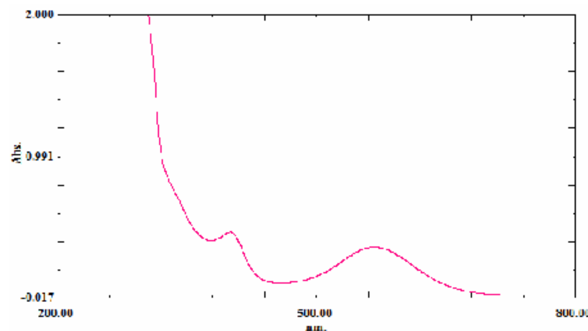

Fig. 3: UV-Visible scan of aqueous solution of derivatized amikacin sulphate (dAS)

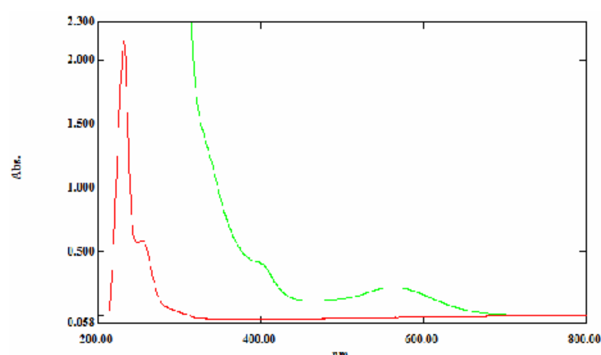

Fig. 4: UV-Visible overlay scan of ninhydrin reagent and derivatized amikacin sulphate (dAS)

Optimized variables in development of color

Firstly, the ninhydrin test was carried out as it is performed in normal laboratory conditions. Various problems during complex formation were encountered that were optimized to achieve good results. Table-1 illustrates the optimized condition for colored complex formation.

Validation parameters

Linearity: The plot of concentration of AS versus absorbance was used to establish the linearity. The method showed linearity at both of the wavelengths, 400 nm and 567 nm, in all the concentration range investigated in this study (0.417-2.500 mg/ml). The plots shown in Fig. 5 and 6 indicated the linear regression equation; Y = 0.2213X – 0.0009 with correlation coefficient (R^2) = 0.9996 at 400 nm and Y = 0.1682X + 0.0043 with correlation coefficient (R^2) = 0.9994 at 567 nm. This indicated that the method was linear at both the wavelengths and could be used to quantify the drug reliably.

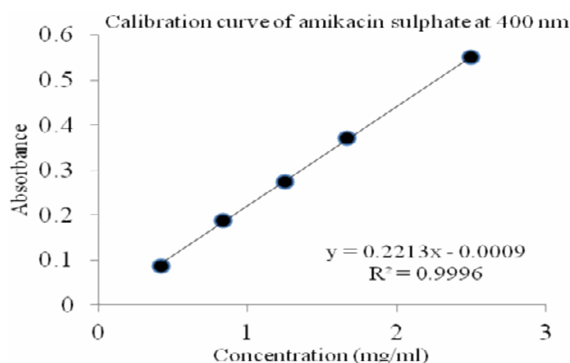

Fig. 5: Calibration curve of derivatized amikacin sulphate (dAS) at 400 nm.

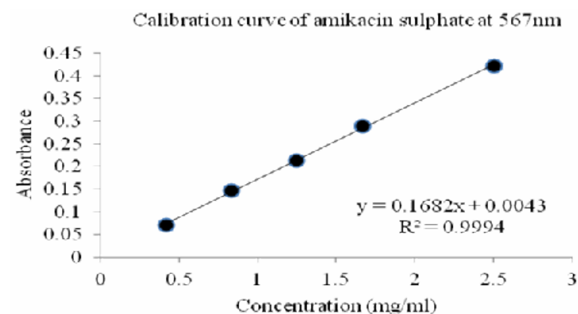

Fig. 6: Calibration curve of derivatized amikacin sulphate (dAS) at 567 nm.

Beer-Lambert Range: The range of calibration was established by considering the practical range necessary, according to the drug concentration in the pharmaceutical products, in order to provide linear, accurate and precise estimations (Fig. 7 and 8).

Table-1: Optimization of variables in the complex formation of amikacin sulphate (AS) and ninhydrin.

Variables	Optimized conditions
Reagent composition	**Usually ethanol and spirit is used to prepare ninhydrin reagent, but to avoid bumping of reaction mixture during heating, a mixture (6:94) of acetone and water used**
Amount of reagent for effective derivatization	**Reage : (2-3ml) produced deep blue color indicative of successful chromophore introduction**
Concentration of analyte	**AS (200 µl ≃ 25 mg) and 2-3 ml of reagent produced required results, very dilute solutions did not give required results**
Heating time	**Heating time (2-5 min), heating at low temperatures (below 50°C) for longer time periods produced yellow or orange color product**
Heating condition	**Direct heating for 2 min in the lowest part of flame and 5 min at 80-100°C in water bath**

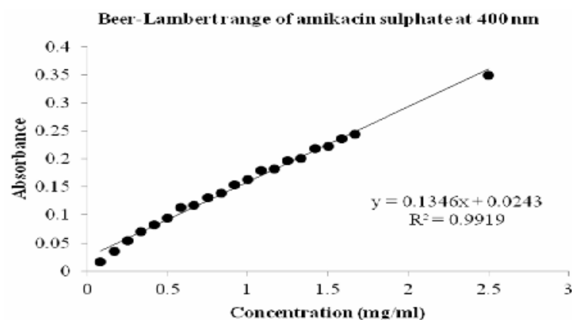

Fig. 7: Curve showing linearity for the whole range (Beer-Lambert range) investigated (0.083 mg/ml-2.500 mg/ml) at 400 nm.

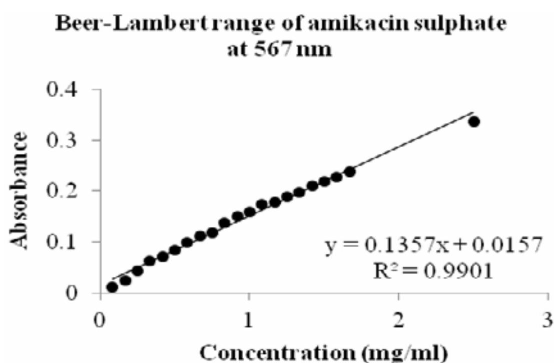

Fig. 8: Curve showing linearity for the whole range (Beer-Lambert range) investigated (0.083 mg/ml-2.500 mg/ml) at 567 nm

Sensitivity: Limit of detection (LOD) and limit of quantification (LOQ): The results shown in Table-2 and 3 indicate linearity, LOD and LOQ values of derivatized AS at both the wavelengths. The method was found linear over the whole range investigated with correlation coefficient 0.997 to 0.9999 at 400 nm and 0.9992 to 0.9995 at 567 nm. LOD value of AS was 0.015 mg/ml at 400 nm and 0.031 mg/ml at 567 nm, while LOQ value was taken as 0.046 mg/ml at 400 nm and 0.094 mg/ml at 567 nm at signal to noise ratio 10:1.

Table-2: Result of calibration, limit of detection (LOD) and limit of quantification (LOQ) of amikacin sulphate (AS) by colorimetric assay method (at 400 nm).

Standard curve	Concentration (mg/ml)	Linear regression equation	Slope	Intercept
1	0.417-2.500	0.2231x - 0.0025 0.9997	0.2239	0.0025
2	0.417-2.500	0.2244x - 0.0058 0.9993	0.2244	0.0058
3	0.417-2.500	0.2223x - 0.006 0.9991	0.2223	0.006
4	0.417-2.500	0.2219x - 0.0053 0.9992	0.2219	0.0053
5	0.417-2.500	0.2225x - 0.0067 0.9993	0.2225	0.0067
	Mean slope (S)		0.223	
	Standard deviation (SD)			0.001
	LOD and LOQ			
	LOD (mg/ml) = 3.3 SD/S			0.015
	LOQ (mg/ml) = 10 SD/S			0.046

Table-3: Result of calibration, limit of detection (LOD) and limit of quantification (LOQ) of amikacin sulphate (AS) by colorimetric assay method (at 567 nm).

Standard curve	Concentration (mg/ml)	Linear regression equation	Slope	Intercept
1	0.417-2.500	0.1682x + 0.0043 0.9994	0.1682	0.0043
2	0.417-2.500	0.1695x + 0.001 0.9992	0.1695	0.001
3	0.417-2.500	0.168x + 0.004 0.9991	0.168	0.004
4	0.417-2.500	0.1668x + 0.0012 0.9991	0.1668	0.0012
5	0.417-2.500	0.1683x + 0.0018 0.9997	0.1683	0.0018
	Mean slope (S)		0.1682	
	Standard deviation (SD)			0.0016
	LOD and LOQ			
	LOD (mg/ml) = 3.3 SD/S			0.031
	LOQ (mg/ml) = 10 SD/S			0.094

Table-4: Recovery, intraday and inter-day accuracy, and precision of the colorimetric assay for the determination of amikacin sulphate (AS)

Concentration (mg/ml)	Mean absorbance	SD	Value found (mg/ml)	Recovery (%)	Accuracy (%)	Precision (% RSD)
At 400 nm						
Intraday accuracy and precision						
0.417	0.086	0.003	0.396	95.07	95.07	3.6
1.250	0.277	0.002	1.256	100.52	100.52	0.76
2.500	0.541	0.021	2.451	98.06	98.06	3.90
Interday accuracy and precision						
0.417	0.087	0.003	0.397	95.25	95.25	3.57
1.250	0.275	0.002	1.248	99.91	99.91	0.95
2.500	0.55	0.02	2.489	99.57	99.57	3.63
At 567 nm						
Intraday accuracy and precision						
0.417	0.071	0.002	0.401	96.04	96.04	2.75
1.250	0.213	0.003	1.245	99.65	99.65	1.18
2.500	0.424	0.002	2.496	99.84	99.84	0.49
Interday accuracy and precision						
0.417	0.072	0.002	0.402	96.52	96.52	3.00
1.250	0.214	0.002	1.248	99.89	99.89	0.92
2.500	0.423	0.002	2.494	99.77	99.77	0.53

Recovery and intra- and inter-day accuracy and precision: The results of recovery, intra- and inter-day accuracy and precision values of the method are shown in Table-4. Mean recovery was found to be 95.07-100.52 % at 400 nm and 96.04-99.89 % at 567 nm) with relative standard deviation less than 5 %, which indicated that the method was reliable. Intra- and inter-day accuracy values were 95.07-100.52 % at 400 nm and 96.04-99.89 % at 567 nm with relative standard deviation less than 5 %, which proved that the method was repeatable and reproducible.

Specificity: Determination of the drug in commercial samples: For determination of specificity- the ability of the assay method to estimate unequivocally the compound of interest in the presence of other components e.g. excipients - commercial injections containing AS were treated like the standard stock solution stated above. The working sample solution was prepared by diluting the stock solution appropriately so that the concentration fell within the range of the calibration curve. The samples of the commercial formulations were analyzed at both the wavelengths and quantified from the calibration curves. Percentage contents determined in commercial samples are given in Table-5. The values determined corresponded to the label claims. These results suggest that the commercial sample of AS injection conformed to the labeled claims of drug content in the formulation. These results proved that the method is reproducible and can be applied for the quantification of AS.

Robustness: The small deliberate changes in the detection wavelength (403, 402, 568 nm), heating time by ± 30 seconds and blank composition (ninhydrin solution or distilled water) had no significant effect on the recovery of the drug which indicates that the method is robust.

Table-5: Percentage contents of amikacin sulphate (AS) in commercial injections.

Name of injection	Wavelength	Amount stated (mg)	Amount calculated (mg)
Grasil	400 nm	250 mg	248.48
	567 nm	250 mg	247.74
	400 nm	500 mg	497.87
	567 nm	500 mg	496.92

Stability of complex: The solutions stored in the laboratory at room temperature, in refrigerator and in freezer were found to be stable for 1 h, 7 days and 1 month, respectively.

Conclusion

It is concluded from the results of the study that the colorimetric method developed for analysis of AS in bulk and injectable dosage form is not only simple, reliable, Moreover, the method requires less time to introduce chromophore as compared to the other reported techniques.

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