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Article

Comparative application of biological and ninhydrin-derivatized spectrophotometric assays in the evaluation and validation of amikacin sulfate injection

Edebi Nicholas. Vaikosen[1,*], Samuel Ogheneruona. Origbo[2], Diepreye Ere[1], Prosper Odaderia[1]

[1]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria, [2]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria

Instrumental techniques are preferred over bioassay methods for antibiotic quantification mainly due to speed and ability to quantify metabolites in biological samples; however, the potency and biological activity of these drugs cannot be assessed. Two methods - agar well diffusion (bio-assay) and spectrophotometric methods were used to evaluate amikacin sulfate injection. Agar plates were inoculated with *S. aureus* inoculum; zones of inhibition from its susceptibility to amikacin were obtained, while spectrophotometric absorption at 650 nm of ninhydrin-derivatized amikacin in phosphate buffer (pH 8) was measured. Methods performance showed linearity from 1 - 16 μgmL^{-1} (bioassay, r = 0.9994) and 10-50 μgmL^{-1} (spectrophotometric, r = 0.9998). Molar absorptivity was 2.595 x 10^4 $Lmol^{-1}cm^{-1}$. Limits of detection and quantification were 1.07 and 3.24 μgmL^{-1} respectively for bioassay method, while corresponding values for spectrophotometric method were 0.98 and 2.97 $\mu g\ mL^{-1}$. Relative standard deviations were ≤ 2.0% for both methods, with recoveries from 95.93 - 100.25%. Amikacin in brands ranged from 97.53 ± 2.68 to 100.84 ± 1.82%, student's t-test was ≤ 2.78 (n = 4) with respect to label claim for both methods. Experimental paired *t*-test ($t = 2.07$; $n = 4$) and *F-test* ($F = 3.94$; n = 4) values indicated no significant difference between both methods, hence comparable and can jointly be used in quality control assessment of antibiotics.

Keywords: Biological activity. Potency. Derivatizaton. Aminoglycosides. Molar absorptivity. Ninhydrin.

INTRODUCTION

Amikacin, (2S)-4-Amino-N-{(1R,2S,3S,4R,5S)-5-amino-2-[(3-amino-3-deoxy-α-D-glucopyranosyl) oxy]-4-[(6-amino-6-deoxy-α-D-glucopyranosyl)oxy]-3-hydroxycyclohexyl}-2-hydroxybutanamide is a broad-spectrum semi-synthetic aminoglycoside antibiotic. It is an alpha-D-glucoside, a carboxamide and an amino cyclitol that is kanamycin A acylated at the N-1 position by a 4-amino-2-hydroxybutyryl group (Figure 1a). It is an antimicrobial and antibacterial agent and like other aminoglycosides its adverse effects include: nephrotoxicity and ototoxicity (Wargo, Edwards, 2014), with the tendency to provoke allergic reactions and the quickly increasing ability to acquire resistances by pathogenic bacteria (CDC, 2019). Although, classified in the same group of antibiotics with tobramycin and gentamicin, it is administered in the treatment of infections caused by bacteria that are resistant to these agents (Mughal *et al.*, 2015). The mechanisms of action of amikacin is by irreversibly binding to components of bacteria that produce vital bacterial proteins, thus, blocking protein synthesis which eventually leads to the stoppage of further bacterial growth (Ullah, Ali, 2017). Amikacin is indicated for infections caused by gram-negative bacteria

***Correspondence:** E. N. Vaikosen. Department of Pharmaceutical and Medicinal Chemistry. Faculty of Pharmacy. Niger Delta University. Wilberforce Island. Bayelsa State. Nigeria. Phone: +2348034626596. E-mail: email-vaikosen@yahoo.co.uk, edebi.vaikosen@ndu.edu.ng. ORCID: 0000-0002-2413-6127

such as *Pseudomonas* species, *Escherichia coli*, Providencia species, Proteus species, *Klebsiella-Enterobacter-Serratia* species, and *Acinetobacter* (Mughal *et al.*, 2015; Kalyani, Rao, 2018). Furthermore, amikacin is also recommended in staphylococcal infections, mostly among Methicillin Resistant *Staphylococcus aureus* (MRSA) infections (Bauman, 2018). Because of its potency and efficacy, amikacin has been recommended as the second-line treatment for multidrug resistant tuberculosis with the failure of first-line drugs (WHO, 2013).

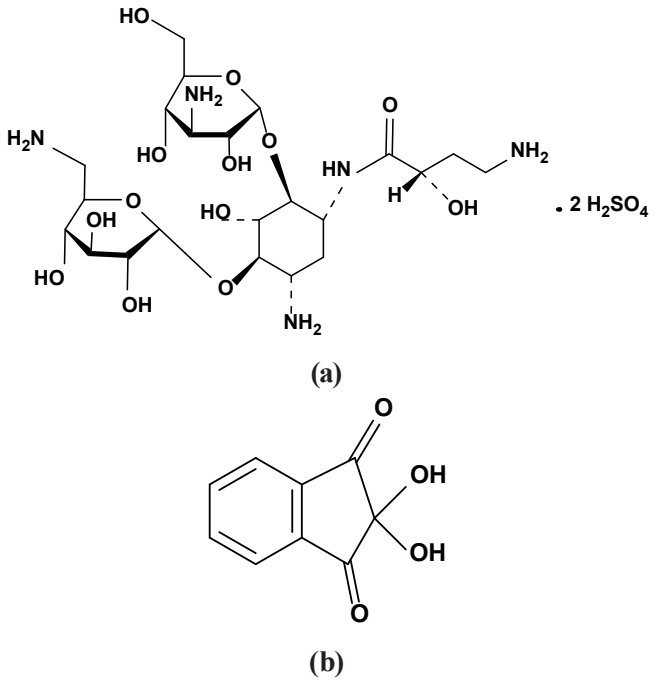

FIGURE 1 - Structures of **(a)** amikacin sulfate and (**b**) ninhydrin.

Since the discovery of antibiotics, it has played a vital role in fight against infectious diseases. Over the years, the use of these drugs has led to a phenomenal rise resistance by microorganisms. Various conditions have led to the increase in resistances to these agents of which has been derived from the organisms, environment and practitioners. The proliferation of falsified and substandard pharmaceuticals have been reported, especially in sub-Saharan African countries having about 42% of global cases (WHO, 2017; Richmond, 2020). The aforementioned has stirred up the need to determine the actual composition of the active ingredients in the drug formulations. The efficacy of an antibiotic is assessed by its potency (Dafale *et al.*, 2016). Therefore, quantification of active pharmaceutical component(s) is very crucial and necessary. Potency of drugs can be determined by chemical and biological methods.

The officially method for the assay of amikacin sulfate injection in international compendia is by HPLC (USP, 2018; BP, 2015). However, various analytical techniques have been reported for amikacin sulfate injection - these includes, spectrophotometry (Al-Sabha, 2010; Mughal *et al.*, 2015; Surya, Gurupadayya, Venkata, 2018), spectrofluorimetry (Omar *et al.*, 2013, Omar *et al.*, 2015; Derayea, Attia, Elnady, 2018), high performance thin layer chromatography (Hubicka, *et al.*, 2009; Kaya, Filazi, 2010), capillary electrophoresis (Yu *et al.*, 2008; Huidobro, Garcia, Barbas, 2009), high performance liquid chromatography (Kalyani, Rao, 2018; Usmani *et al.*, 2019; Korany *et al.*, 2014), TLC-GC/FID (Anyakudo, Adams, Schepdel, 2020), FTIR derivative spectroscopy (Ovalles *et al.*, 2014) and molecular surface plasma by resonance sensors (Yola, Atar, Eren, 2014).

UV-Visible spectrophotometry has found usefulness in pharmaceutical and biomedical analysis for qualitative and quantitative purposes over the years. This is because it is versatile, robust, simple, quick and cheap for both research and routine analysis (Vaikosen *et al.*, 2020; Peraman, Reddy, 2019). The presences of chromophores in pharmaceuticals make them easily to be assayed in the UV region, while their absence requires the use of chromogens to derivatized them to form coloured complexes that absorb at higher wavelength in the visible region. Amikacin, like other aminoglycosides is non-chromophoric and this makes direct UV spectrophotometric determination not feasible (Anyakudo, Adams, Schepdel, 2020; Vaikosen, Ebeshi, Worlu, 2019). Structurally, amikacin has four primary amine groups, one secondary amine group, one primary OH group, seven secondary OH groups and one carbonyl group – these functional groups makes it highly susceptible for quantitative derivatization in the visible region (Baietto *et al.*, 2010). Ninhydrin, 2,2-dihydroxyindane-1,3-dione (Figure 1b) is a known chromogen, that reacts with primary amines or amino compounds – this includes amino acids, peptides, proteins, and even ammonia (Friedman, 2004). This property has made it gained wide adaptations in analytical chemistry and biochemistry for quantitative and quantitative purposes. The presence of free primary amino

in most aminoglycosides such as amikacin, enable them to react with ninhydrin to form the derivatized schiff base of the drug for quantitative UV spectrophotometry through oxidative-condensation reaction.

Biologically, the use of agar diffusion to assess biological activity of substances or antibiotics has evolved over time, from Fleming through Heatley (Heatley, 1944, Zuluaga *et al*., 2009), to the present day assessment using dose concentration-response relationship. This is based on growth inhibitory effect on microorganism when exposed to varying concentrations of drug substance. Often, researchers prefer the use of physico-chemical instrumental methods over bioassay methods because of their accuracy, precision and speed and particularly, when handling biological samples (like faeces, urine, serum or plasma) and pharmacokinetics or pharmacodynamics. In addition, the inability of microbiological assay to quantify metabolites and impurities other than the active pharmaceutical ingredients in a sample is a major limitation of its application (Zuluaga *et al*., 2009). Despite these shortcomings with the agar diffusion method, it is pertinent to mention that the official compendia and analytical methods are physicochemical only; and do not in any way address the potency and activity of antimicrobials neither does it predicts the loss of activity (Cazedey, Salgado, 2013). This shows that instrumental methods, such as, GC-MS, HPLC and UV spectrophotometry cannot provide a true reflection of the biological activity of antibiotics and its' intended use.

Hence an attempt has been made to develop a simple, quick, cheap, sensitive, accurate and reproducible amikacin-ninhydrin derivatized spectrophotometric method for routine quality assessment in conjunction with the simple and cheap agar well diffusion method.

MATERIAL AND METHODS

Material and Chemicals

Apparatus

All spectra were ran on JENWAY 6305 model UV-Vis Spectrophotometer, while analytical weighing balance (Saturious), pH meter (Orion) and Micro-pipettors (Eppendorf) were all calibrated before used. Other equipment such as autoclave (Fisher Scientific), oven (Thermo Fisher Scientific), incubator (Memmert) and lamina flow (Biobase) were functional before been applied.

Chemicals and reagents

Chemicals used were of spectroscopic or analytical grade. The chloroform and methanol were manufactured by JHD of China, Ammonia solution was manufactured by BDH chemicals Ltd Poole England, Acetonitrile was manufactured by sigma- Aldrich of Switzerland and ninhydrin (98.5%) was manufactured by KEM LIGHT SPECHEM LAB LIMITED, Mumbai, India. The amikacin sulfate standard was purchased from Sigma-Aldrich Chemie, GmbH, Germany. Potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4) were manufacture by Fisher Scientific UK, Ltd, while Oxoid Mueller Hinton agar (OMHA) was manufactured by Thermo Fisher Scientific Inc, UK.

Preparation of reagents and solutions

Ninhydrin reagent

Ninhydrin reagent, 0.02 mol L^{-1} was prepared by dissolving 0.25 g of ninhydrin powder in 30 mL of 2% (w/v) sodium carbonate solution and making the volume up to the 50 mL mark using the same sodium carbonate solution.

Phosphate buffer solution

Phosphate buffer was prepared by dissolving 16.73 g K_2HPO_4 and 0.523 g KH_2PO_4 in sufficient water to produce 1000 mL, sterilized and adjusted to pH 8, with either 8 mol L^{-1} phosphoric acid or 10 mol L^{-1} KOH.

Standard Amikacin solution

A stock solution of amikacin sulfate standard 1000 µg mL^{-1}, was prepared by dissolving 50 mg equivalent of amikacin reference standard in 1 mL of acetonitrile

in 50 mL volumetric flask and then made to mark with the phosphate buffer (pH 8.0) solution.

Preparation of agar and inoculation plates

The Muller Hinton agar (MHA) used in this study was manufactured by Oxiod Limited, UK and solution was prepared as prescribed by the manufacturer. Sterilized molten agar 40 mL was poured into series of 100 x 20 mm petri dishes in a laminar flow (an aseptic surrounding) to a height of 4 mm and allowed to solidify.

Test micro-organism and inoculum

The test micro-organism recommended for the biological assay of amikacin is *Staphylococcus aureus* (BP, 2015; USP, 2018) and the strain ATCC 29737 was obtained from the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, Niger Delta University. Cultures of *Staphylococcus aureus* ATCC 29737 were prepared and maintained on Mueller Hinton medium (Oxiod, UK). However, prior to its use for the study, a comparative susceptibility test with other microorganisms (*E. coli* and *P.* a*eruginosa*) was carried out to ascertain its appropriateness as test organism for amikacin.

Samples

Three brands of amikacin sulfate injections (each containing 500 mg amikacin per 2 mL) used for this study were purchased from reputable Pharmacies in Yenagoa, Bayelsa State, Nigeria. Two of the drugs were branded, while the third was unbranded. The branded injections were manufactured by Ciron Drugs and Pharmaceutical Pvt Ltd., Tarapur Boisar, Maharashtra, India (AMK_1); Ranbaxy (Alfakim), Manufactured by - Nitin Lifesciences Ltd., Poanta Sahib, India (AMK_2) and the unbranded (AMK_3).

Spectrophotometric method

General procedure and determination absorption spectra

To an aliquot, 1.0 mL stock solution of amikacin standard in 10 mL volumetric flask, 0.2 mL of the ninhydrin reagent was added and heated in a boiling water bath for 10 minutes to obtain a purple complex. This was cooled and made to mark with phosphate buffer solution. The resultant complex was scanned from 400 - 850 nm against a reagent blank treated the same way without AMK solution.

Linearity and validation of spectrophotometric method

A five point calibration graph was prepared by transferring aliquots of stock solution of amikacin standard into a series of 10 ml volumetric flasks using a micro-pipette to obtain working concentrations ranging from 10 - 50 $\mu g\ mL^{-1}$. To each flask, 0.2 mL of the ninhydrin reagent was added and heated in a boiling water bath for 10 minutes to obtain a purple complex. The absorbance of each concentration was measured at 650 nm against a reagent blank.

Validation of proposed methods

The assessment and validation of method performance was carried out using criteria such as; precision, accuracy, specific interference, sensitivity, ruggedness and robustness – these were determined in addition to the linearity range. The recovery studies were evaluated at three concentration levels with 10, 15 and 20 $\mu g\ mL^{-1}$ of the reference standards. The inter-day and intra-day precisions were carried out at the aforementioned concentrations in three replicates on 3 days within a week and on the same day respectively.

The limit of detection (LoD) and the limit of quantification (LoQ) were determined by the expressions; $LoD = 3.3\sigma/b$; $LoQ = 10\ \sigma/b$ (where σ is the standard deviation of the intercept of regression line, and 'b' is the slope of the calibration curve). Both were used to measure the sensitivity of the method (Bohm, Stachel, Gowik, 2010; Shrivastava, Gupta, 2011).

Extraction amikacin from injection

An equivalent of 500 mg amikacin in injection vial was rendered alkaline with 10 mL volume of 5% (v/v) ammonia solution in a 250 mL separatory funnel (Vaikosen,

Ebeshi, Worlu, 2019). The liberated base was extracted with 4 portions of 10 mL chloroform. The combined chloroform extract was passed through anhydrous Na_2SO_4 supported on a filter paper. The filtrate obtained was evaporated using a rotatory evaporator to a residue of about 2 mL and to dryness by a stream nitrogen gas. Residue was re-dissolved in 2 mL methanol and transferred into a 25 mL volumetric flask and made to mark with acetonitrile (Solution A).

Biological method

The assay is concentration dependent and it is hinged on growth response due to differential inhibitory effect from concentrations of AMK standard on reference bacteria (*S. aureus* ATCC 29737) seeded in agar. The obtained linear concentration-growth-response relationship is then compared to the quantity present in the sample under examination (USP, 2018; BP, 2015).

Preparation and standardization of micro-organism

The micro-organism was standardized in accordance to BP specification (BP, 2015). Prior to use, *Staphylococcus aureus* inoculum was grown in a brain heart infusion (BHI) broth in a conical flask, at 35 ± 2 °C for 24 hours. An aliquot or a peal of the test organism was transferred into 2 mL NaCl solution [0.90 % (w/v)] with the aid of a wire loop to obtain a cell density of 1 x 10^8 CFU mL^{-1} and the turbidity of solution was compared with McFarland's standard and also by measuring the absorbance at 580 nm to obtain 1.0 ± 0.1). Standardized suspension, 1.0 mL, was further diluted to 100 mL of sterile culture medium at 48 °C to obtain the *Staphylococcus aureus* inoculum for assay.

Preparation of amikacin reference standard

Amikacin sulfate reference standard 50 mg, was transferred into a sterile 10 ml volumetric flask, 2 mL of sterilized phosphate buffer solution (pH 8.0) was added to dissolve the standard and then made to mark to give a stock solution of 5,000 µg mL^{-1} using the same buffer solution. Working concentrations ranging from 1 - 28 µg mL^{-1} were prepared from the stock solution.

Preparation of amikacin samples

Amikacin sulfate injection, 0.01 mL (equivalent to 2.5 mg amikacin) was transferred from vial into a sterilized 50 mL volumetric flask with the aid of a micropipette. This was then diluted to mark with sterilized phosphate buffer solution (pH 8.0) to give a sample stock solution of 50 µg mL^{-1} amikacin. Working concentrations ranging from 1 - 16 µg mL^{-1} were prepared by serial dilution from this stock solution using the sterilized phosphate buffer.

Agar well diffusion bioassay method

The agar well diffusion method for susceptibility testing was adopted using a 100 x 20 mm petri dish. Each agar plate (sterilized) was first inoculated aseptically using a sterile swab. A sterile cotton swab was aseptically dipped into *S. aureus* (1 x 10^8 CFU mL^{-1}) broth culture, the excess liquid was then removed by gently pressing or rotating the soaked swab against the inside of the tube. A streak of the inoculum (swab) was made on the agar plate such that a uniform lawn was formed on agar surface. The plate was allowed to dry for 5 minutes. Four equidistant wells were aseptically made with a 6 mm diameter sterile cork borer. The base of each well was sealed with 2 drops (approximately 0.1 mL) of the molten agar and allowed to solidify. To each well, 100 µL amikacin standard and sample solutions (with concentrations 2, 4, 8 and 16 ug mL^{-1}) were delivered randomly and labelled appropriately in triplicates. Inoculated plates were then incubated at 37 °C for 18 h. The diameters of the zone of inhibition for each concentration were then measured for both standard and sample using antibiotic zone scale-C model PW 297.

To validate the agar diffusion method, the aforementioned parameters (calibration curve, precision, accuracy, LoD and LoQ) under spectrophotometric methods were determined (Zuluaga *et al.*, 2009; Solano *et al.*, 2011; ICH, 2005).

The calibration curve was obtained by plotting the logarithm of 5 concentrations of amikacin standard (1, 2, 4, 8 and 16 µg mL^{-1}) against mean diameters of zone of inhibition (mm). Assay was performed in triplicates and the linearity was determined by applying the least-square method.

The precision of the method was evaluated by repeatability (intra-assay) and intermediate (inter-assay) determinations and were expressed as relative standard deviation (%RSD). Both determinations were carried out in triplicates at three concentrations - 2, 4, and 8 μg mL^{-1} amikacin standard solutions.

To evaluate the accuracy, the percentage recovery was performed at three levels of concentrations - 4, 8 and 16 μg mL^{-1} with two brands of injections (AMK_1 and AMK_2) and amikacin reference standard. This was achieved by transferring 2.5 mL aliquots of 4 μg mL^{-1} amikacin injection solution into three 5 mL volumetric flasks and adding equal volumes of 4, 12 and 28 μg mL^{-1} amikacin standard solutions to mark and homogenizing gently. Each concentration was assayed aseptically in quadruplicate.

Procedure for application of assay method to pharmaceutical preparations

Spectrophotometric method

To 0.5 mL aliquot of solution A (AMK extracted from injection) in 10 mL volumetric flask, 0.2 mL of the ninhydrin reagent was added, diluted to 2 mL water and heated in a hot water bath (80 - 100 °C) for 10 minutes. This was then cooled and made to volume with phosphate buffer. Absorbance of resultant AMK-ninhydrin purple complex was measured at 650 nm.

Agar diffusion method

Amikacin sulfate injection, 10 μL, was transferred into a 100 mL volumetric flask containing 20 mL, sterilized phosphate buffer solution (pH 8.0), homogenized and made to mark with the same buffer solution with the buffer to obtain a stock concentration of 25 μg mL^{-1}. Working concentrations of 2, 4, 8 and 16 μg mL^{-1} were prepared and plates were inoculated and incubated at 37 °C for 18 -20 hrs. The diameters of the zone of inhibition for each concentration were then measured and mean values were used to calculate amount of AMK in drugs.

RESULT AND DISCUSSION

Visible spectra of derivatized-amikacin

Figure 2, shows the spectra of derivatized amikacin by condensation reaction using ninhydrin solution at a pH of 8.0 (phosphate buffer) in the visible region. The absorption of the Ruhemann's purple complex showed three distinctive peaks at 550, 650 and 800 nm, with peaks at 800 nm being the maximum.

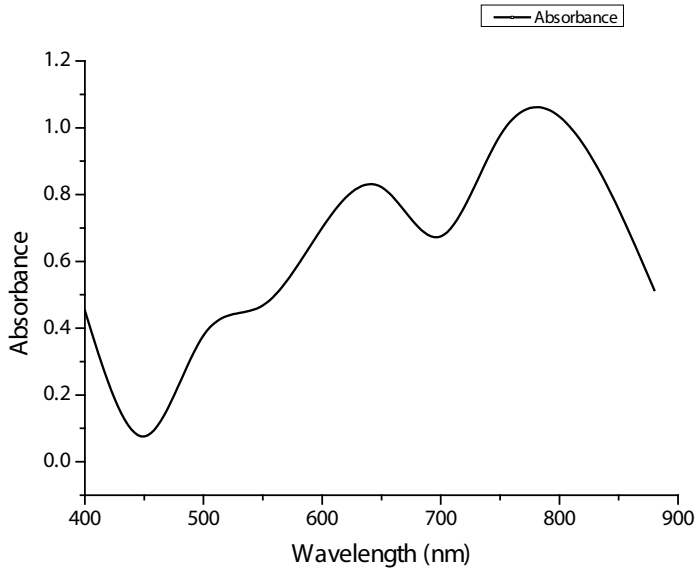

FIGURE 2 - Absorption spectrum of amikacin-ninhydin complex.

Equation and mechanism of reaction

The suggested equation of reaction for the condensation reaction of amikacin and ninhydrin is presented in Scheme 1, while the proposed mechanism of the reaction is shown in Scheme 2. The stoichiometric equation for the reaction between amikacin and ninhydrin was 1:2 ratio, with the formation of the Ruhemann's purple complex, two molecules of water and oxi-deaminated amikacin in which the α-NH_2 group in the 4-amino-2-hydroxybutyryl group was replaced by – COOH to form a 4-amino-2-hydroxybutyl carboxylic acid moiety.

Microorganisms for bioassay

The agar plates for zones of inhibition for *S. aureus*, *P. aeruginosa* and *E. coli* are presented in Figure 3 – with the *S. aureus* being the most appropriate test organism. The order of comparative susceptibility test with respect to AMK was *P. aeruginosa* < *E. coli* < *S. aureus*.

FIGURE 3 - Agar plates susceptibility test with AMK.

Analytical performance

Linearity, range and sensitivity

The analytical performance of both methods were assessed and validated in accordance with ICH guidelines (ICH, 2005).

Table I, shows the analytical characteristics exhibited by spectrophotometric and agar well diffusion methods. The calibration curves for AMK reference standard showed linearity for concentrations ranging from 10 - 50 µg mL^{-1} and 1 – 16 µg mL^{-1} for ninhydrin-spectrophotometric and bioassay methods respectively (Figures 4a and 4b). The intercept and slope obtained from the regression equations (y = bx + c), were 0.005 and 0.061 respectively for the spectrophotometric method, with corresponding values of 7.308 and 16.540 for agar diffusion method. Beer's plot was applied on the spectrophotometric method between concentration of drug and absorbance (n = 5), while for bioassay, the logarithm of the concentrations was plotted against the diameter of zones of inhibition (mm) (n = 5). The correlation coefficient (r) were 0.9998 and 0.9994 for spectrophotometric and bioassay methods respectively. These values indicated high sensitivity of methods. The LoD and LoQ were 0.98 and 2.97 µg mL^{-1} for spectrophotometric method respectively, while 1.07 and 3.24 µg mL^{-1} were obtained for the bioassay method. These values simply confirmed the repeatability and reliability of both methods, with spectrophotometric method having lower LoD and LoQ values. The high molar absorptivity of 2.595 x 10^4 L mol^{-1} cm^{-1} obtained for the AMK-ninhydrin derivatized condensation product also portrayed high sensitivity of the spectrophotometric method.

TABLE I - Analytical characteristics of methods

PARAMETER	Observed value	
	Spectrophotometry	Agar diffusion
Absorption wavelength (nm)	550, 650, 800 (λ_{max})	-
Concentration range (µg mL^{-1})	10 - 50	1 - 16
Molar absorptivity (L mol^{-1} cm^{-1})	2.595 x 10^4	-
Limit of detection (µg mL^{-1})	0.98	1.07
Limit of quantification (µg mL^{-1})	2.97	3.24
Regression equation		
Slope	0.005*	7.308**
Intercept	0.061	16.540
Correlation coefficient (r)	0.9998	0.9994

Legend: *Beer's concentration plot ** Agar well diffusion, Log concentration plot

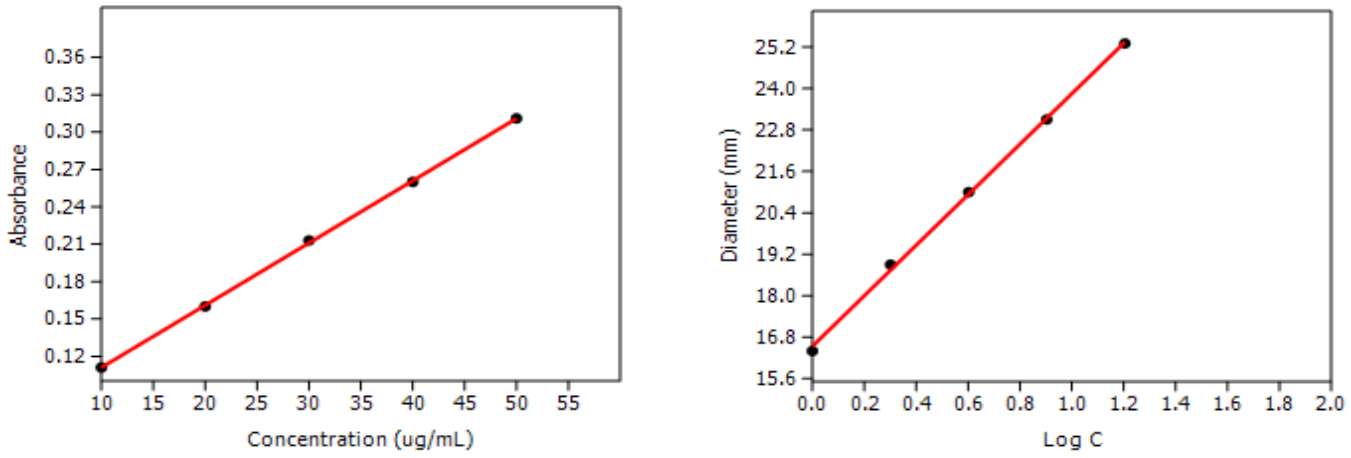

Figure 4 - Calibration curves for (a) ninhydrin spectrophotometric and (b) Agar plate diffusion methods

Precision and accuracy

The intra-day and inter-day precision for both methods are presented in Table II. The relative standard deviations (%RSD, n = 3) for the spectrophotometric method ranged from 0.5 - 1.88 % for both inter- and intra-day precision, while the bio-assay method ranged from 1.43 – 2.00%. The standard errors of the mean (SEM) for both methods were ≤ 0.22 for all runs. These values indicated high reproducibility, with satisfactory precision and accuracy of both methods.

TABLE II - Evaluation of precision and accuracy of methods

Analyte Drug	Taken (μgmL^{-1})	Intra-day determination (n=3)			Inter-day determination (n=3)		
		Found±Sd (μgmL^{-1})	RSD%	SEM	Found±Sd (μgmL^{-1})	RSD%	SEM
Spectroscopic	10	9.90±0.05	0.50	0.03	10.05±0.15	1.54	0.09
	15	15.07±0.08	0.57	0.05	14.82±0.25	1.70	0.15
	20	19.79±0.38	1.88	0.22	19.93±0.25	1.23	0.14
Bioassay	2	2.02±0.04	1.89	0.02	2.06±0.04	1.78	0.02
	4	4.11±0.08	1.94	0.04	4.03±0.06	1.43	0.03
	8	7.96±0.13	1.69	0.08	7.73±0.16	2.00	0.09

Robustness and recovery studies

The robustness and reliability of both methods were assessed under recovery studies - by assessing the effect of minor variations in some experimental conditions using two brands of the injection and spiked concentrations of pure AMK (Tables IIIa and IIIb). Calculated percent recovery ranged from 95.11 ± 3.88 – 100.25 ± 2.663 % for both brands in the ninhydrin-spectrophotometric method, while the bioassay method ranged from 97.67 ± 2.15 - 101.07 ± 1.24 %. These values implied that both variables do not have significant effect on the assay of AMK and both methods were considered robust and reliable for routine analysis.

TABLE IIIa - Recovery study of amikacin by spectrophotometric method

Amikacin brand	Amt. of drug in injection (μgmL^{-1})	Amt. of pure amikacin added (μgmL^{-1})	Total found (μgmL^{-1})	Recovery of added amikacin (%)
AMK_1	10	10	19.59± 0.37	95.93 ± 3.88
	10	15	24.99 ± 0.14	99.91 ± 0.93
	10	20	30.01 ± 0.28	100.07 ± 1.42
AMK_2	10	10	19.71 ± 0.33	97.10 ± 3.38
	10	15	24.69 ± 0.50	97.93 ± 3.37
	10	20	30.05 ± 0.53	100.25 ± 2.63

Table IIIb - Recovery study of amikacin potency by agar diffusion method

Amikacin brand	Amt. of drug in injection ($\mu g\ mL^{-1}$)	Amt. of pure amikacin added ($\mu g\ mL^{-1}$)	Mean & range (zone of inhibition) (mm)	Total quantity found (μgmL^{-1})	Recovery of added drug (%)
AMK_1	2	2	20.7 (20.7 - 21.2)	3.95 ± 0.09	97.67 ± 2.15
	2	6	23.0 (22.7 - 23.4)	7.96 ± 0.12	99.39 ± 1.49
	2	14	25.2 (24.9 - 25.6)	16.15 ± 0.20	101.07 ± 1.24
AMK_2	2	2	20.9 (20.5 - 21.5)	3.99 ± 0.09	99.58 ± 2.30
	2	6	23.1 (22.7 - 23.6)	8.00 ± 0.18	99.94 ± 2.22
	2	14	25.1 (24.9 - 25.2)	16.05 ± 0.10	100.36 ± 0.62

Assay of dosage form

Three brands of amikacin injection (AMK_1, AMK_2, AMK_3) were assayed by the proposed ninhydrin-spectrophotometric and the agar diffusion methods. Table IV, shows the results obtained for both methods. The pharmacopeia specification for amikacin injection ranges from 90.0 to 110.0% of the claimed amount (BP, 2015; USP, 2018). The amounts of drugs found in the injections were within the stipulated range and were in good agreement with label claims on all brands. Percentage content of amikacin ranged from 99.70 ± 1.82% to 100.30 ± 1.42% for spectrophotometric assay, while the biological method was from 97.80 ± 1.43% to 99.90 ± 1.51%. In terms of potency, the amount of amikacin in the injections were from 978 ± 14 – 999 ± 15 µg mg^{-1} amikacin sulfate (Figure 5) and they were considered satisfactory.

TABLE IV - Application of bioassay and spectrophotometric methods to formulation and statistical analysis

Amikacin brand (inj)	Label claim (mg mL^{-1})	Agar diffusion*		Spectrophotometry		**Paired t-test and F-test (n = 4)
		Amt. found (%)	Std error of mean	Amt. found (%)	Std error of mean	
AMK_1 (Crion)	250	98.10 ± 1.56 t = 2.78	±1.95	100.30 ± 1.42 t = 0.37	±1.78	t = 0.40; F = 1.32
AMK_2 (Ranbaxy)	250	97.80 ± 1.43 t =2.39	±1.79	99.89 ± 1.58 t = 1.14	±1.98	t = 0.93; F = 1.11
AMK_3 (Unbranded)	250	99.90 ± 1.51 t = 0.42	±1.88	99.70 ± 1.82 t = 2.31	±2.27	t = 2.07; F =3.94

* Percent potency converted to amount in percent; Student *t-te*st; **Paired t-test between agar diffusion and spectrophotometric methods. Theoretical value for t-distribution and F at 95% confidence limit for four determinations were 3.18 and 9.28 respectively

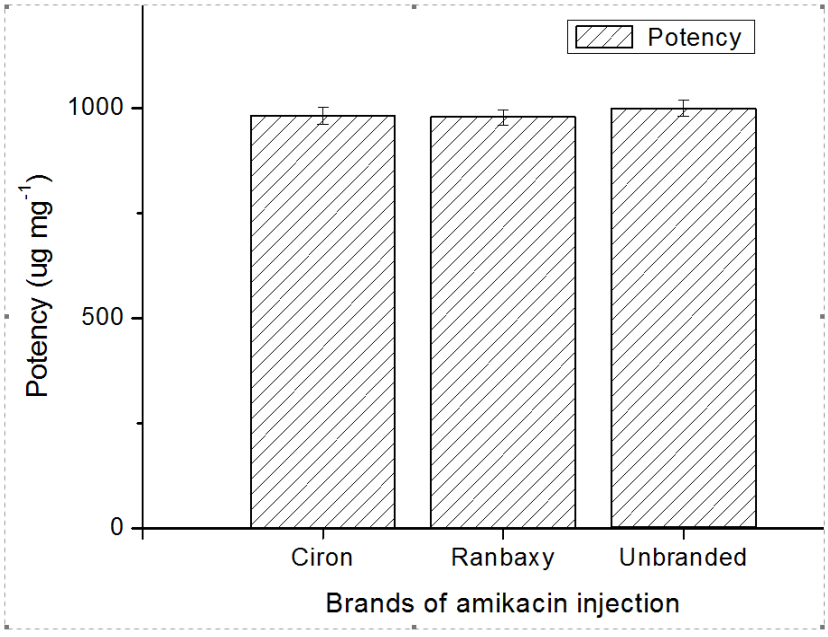

FIGURE 5 - Potency of amikacin sulfate injection.

Comparative analysis

Both assay techniques were compared using statistical analysis (Table IV). The student *t*-test was applied to ascertain if there are or no significant difference between the mean contents of AMK obtained for the two proposed methods and the label claim on the three brands of injection. The student's t-test values for both methods were ≤ 2.78, while the tabulated value for 4 replicates at 95% confidence is 3.18. This shows that there was no significant difference between the label claim on brands and values obtained in applying both spectrophotometric and biological methods (Karthik, 2016). However, the spectrophotometric method showed a better closeness to the label claim when compared to the bioassay method. In addition, the paired *t-test* and variance ratio *F-test* between both assay methods, showed that experimental values ranged from 0.40 to 2.07 and 1.11 to 3.94 respectively (i.e., $t \leq 2.07$; $F \leq 3.94$) - none exceeded the critical values ($t = 3.18$; $F = 9.14$) for three degrees of freedom. This also implies that there was no significant difference between both methods at the confidence level of 95% (Miller, Miller, 2005).

The LoD and LoQ values for both methods suggested that the ninhydrin-derivatized spectrophotometric method was more sensitive, with concentration differentials of 0.09 (9.2%) and 0.27 (9.1%) μg mL^{-1} respectively. The RSD% also indicated higher accuracy and precision for spectrophotometric method than the agar well diffusion technique (Table II). However, lower SEM values for the bioassay method compared to spectrophotometric technique depicts better precision with respect to the actual amount of drug assayed (McHugh, 2008).

The spectrophotometric technique was fast and time saving, simple, economical in terms of consumables and in addition more samples can be assayed within a day compared to agar diffusion method. However, the agar diffusion method was found to be more specific and demonstrated the potency and biological activity of the drug – which portrays its intended use as antibiotics against bacteria or the test organism. It was also found to be relatively cheap and simple. It is pertinent also to mention that both proposed methods do have limitations, when a large number of samples are involved – especially with biological or fluid samples. The use of HPLC assay method is preferable over both proposed methods with respect to the aforementioned, since hundreds of samples can be analysed within 24 hours using an auto-sampler. The proposed ninhydrin method have also shown to be a more convenient, simple, less expensive, and less time consuming with the Ruhemann's purple complex persisting for over 24 h compared to other spectrophotometric methods that involves charge transfer and extraction of coloured complexes.

Based on these limitations and advantages discussed for both proposed assay methods, it is imperative that both methods should be applied complimentarily in the assay of antibiotics during quality control assessment.

CONCLUSION

In this study, two analytical methods - agar well diffusion (bio-assay) and spectrophotometric methods were used to evaluate amikacin sulfate injection. Both methods were successfully validated for linearity, accuracy, precision and reproducibility. Comparatively, the spectrophotometric method was simple, quick, time saving and could be used for quantification of impurities and metabolites in biological samples; however, it is inferior to the bioassay method (agar well diffusion) in terms of assessment of biological activity, potency and loss of biological activity of the antibiotics and its intended use. Furthermore, it is the most appropriate method to evaluate the potency of antibiotics (Cazedey, Salgado, 2011). It is also pertinent to mention that the paired *t-test* and *F-test* (n = 4) indicated no significant difference between both methods and thus statistically comparable. In addition, both methods could be adjudged complementary and should be applied concomitantly for quality audit and assessment of antibiotics such as amikacin.

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