Iranian Journal of Analytical Chemistry
Volume 5, Issue 2, September 2018 (39-55)

مجله ایرانی شیمی تجزیه
دوره ۵، شماره ۲، مهر ماه ۱۳۹۷ (۵۵-۳۹)

روش‌های تجزیه‌ای برای تعیین آمیکاسین در نمونه‌های دارویی و سیالات بیولوژیکی: مقاله مروری

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تاریخ دریافت: ۲٤ مرداد ۱۳۹۷ تاریخ پذیرش: ۲۸ شهریور ۱۳۹۷

Analytical Methods for the Determination of Amikacin in Pharmaceutical Preparations and Biological Fluids: A Review

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Received: 15 August 2017 **Accepted:** 19 September 2018
DOI: 10.30473/ijac.2018.41591.1133

چکیده

چکیده: آمیکاسین متعلق به خانواده‌ی آمینوگلیکوزیدها از دسته آنتی‌بیوتیک‌ها می‌باشد. آمیکاسین طیف وسیعی از آنتی‌بیوتیک‌هاست که در برابر عفونت‌های باکتریایی گرم مثبت و گرم منفی موثر می‌باشد. ساختمان شیمیایی آمیکاسین شامل گروه‌های هیدروکسیل، گلیکو پیرانوزیل و آمینی می‌باشد. به هر حال فقدان کروموفر در ساختار آمیکاسین، شناسایی آن را در ناحیه ماوراء بنفش–مرئی (۲۰۰ تا ۸۰۰ نانومتر) مشکل می‌سازد. بنابراین، مشتق‌سازی از آمیکاسین صورت گرفت تا یک گروه کروموفر در ساختار آن وارد گشته و به شناسایی آن در ناحیه ماوراء بنفش–مرئی کمک کند. روش‌های متعددی برای تعیین آمیکاسین در نمونه‌های بیولوژیکی و دارویی توسعه و گزارش شده است. این روش‌ها شامل تکنیک‌های کروماتو گرافی از جمله کروماتوگرافی لایه نازک، کروماتوگرافی مایع، کروماتوگرافی مایع برهم‌کنشی آبدوستی، کروماتوگرافی مایع با کارآیی بالا، کروماتوگرافی مایع با کارآیی فوق‌العاده بالا، کروماتوگرافی یونی، طیف‌سنجی ناحیه مرئی–ماوراء بنفش، اسپکتروفلوئوریمتری، اسپکترومتری رزنانس مغناطیسی هسته، اسپکترومتری جرمی، کمی لومینسانس، روش پراکندگی رالی، روش های الکتروشیمی مانند پلاروگرافی، ولتامتری چرخه‌ای، آمپرومتری، سنجش‌های میکروبی و ایمن‌سنجی می‌باشد. این مقاله کاربرد این روش‌ها را در تجزیه آمیکاسین در نمونه‌های دارویی و بیولوژیکی مشخص می نماید.

واژه‌های کلیدی

آمیکاسین؛ تجزیه؛ کروماتوگرافی؛ طیف‌سنجی؛ روش‌های الکتروشیمی؛ ایمن‌سنجی.

Abstract

Amikacin (AM) belongs to the family of aminoglycoside antibiotics. It is a broad spectrum antibiotic, effective against both Gram-negative and Gram-positive bacterial infections. The chemical structure of AM consists of hydroxyl (OH), glucopyranosyl and amino (NH_2) groups. However, the lack of chromophore in the AM structure made it difficult to detect it in the UV-visible region (200–800 nm). Therefore, derivatization of AM is carried out to introduce a chromophore in its structure which helps in its detection in the UV-visible region. Several methods have been developed and reported for the determination of AM in pharmaceutical and biological samples. These methods include chromatographic techniques such as thin layer chromatography (TLC), liquid chromatography (LC), hydrophilic interaction liquid chromatography (HILC), high performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC), ion chromatography (IC); spectrometry such as UV-visible spectrometry, spectrofluorimetry, nuclear magnetic resonance (NMR) spectrometry, mass spectrometry (MS); chemiluminescence; Rayleigh scattering method; electrochemical methods such as polarography, cyclic voltammetry, amperometry; immunoassay and microbial assay. This review highlights the application of these methods in the analysis of AM in pharmaceutical and biological samples.

Keywords

Amikacin; Analysis; Chromatography; Spectrometry; Electrochemical Methods; Immunoassay.

1. INTRODUCTION

The development of a large number of drugs in a variety of dosage forms provides numerous ways to treat human ailments. However, the therapeutic activity of these drugs is strictly associated to their purity, quality, and dose to be administered. In order to obtain maximum therapeutic benefit from

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drugs, various physical and chemical methods have been developed to evaluate the contents of active ingredients in their respective dosage form. Therefore, the analysis of a pure drug in the presence of formulation ingredients, degradation products, impurities, and metabolites is extremely crucial for product formulation studies. Since the pharmaceuticals may develop impurities/degradation products at various stages of their manufacture, transportation, and storage, it becomes necessary to detect such products and quantify the active substance.

Amikacin (AM) or amikacin sulfate is a semi-synthetic aminoglycoside antibiotic which was derived from kanamycin B to resolve the problem of drug resistance. It is widely used in human and veterinary medicine because of its good activity against Gram-positive and Gram-negative bacteria. A number of different analytical techniques have been utilized to study AM, its salt AM sulfate, and its degradation / metabolic products leading to a better understanding of the stability and mode of action of the active drug both in the pure form and in formulated products. The various analytical methods which have been reported in the literature for the quantitative determination of AM are discussed below.

2. CHROMATOGRAPHIC TECHNIQUES

2. 1. Thin-Layer Chromatography (TLC)

TLC is a very commonly used technique for the identification of pharmaceutical compounds, separation of non-volatile mixtures, determination of purity and to follow the progress of a reaction. This technique has also been used for the determination of aminoglycosides (AGs) in pharmaceutical dosage forms (injections, capsules, eye drops, solutions, ointments, etc). The AGs including AM have been separated on silica gel plates and ninhydrin was used for the location of the spots while analysis was carried out in situ using TLC. This method showed good linearity, repeatability with a limit of detection (LOD) of 60–200 ng [1].

2. 2. Liquid Chromatography (LC)

Liquid chromatography is a technique used to separate a sample into its individual components. The separation occurs on the basis of interaction of the sample with the mobile and stationary phases. Because there are many stationary/mobile phase combinations that can be employed for the separation of a mixture, there are several different types of chromatography that are classified on the basis of the physical states of those phases.

2. 2. 1. Analysis in biological fluids

In biological fluids, AM has been determined by a selective LC method with online post-column derivatization. In this method, reversed phase (RP)-column with pentane sulfonic acid (ion-pairing reagent) was used in the presence of tobramycin as the ion-exchanger as well as the internal standard (IS). AGs were eluted with alkaline sodium sulfate solution after complete removal of plasma proteins and then injected into the system. The separated material was then derivatized by ortho-phthalaldehyde (OPA) and mercaptoethanol in borate buffer (pH 10.4) at 50°C. The fluorimetric detection was carried out at λ_{ex} = 340 and λ_{em} = 418 nm with a t_R of 8 min at a flow rate of 1.2 ml/min. The limit of quantification (LOQ) for AM in plasma sample was found to be 25 ng/ml [2]. A new simple LC method along with tandem mass spectrometry (LC–MS/MS) has been developed for the determination of AGs including AM in human plasma. The separation of the antibiotics was based on ion-pair chromatography on an RP–C_{18} column. It was successfully applied to the determination of AM in human plasma samples previously deproteinized by using heptafluorobutyric acid (HFBA) and injecting 1 µl of supernatant into the chromatographic system. The method showed good accuracy and precision [3].

A study has been conducted for the determination of AM in epithelial lining fluid (ELF) via a bronchoalveolar lavage (BL) in newborns. In this method, the BL fluid was taken from ventilated neonates who were treated with AM (intravenous injections). The determination of AM was carried out by LC with pulsed electrochemical detection (LC–PED) and capillary electrophoresis with capacitively coupled contactless conductivity detection (CE–C^4D) [4]. Another simple and sensitive quantitative ion-pair LC/MS method for the determination of AM, gentamicin, and vancomycin in newborn human plasma was developed. In this method, the plasma proteins (25 µl) were precipitated by using trichloroacetic acid and the detection was carried out by ion-pair RP–LC coupled with electron spray ionization tandem mass spectrometry (ESI–MS). The t_R for each sample was 7.5 min and the calibration curve was found to be linear in the concentration range of 0.3–5.0 and 1.0–100 mg/l for AM and vancomycin, respectively. The LOQ accuracy was in the range of 103–120% with the precision of >19% and for above LOQ concentration the accuracy was found to be in the range of 98–102% with a precision of >6% [5]. The quantification of AM and kanamycin has been carried out using LC–MS/MS in human serum and apramycin as an IS. The method showed good accuracy, precision, recovery, and selectivity with an LOQ of 250

ng/ml and 100 ng/ml for AM and kanamycin, respectively [6].
LC and enzyme immunoassay methods have also been developed for the assay of AM in a raw milk sample. The anti-AM antibodies were developed by the immunization of rabbits with AM-bovine serum albumin (BSA) conjugate. It has been found that immunoassay has an IC–50 value of 1.30 ng/ml with spiked recoveries of 25–1000 ng/ml, recoveries of 69.8–93.3% and CVs value of 8.5–17.6% [7]. An automated solid phase extraction (SPE) and LC–MS/MS method has been developed for the simultaneous determination of 15 AGs in pigs, chicken, cattle, kidney, cow milk and hen eggs. Monopotassium buffer (ethylenediaminetetraacetic acid) was used for the extraction of the homogenized sample while cleaning was carried out by automatic SPE by carboxylic acid cartridges. The analytes were separated by a particular column for AGs and eluted with trichloroacetic acid and acetonitrile. The calibration curve was found to be linear for AGs in the concentration range of 20–1000 µg/kg with reasonable recoveries of 71–108%. The LOD for all AGs was found to be 8.1–11.8 µg/kg [8].

2.2.2. Analysis by fluorescence detection
Another LC method for the determination of AGs (AM, gentamicin, neomycin) has been developed and validated. The determination of AGs was carried out by a pre-column reaction with 7-fluoro-4-nitrobenzene-20-oxo-1,3-diazole and the derivatives formed were analyzed using an RP–LC Ultrasphere C_{18} column with fluorescence detection at λ_{ex} = 465 and λ_{em} = 531 nm. The method showed good linear response for AGs in the concentration range of 0.65–10 µg/ml with a correlation coefficient of greater than 0.9999 [9].

2.2.3. Analysis by evaporative light scattering detection
A direct determination of AM and its precursor (kanamycin) has been made by a newly developed and validated method on an RP–LC column using evaporative light scattering detection (ELSD). The technique employed ion-pairing acidic reagents of increased molecular mass, increased mobile phase volatility and decreased peak width and asymmetry that resulted in a greater ELSD response. In this method, Thermo Hypersil Beta Basic C_{18} column with a mobile phase consisting of a mixture of water and methanol (60:40, v/v) and 3.0 ml/l of fluoropentanoic acid (18.2 mM) was used. For ELSD the optimal experimental conditions were nitrogen pressure of 3.5 bars and evaporation temperature at 50 °C. The calibration curves for AM and kanamycin were found to be in the range of 7–77 and 8–105 µg/ml, respectively, with LODs of 2.2 and 2.5 µg/ml, respectively [10].

2. 3. Hydrophilic Interaction Liquid Chromatography (HILC)
HILC is a variant of normal phase liquid chromatography that uses hydrophilic stationary phase with reversed-phase type eluents and is particularly applicable when using electrospray mass spectrometry due to ionization of the analyte in the solution. For the determination of six AGs in serum, HILC with mass spectrometry (MS) has been applied. An automatic and specific method was developed for the quantification of AM, gentamicin, kanamycin, neomycin, paromomycin, and tobramycin. An automated SPE was used to prepare samples and HILC was used for the separation of analytes from endogenous compounds. Electrospray ionization with tandem mass spectrometry (ESI–MS) was used for the detection of AGs with LOQ value of 100 ng/ml [11].

2. 4. High-Performance Liquid Chromatography (HPLC)
HPLC is basically an improved form of column chromatography which is used to separate, identify and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent (mobile phase) containing the sample mixture through a column filled with a solid adsorbent material (stationary phase). The bulk and pharmaceutical products of AGs prepared either through fermentation (kanamycin, gentamicin, sisomicin, and tobramycin) or synthetically (AM) are determined by HPLC. A C_8 RP–column has been used and the method is based on derivatization of AGs with 2,4,6-trinitrobenzenesulphonic acid followed by UV detection at 350 nm [12]. A simple, accurate, precise, rapid, economical and stability-indicating pre-column derivatization HPLC method has been developed for the determination of AM. In this method the solution of AM was derivatized before analysis and acetonitrile with water in a ratio of 50:50 (v/v, pH 6.0) was used as the mobile phase. The t_R for the determination of AM was found to be 4 min and the method showed good linearity in the concentration range of 12.5–125%. The accuracy of the method was 99.88±0.42% while the LOD and LOQ values were found to be 5.35 and 16.21 µg/ml, respectively [13].
A simple, rapid and sensitive HPLC method has been employed to study AM in dog plasma. For this purpose, deproteinization of the sample was carried out using 10% (w/v) trichloroacetic acid and centrifugation of the sample was performed to obtain the supernatant. A 100 µl quantity of

supernatant was injected into an RP-column with an ion-paring mobile phase (octyl sulfate). Post-column derivatization was carried out with OPA, 2-mercaptoethanol and fluorescence detection was performed at an excitation wavelength of 340 nm and emission at 415 nm. The overall recovery was found to be 99.5–105% with a concentration range varying from 0.2–2.0 µg/ml and a regression of 0.999. The %RSD was found to be in the range of 4.7–1.1 while LOD was 25 ng/ml [14]. Similarly, a simple and sensitive pre-column derivatization method has been developed for the determination of AGs using OPA as the derivatizing agent while the derivatives of AGs were stabilized by β-mercaptopropionic acid. The quantification was carried out by RP–HPLC in the presence of optimal conditions such as OPA concentration, pH, and temperature. The fluorescent derivative formed from sisomicin was found to be stable for 6 h in methanol (50%). The plasma samples when spiked with sisomicin were found to produce a linear response in the concentration range of 136–900 µg per injected volume of 40 µl with LOD of 0.06 µg/ml. The method was successfully applied to the determination of whole blood samples of the rabbit after subcutaneous injection of 1 mg/kg of AGs. The LOD for sisomicin and netilmicin for dried blood spots (DBS) was observed as 0.053 and 0.50 µg/ml, respectively [15].

A simultaneous method for the determination of daptomycin, AM, gentamicin and rifampicin in human plasma has been developed. The sample was prepared by the precipitation of protein with acetonitrile followed by direct injection into the HPLC column coupled with mass detection. Drug retention times for daptomycin, AM, gentamicin and rifampicin were found to be 10, 2, 3.5 and 11.5 min, respectively. The method showed good linearity for the drugs in the range of clinically relevant concentrations in human plasma. The values of LOQ for the drugs were found to be 1.56, 2.34 and 0.63 for both gentamicin and rifampicin, respectively [16]. A simple, rapid and sensitive HPLC method has been developed with fluorimetric detection (HPLC–FLD) for the determination of AM in human plasma. OPA in the presence of N-acetyl-L-cysteine (NAC) at pH 9.5 was used as a derivatizing agent for AM for 5 min at 80 °C. An RP–LC column with phosphate buffer (0.05M) and acetonitrile in the ratio of 80:20 (v/v) was used as mobile phase at a flow rate of 1.0 ml/min. This method showed good linearity in the concentration range of 0.17–10 µg/ml with a correlation coefficient of 0.9995 [17]. Similarly, another simple, rapid and sensitive method was developed for the determination of AM in human plasma and urine samples. The centrifugation of the body fluids was carried out after dilution with an ethanol/sodium carbonate mixture. After centrifugation, an aliquot was directly injected into the RP–C_{18} column and the detection was based on the formation of a complex with Cu (II). The catalyst used for the formation of the complex was luminal/hydrogen peroxide. The study showed good linearity in the range of 0.15–2.0 µg/ml and the LOD was observed to be 50 µg/ml in plasma or urine. The %RSD for intra- and inter-day was 9% whereas the percent recovery in plasma and urine was found to be 92% [18]. Another RP–HPLC method has been developed for the quantitative determination of 11 drugs in human urine. The drugs included imipenem, paracetamol, dipyrone, vancomycin, fluconazole, cefazolin, prednisolone, dexamethasone, furosemide, ketoprofen, and AM. The separation of the drugs was carried out by C_{18} column and detection was made by diode array (DAD) and fluorescence (FL) detectors. The mobile phase used in different proportions included methanol, acetonitrile and 0.05% trifluoroacetic acid in water while ortho-phthaldehyde-3-mercaptopropanoic acid (OPA–3–MPA) was used as a fluorescent agent for AM in RP–HPLC–FL. The t_R for all the tested drugs was found to be in the range of 4.01–18.98 min. The method showed good linearity in the concentration range of 0.03–52 µg/ml for all drugs [19]. Determination of AM has also been carried out in serum by an HPLC method in which AM was separated from serum via adsorption on silica gel followed by its derivatization by OPA and eluted via ethanol. The derivatized drug was quantified by fluorimetry and the method showed good linearity for AM in the concentration range of 1–15 µg/ml [20]. A simple HPLC method for the assay of gentamicin, AM, and tobramycin in serum has also been developed in which the AGs were extracted from serum using a CM–Sephadex and were determined by RP ion-chromatography. The post column derivatization was carried out by OPA that was used for the formation of fluorescent products and their detection. The method showed good linearity for the determination of AGs in the studied concentration range with a CV of <4% [21]. In another study, HPLC has been used extensively for the determination of AM in serum. The drug was extracted by a disposable cation exchange column and the method was based on the derivatization of AM by 1-fluoro-2,4-dinitrobenzene, which was then analyzed by RP–HPLC with UV detection at 365 nm. Kanamycin was used as an IS and the % recovery for AM was found to be 72%. The precision of the method expressed in CV was about 3% [22]. The determination of AM in µl quantities in biological fluids using 1-fluoro-2,4-dinitrobenzene as a derivatizing agent has been performed through

HPLC. During the derivatization process formation of stable chromophore also took place. An ultrasphere–ODS C_{18} column protected with a guard column and acetonitrile with water in the ratio of 68:32 (v/v) has been used as a mobile phase. The method showed good linearity in the concentration range of 2–64 µl with an average CV of less than 5% [23].

A pre-column derivatization method for the determination of AM in the presence of the synthetic precursor, kanamycin sulfate, in bulk and pharmaceutical dosage forms by HPLC has been developed and validated. In this method, the pre-column derivatization was based on Hantzsch condensation reaction and the colored products formed were separated by RP–HPLC. The separation was carried out on a Spherisorb C_{18} ODSL column (250×4.6 mm, 5 µm) using the mobile phase of a mixture of acetonitrile (0.1 M) and sodium acetate buffer (pH 5.0, 25:75, v/v). The column temperature was maintained at 35 °C with a flow rate of 2 ml/min and detection of AM and kanamycin was carried out by using DAD at 330 nm. The method showed good linearity, accuracy, precision, specificity, and robustness [24]. HPLC with post-column derivatization has also been utilized for the determination of AM in parenteral dosage forms. In this method, Waters Sunfire C_{18} column (4.6×150 mm, 5 µm) with a mobile phase of sodium sulfate and methanol (97:3, v/v) was used at a flow rate of 1 ml/min. The temperatures for the column and post-column derivatizations were 25 and 45 °C, respectively, with a fluorescence detection at λ_{ex}=360 and λ_{em}= 440 nm. The method showed good linearity in the concentration range of 2.09–41.81 mg/l with an average recovery of 98.6% [25]. Another RP–HPLC method has been developed for the simultaneous determination of cefepime and AM in injections. In this method, a C_{18} (25 µm) column has been used with the mobile phase of acetonitrile:water (10:90, v/v) at a flow rate of 1 ml/min. The detection was carried out at 212 nm with a t_R of 2.51 and 6.23 min for AM and cefepime, respectively. The method showed good linearity for cefepime and AM in the concentration range of 20–100 µg/ml. The recovery for cefepime and AM was found to be 98.22±0.56% and 99.63±0.57%, respectively [26]. An HPLC method has been developed and validated for the assay and particle size distribution (PSD) of AM in an aerosol. The performance of a charged aerosol detector (CAD) has been compared to a UV detector. In this method, weighed ($1/X^2$) least squares regression has been used for the preparation of calibration curve. The method showed good linearity in the studied concentration range with a correlation coefficient of 1.0. The accuracy of the method has been found in the range of 100–101% with an LOQ value of 4% [27].

An RP–HPLC method has been employed for the determination of colistin and AGs such as AM, kanamycin, gentamicin, and neomycin by post-column derivatization followed by fluorescence detection. In this method, the extraction of analytes was carried out by sonication followed by shaking with dilute HCl and the post-column derivatization by using orthophthalaldehyde-2-mercaptoethanol. The colistin assay was carried out using acetonitrile–aqueous sodium sulfate-triethylammonium phosphate (pH 2.8) as a mobile phase in a ratio of 22:78 (v/v) whereas for the separation of AGs two eluents were tested, tetrahydrofuran-aqueous sodium sulfate (3:97, v/v) and acetic acid-pentanesulfonate (1.5:98.5, v/v). The method was successfully employed on pharmaceutical preparations and could also be applied for the assay of these antibiotics in other samples such as meat and animal serum for residues and pharmacokinetic studies. The method showed good linearity in the concentration range of 0.4–320 mg/l with LOD values of 0.15, 0.22, 0.18, and 0.20 mg/l for AM, kanamycin, gentamicin, and neomycin, respectively. The recoveries were also found to be 92.4, 84.6, 89.1 and 91.1% for neomycin, gentamicin, AM, and kanamycin, respectively [28].

Chromatographic separation with indirect fluorescence detection has also been developed for the analysis of AM. The method was based on the displacement reaction between the AGs and Cu (II)-L-tryptophan complex. Since AM has a greater affinity towards Cu (II) ion and results in the displacement of L-tryptophan (L-Trp) from the Cu (L-Tryp) complex. The resultant increase on L-Trp fluorescence was measured, which is quenched when coordinated with Cu (II) was due to the presence of AG. It has been found from fluorescence titration that there was a stoichiometric ratio of 1:1 between the reaction of AGs and Cu $(L\text{-}Trp)_2$ complex. The HPLC detection was carried out by mixing the buffered Cu $(L\text{-}Trp)_2$ solution with the mobile phase whereas the separation was carried out using a column packed with a polymeric strong cation exchanger. The calibration curve was found to be linear for AM in the concentration range of 25–586 ng with LOD of 4.2–14.5 ng [29].

An HPLC method with evaporative light scattering detection (ELSD) for the determination of AM and related substances has been developed. The method employed a C_{18} column with the mobile phase of water, ammonium hydroxide and glacial acetic acid (96:3.6:0.4, v/v/v) at pH 10.0. The flow rate for the mobile phase was 0.8 ml/min, the temperature for the drift tube was 110 °C and the

flow rate of gas was 3 l/min. The calibration curve was found to be linear in the concentration range of 0.05–2.5 mg/ml with the regression of 0.9998 and recovery of 99.6–101.2% [30]. Similar parameters except for a few changes in pH (10.0–10.2) and temperature (30 °C) have been employed while determining AM by an HPLC–ELSD method. The method showed good linearity for AM in the concentration range of 0.1–1.5 mg/ml with an average recovery of 99.1% with RSD of 0.21% [31]. An HPLC–ELSD method for the determination of AM in lotions has also been developed using an Agilent ZORBAX Extend C_{18} (4.6×250 mm, 5 µm) column with a mobile phase of water:ammonia:glacial acetic acid (96:3.6:0.4, v/v) at a pH of 10.0 and a flow rate of 1 ml/min. The temperature of the drift tube was 50 °C with a pressure of 3.5×10^5 Pa for the nebulizing gas. The method showed good linearity for AM in the concentration range of 0.6–6.0 mg/ml with a CV of 0.9999 and an average recovery of 100.2% [32].

A new method for the determination of AM through derivatization with 6-aminoquinoyl-N-hydroxysuccini-midyl carbamate (AQC) by HPLC with UV detection has been reported. The derivatization was carried out at room temperature with borate buffer (pH 8.0) and the method showed 0.068 µg/ml LOD with a precision of <1% [33]. The simultaneous determination of AM, tobramycin, and kanamycin has been carried out by micellar electrokinetic chromatography (MEKC) with UV detection. A tris buffer (180 mM, pH 9.1) with 300 mM sodium pentane sulfonate (SPS), as an anionic surfactant, was used. The method was found to be linear in the concentration range of 0.1–0.5 mg/ml for AM and 0.4–2.0 mg/ml, each for tobramycin and kanamycin. The value of LOD for AM was found to be 0.08 mg/ml and for tobramycin and kanamycin each 0.2 mg/ml [34].

The determination of AM and its impurities through two-step liquid chromatography with pulsed electrochemical detection has been carried out. The mobile phase used in the method comprised of 1.8 g/l, sodium 1-octane sulfonate, 14 ml/l tetrahydrofuran, 50 ml/l of phosphate buffer (pH 3.0), and 20 g/l sodium sulfate. A solution of 0.5 M sodium hydroxide was also added to enhance the post-column detection. The method showed good sensitivity, accuracy, efficiency, and linearity in the concentration range studied [35].

A strong cation exchange chromatography (CEX–HPLC) with sensitive chemiluminescence (CL) detection has been developed for the determination of AGs in water. The method reduces the necessity of derivatization and was based on the inhibitory effect of AGs on the CL reaction between luminol and H_2O_2 catalyzed by Cu (II). In this method, AGs were successfully separated within 10 min on an SCX-column using a mixture of 5.0×10^{-3} mol/l sodium acetate and 0.65 mol/l of NaCl (pH 6.1) as the mobile phase. The LOD of the AGs was found to be in the concentration range of 0.7–10 µg/l with an RSD of 2.7–5.4% [36].

The development and validation of an HPLC method have been carried out for the determination of AM in water samples by solid phase extraction (SPE) and pre-column derivatization. In this method, the AM residues were extracted from water samples by SPE cartridge and extracts were treated with a derivatizing agent (4-chloro-3,5-dinitrobenzotrifluoride) at a temperature of 70 °C for 20 min. The derivative formed was separated using a C_{18} column and detection was carried out at 238 nm. The method showed good linearity in the concentration range of 0–500 µg/l with LOD and signal-to-noise ratio of 0.2 µg/l and 3, respectively. Percent recoveries of AM were found to be 87.5–99.6% for 3 different samples of water with RSD of 2.1–4.5% [37].

2.4. 1. Ultrahigh-performance liquid chromatography (UHPLC)

UHPLC is a powerful tool for increasing HPLC sample throughput, chromatographic efficiency and sensitivity. A UHPLC method has been developed for the determination of AM in human serum. The method was based on the derivatization of AM with fluoroenylmethyl chloroformate (FMOCCl) and glycine while pre-column derivatization was carried out by FMOCCl in the presence of 0.2 M borate buffer. Glycine (0.1 M) was used as a stabilizing agent to stabilize the formed fluorescent complex and fluorescence detection for AM was carried out at 315 nm on excitation at 265 nm wavelength. Acetonitrile and water in the ratio of 70:30 (v/v) at a flow rate of 0.4 ml min^{-1} were used as a mobile phase. The developed method showed good linearity in the concentration range of 0.5–10 µg ml^{-1} with a correlation coefficient of 1.0 [38]. A similar technique with tandem mass spectrometry (MS) has also been used for the identification and quantification of nine AGs in bovine kidney, liver and muscle tissues. The AGs include neomycin, streptomycin, dihydrostreptomycin, kanamycin, gentamicin, apramycin, AM, and hygromycin while tobramycin was used as an IS. The recoveries were found to be 70–120% for all AGs except for hygromycin that was 61% [39].

2. 5. Ion Chromatography

AM in raw materials and pharmaceutical preparations has been determined by ion chromatography. In this method, carbopac MA1 column with a mobile phase of deionized H_2O and

400 mmol/l of NaOH at a flow rate of 0.4 ml/min was used. The calibration curve was found to be linear in the concentration range of 0.9–4.8 µg/ml with a LOD of 3.2 µg for AM [40].

3. SPECTROMETRIC TECHNIQUES

Several spectrometric techniques have also been reported for the analysis of AM which are presented in the following sections:

3. 1. Ultraviolet and Visible Spectrometry

The quantitative determination for AM, kanamycin, neomycin, and tobramycin has been carried out by a spectrometric assay. The method is based on the Hantzsch reaction by forming dihydrolutidine derivatives that were measured spectrometrically. The excipients used in the study such as EDTA, phenol, sodium bisulfate and sodium citrate did not interfere while salts of ammonia did interfere in the assay. The method showed good linearity in the studied concentration range with RSD of 1.64, 1.88. 2.10, and 1.93% for AM, kanamycin, neomycin, and tobramycin, respectively [41]. It has been reported that certain AGs react with 2,3,5,6-tetrachloro-p-benzoquinone in borate buffer at pH 9.0 to form charge transfer complex. These complexes show absorption maxima at 350 nm with molar absorptivities of 3.78, 4.69, 5.0 and 7.08×10^5 for kanamycin, neomycin B, AM, and tobramycin, respectively. Good accuracy and sensitivity have been shown in the high-intensity bands of charge transfer complexes [42]. The simultaneous determination of neomycin, kanamycin, tobramycin, and AM has also been carried out by spectrometry. The method is based on the Rimini test with disodium pentacyano-nitrosylferrate (II) for primary and secondary aliphatic amines. The results showed that Beers Law is obeyed in the concentration range of 1.7×10^{-4}–1.9×10^{-3} M for neomycin, 2.6×10^{-4}–2.0×10^{-3} M for kanamycin, 2.5×10^{-4}–1.6×10^{-3} M for tobramycin, and 2.8×10^{-4}–1.7×10^{-3} M for AM. The method has been used to check the purity of the antibiotics with good accuracy, precision and relatively fast speed [43]. Tobramycin and other AGs such as AM, neomycin, gentamicin, and kanamycin were analyzed by a spectrometric method using derivatizing agents such as OPA, fluorescamine and dansyl chloride. The results obtained from this method for the determination of tobramycin and AGs were compared with results obtained from the previously developed method. It was found that OPA and fluorescamine showed good and reproducible results as compared to that of dansyl chloride [44].

The spectrometric determination of AM via charge transfer complex formation using tetracyanoethylene (TCNE, 0.129 g/100 ml) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 0.0127 g/100 ml) reagents has been carried out. Absolute ethanol, borate buffer (0.05 M, pH 9.0), sodium carbonate solution (0.0528 g/100 ml) and the standard solution of AM (100 µg/ml) were used. Sodium carbonate solution (5×10^{-3} M) with TCNE (1.2 ml) was added to the AM solution and the absorbance of the colored solution was measured at 330 nm (20 °C). For the analysis of the complex with DDQ, to a 200 µg/ml AM 0.5 ml of borate buffer (pH 9.0), 1.5 ml of DDQ (10^{-3} M) was added and the solution was kept for 30 min in a water bath at 40 °C. The absorbance of the colored complex was measured at 340 nm. The method showed good linearity in the concentration range of 0.4–6.8 and 0.8–20.0 µg/ml for TCNE and DDQ complexes, respectively. The LOD and LOQ for TCNE and DDQ complexes were found to be 0.06 and 0.18, 0.19 and 0.63 µg/ml, respectively while the recovery for AM in TCNE and DDQ complexes was 96.54 and 99.47%, respectively [45]. Another spectrometric method has been developed for the determination of AM after derivatization. The stability of AM has been evaluated with 0.9% sodium chloride injection. Acetylacetone-formaldehyde was used as a derivatizing agent and boric acid-acetic acid solution was used as a buffer. The detection was carried out at 339 nm. The method showed good linearity in the concentration range of 0.2–1.5 g/l with a correlation coefficient of 0.9991 [46]. A UV spectrometric method for the simultaneous estimation of cefepime hydrochloride and AM has been reported. The wavelengths of 257 and 191 nm were selected for cefepime HCl and AM analysis, respectively. The linearity was observed in the concentration range of 5–11 µg/ml for AM and 20–44 µg/ml for cefepime HCl. The recoveries were found to be in the range of 98.08–101.87% for both cefepime HCl and AM, respectively [47].

A UV spectrometric method has been developed for the analysis of AM, kanamycin, neomycin, and streptomycin in bulk and in pharmaceutical dosage forms. In this method, nitrosation of the primary amino groups was followed by cyanoacetamide reaction in ammonia at 100 °C (30 min). The products formed in this reaction have marked absorption maxima at 270 nm. The method showed good linearity for AM, kanamycin, and neomycin in the concentration range of 4–40 µg/ml and for streptomycin 8–80 µg/ml [48]. The quantitative determination of AM sulfate in injection has been carried out by a UV spectrometric method. Under optimal conditions of temperature and pH, AM reacts with ninhydrin to form a coordinated compound that can be analyzed at 568 nm. The method showed good linearity in the concentration

range of 15–27 µg/ml with regression of 0.9996 while RSD of 0.83% was found for 99.56% average rate of recycling [49]. A derivative spectrometric method for the determination of cefepime HCl and AM in combined parenteral dosage forms have been developed. The method was based on the respective zero crossing point (ZCP). The first-order derivative spectra were obtained in 0.1 N HCl and the determinations were carried out at 220 nm (ZCP of cefepime HCl) for AM and at 294 nm (ZCP of AM) for cefepime HCl. The method showed linearity in the concentration range of 20–100 µg/ml for both cefepime HCl and AM. The LOD for cefepime HCl and AM was found to be 0.52 and 0.65 µg/ml, respectively while the LOQ was found to be 1.58 and 1.97 µg/ml, respectively. The recovery for both drugs was 99.53 and 98.94%, respectively [50].

In another study, two spectrometric methods have been proposed for the determination of AM, gentamicin, kanamycin sulfate, streptomycin sulfate, neomycin sulfate, and tobramycin in pure form and in pharmaceutical dosage forms. The methods were based on proton transfer from the acid such as 2,4,6-trinitrophenol (TNP), or 2,4-dinitrophenol (DNP) to the primary amino group of AG which acts as a base with the formation of yellow ion-pair complexes. Both methods showed good agreement for the Beer's law plot in the concentration range of 2.5–140 and 2.5–100 µg/ml for 2,4-DNP and 2, 4, 6-TNP complexes, respectively. The methods showed good linearity, accuracy, and precision for the determination of AG [51]. A new spectrometric method has also been developed for the determination of AM using vanillin in the pure and commercial dosage forms. The absorption maximum of AM was found to be 400 nm with a molar absorptivity of 5.27×10^3 l/mole/cm. The method showed good linearity in the concentration range of 10–50 µg/ml with a correlation coefficient in the range of 0.9991–0.998. The Sandell's sensitivity for this method with vanillin was found to be 0.004 at 0.45 µg/ml. The recovery for AM in the presence of vanillin was in the range of 103–106.4% with RSD of 0.004–0.005% [52]. Another validated spectrometric method for the determination of AM in pharmaceutical formulations has been developed that is based on the measurement of absorption maximum at 530 nm in aqueous solution. The method showed good accuracy, precision, and linearity in the concentration range of 5.0–8.0 mg/100 ml [53].

3. 2. Spectrofluorimetry

A spectrofluorimetric study has been carried out for the assay of AGs such as neomycin, tobramycin, AM, and kanamycin by using 2-hydroxyl-1-naphthaldehyde as a fluorogenic reagent. The method is based on the reaction between the fluorogenic reagent and AGs via their amino groups. The product formed showed maximum fluorescence intensity at 434 nm at the excitation wavelength of 366 nm. The method showed good linearity between fluorescence intensity and the concentration of neomycin and tobramycin, and AM and kanamycin of 0.5–5 µg/ml and 0.25–4 µg/ml, respectively. The recoveries were found to be in the range of 99.67–100.26% with the standard deviation of ±1.32 to ±1.69. The LOD for AGs was found to be 10 ng/ml [54]. A similar study has been conducted for the determination of AM through fluorimetric method. It is based on the charge transfer complex formation between AM as a donor and chlorovanillic acid (CA) as an acceptor. It has been found that AM reacts with CA in absolute ethanol at 25 °C that leads to the formation of a charge-transfer complex. The stability of the complex was about 50 minutes showing the excitation and emission peaks at 135 and 53 nm, respectively. The method showed good linearity in the concentration range of 80–200 µg/l, LOD was 28.9 µg/l while the recovery was found to be in the range of 95.5–101.4% with RSD of 0.47% [55].

The determination of certain AGs such as AM, tobramycin, neomycin sulfate, gentamicin, kanamycin sulfate, and streptomycin sulfate through fluorimetric technique has also been carried out. The method is based on the formation of a charge transfer complex between AG and safranin in a buffered medium (pH 8.0). The complexes were then extracted with chloroform and showed excitation maxima at 519–524 nm and emission maxima at 545–570 nm. The method showed good linearity in the concentration range of 4–60 pg/ml for AM, 4–50 pg/ml for gentamicin, neomycin sulfate, and kanamycin sulfate, 4–40 pg/ml for streptomycin sulfate and 5–50 pg/ml for tobramycin with good accuracy, precision, specificity and robustness [56]. A fluorimetric method has been used for the determination of AM. The excitation and emission wavelength were found to be 295 and 367 nm, respectively. The method is based on the conversion of an amino group to nitryl followed by its hydrolysis in acidic conditions to produce reducing sugar. The reaction of reducing sugar in the presence of ethylenediamine in phosphate buffer solution produces a strong fluorescent substance. The method has been found to be linear in the concentration range of 2×10^{-5}–5×10^{-6} mol/l while LOD was found to be 4.2×10^{-7} [57].

A spectrofluorimetric method has been reported for the determination of AM, neomycin sulfate, and tobramycin in pure form, pharmaceutical

formulations and human plasma. The method is based on the condensation of the drugs with ninhydrin and phenylacetaldehyde in a buffered medium (pH 6.0) which resulted in the formation of a fluorescent derivative and exhibited the excitation and emission maxima at 395 and 470 nm, respectively. The method was linear in the concentration range of 98.33–101.7% for the drugs in different dosage forms [58]. A study has been carried out on the lipoic acid capped europium nanoparticles (NPs) based on the spectrofluorimetric detection of primary amines. The method is based on fluorescence quenching by the formation of a complex between lipoic acid and the amines on the surface of europium NPs. The AGs determination was carried out by fluorescence quenching at 315 nm at pH 5.0 in pharmaceuticals and milk. The method for the determination of AGs including AM, gentamicin, and tobramycin showed good linearity in the concentration range of 0.75–27.5, 0.4–26 and 0.20–25 µg/ml, respectively [59].

3 .3. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy has been performed on AGs antibiotics (kanamycin, AM) in alkaline media. Since the antibiotics are hydrophilic in nature that contains aminated cyclitol linked with amino sugar and could bind to in vivo or in vitro negatively charged membranes. NMR and fluorescence depolarization studies have been carried out for the evaluation of the effect of AGs on phosphate heads and fatty acyl chains of phospholipids [60]. Another study has been conducted for the evaluation of AGs for their interaction with 3'-phosphotransferase [APH(3')-IIIa] and ATP. For the purpose of detection of intra- and inter-ring nuclear overhauser effect spectroscopy (NOES) of butirosin A and AM in ternary complexes with APH(3')-IIIa and ATP was studied by NMR method of transferred NOES (TRNOES). The enzyme bound structures for butirosin A illustrated that 2,6-diamino-2,6-dideoxy-D-glucose and D-xylose are responsible for the limited motions and stacking arrangement. The spectra of TRNOES predicted that AM having 6-amino-6-deoxy-D-glucose ring was flexible when it binds to APH (3')-IIIa [61]. NMR, UV-vis, circular dichroism (CD) and electron paramagnetic resonance (EPR) spectrometric techniques have been used to evaluate protonation and Cu (II) coordination properties of AM. Five coordinate, mononuclear and tetragonal complexes that range from Cu (H_3A) to Cu (H_2A) were found to be formed. The investigation has also been carried out on the effect of AM on Cu (II) binding by physiological Cu (II) carriers, histidine, albumin and easing of oxidation of 2-deoxyguanosine by Cu (II) AM complexes. It has been found that the complexation of Cu (II) by AM does not affect Cu (II) homeostasis in blood and could affect the intracellular activity of drug [62].

A ^{13}C NMR study has also been carried out for the evaluation of Cu (II) complex of AM at pH 5.5 using fast exchange condition to measure spin-lattice relaxation. The pseudo-tetrahedral complex in the 1:1 ratio was determined by the relaxation rates analysis and UV-visible spectrometry. The amino nitrogen of AM along with amide nitrogen and a hydroxyl group in aminopropylcarbonyl side chain were the carbon binding sites that were used to determine the structural model of complexation using the Cu-carbon bond distance [63].

3. 4. Mass Spectrometry (MS)

A study has been carried out for the determination of mass spectra of 11 AGs using californium-252 plasma desorption (^{252}CFPD) mass spectrometry. The spectral data confirmed that the AGs residues isolated from food products were of animal origin. The mass spectra by using time-of-flight ^{252}CFPD MS of 11 AGs that included AM, neomycin, kanamycin, paromomycin, tobramycin, apramycin, streptomycin, dihydrostreptomycin, netilmicin, sisomicin, and gentamicin gave positive ion spectra. The AGs antibiotics were derivatized to produce extractable chromophoric compounds except for streptomycin and dihydrostreptomycin that exhibited nitrophenyl derivatives. The spectra were taken in both negative and positive ion modes [64]. A mass spectrometric study has also been carried out to characterize thiosoindole derivatives of AGs antibiotics. The drugs were derivatized with 1,2-phthalic dicarboxaldehyde in the presence of thiol resulting in the introduction of UV chromophoric thioisoindole moiety. MS studies for molecular identity have been carried out and it has been found that in gentamicin and kanamycin the amino groups were fully derivatized as compared to that of AM and tobramycin in which partial derivatization occurred and the product was formed on the thioisoindole group [65].

3. 5. Colorimetric Methods

A new, selective and sensitive colorimetric biosensor has been developed for the determination of gentamicin, AM, and tobramycin via gold nanoparticles (Au NPs) which act as sensing agents. The method showed a rapid color change from red to blue due to the aggregation of Au NPs via gentamicin, AM, and tobramycin. This colorimetric sensor showed good linearity in the concentration range of 2.67–33.93, 13.33–66.67 and 20–180 ng/ml for gentamicin, AM, and

tobramycin, respectively [66]. Since AM does not have any chromophore or conjugated system for its UV and fluorescent light detection (FLD), therefore, a colorimetric method has been developed in which aqueous ninhydrin and AM were treated (2–5 min) and they produced a Rubemann purple colored derivative that could be detected at two wavelengths, 400 and 567 nm. The method showed good linearity in the concentration range of 0.417–2.500 mg/ml and accuracy of 95.07–100.52% at 400 nm and 96.04–99.89% at 567 nm [67].

In another attempt, some AGs (gentamicin, tobramycin, AM) were determined in milk by the colorimetric silver nanoparticles (Ag NPs) used as a sensor. Ag NPs were prepared by chemical reduction method using sodium borohydride, a reducing agent, and sodium dodecyl sulfate as a stabilizing agent. It was found that the yellow color of Ag NPs converted into orange and red color in proportions that depended upon the AGs concentration. The quantitative determination was carried out by measuring the decreasing absorbance at 394 nm. The method showed good linearity in the concentration range of 20–60 ng/ml, 23–60 ng/ml, and 60–100 ng/ml for gentamicin, tobramycin, and AM, respectively [68]. A colorimetric method has been developed for the determination of AM in trace amounts. The method is based on the aggregation of Au NPs in the presence of AM which leads to the change in the ratio of A640/A520. The method showed good linearity in the concentration range of 2.0×10^{-7} to 5.0×10^{-6} mol/L while the LOD of the method was found to be 6.0×10^{-9} mol/l with RSD of 1.9%. The proposed method has been applied to AM injection and the recovery was found to be in the range of 100–105.0% [69].

4. CAPILLARY ELECTROPHORESIS (CE)

The CE coupled mass spectrometric studies have been carried out for the determination of pK_a values of amino groups of AM and its derivatives. The values of pK_a were found to be 8.4, 6.7 and 9.7 by measuring electrophoretic motilities of the molecules as a function of pH [70]. A copper (Cu) microparticle-modified carbon fiber microdisk array electrode was developed and used in CE for the simultaneous determination of five AGs including netilmicin, tobramycin, lincomycin, kanamycin, and AM. The array electrode showed high catalytic activity for AGs, good stability and reproducibility by using optimal conditions such as separation voltage of 6.2 kV and electrophoretic medium on which AGs were separated within 20 min [71]. A capillary electrophoretic method has also been developed in which capacitively coupled contactless conductivity detection (CE–C4D) was used for the determination of AM via ammonium acetate (20 mg/l) used as an IS. The method showed good linearity in the study concentration range with a regression of 0.9996. The LOD and LOQ were found to be 0.5 mg/l and 1.7 mg/l, respectively [72].

The determination of AM in injection solution has also been carried out by CE with high-frequency conductivity. AM is separated and detected within 5.5 min by using 5.0 mmol/l lactic acid, C2H5OH (30%) and 20.0 kV separation voltage. The method showed good linearity for AM in the concentration range of 5–150 mg/l with LOD of 1.5 mg/l [73].

4. 1. High-Performance Capillary Electrophoresis (HPCE)

An HPCE method has been developed for the determination of AM in human plasma and has clinical laboratory applications. The method of ultrafiltration followed by derivatization with 1-methoxy-carbonylindolizine-3,5-dicarbaldehyde (fluorescence derivatizing agent) at room temperature has been carried out. The aliquot was directly injected into fused silica capillary at anode side using dynamic compression injection at 50 hPa for 6 s. The electrophoresis was carried out with 40 mM SDS–20 mM phosphate-borated buffer at pH 7.0. It has been found that the derivative has a retention time of 16.7 min on measurement at 482 nm. The method showed good linearity in the concentration range of 5–100 mg AM/ml with a LOD of 0.5 µg AM/ml [74].

5. CHEMILUMINESCENCE METHODS

Another attempt has been made to determine AM by chemiluminescence method based on the inhibition of chemiluminescence emission generated by the oxidation of luminol in an alkaline medium using H_2O_2 catalyzed with Cu (II) on interaction with AM. The interaction was due to the formation of a complex between the catalyst and AM and the method was found linear in the concentration range of 9.89–20 mg/l with LOD of 2.97 mg/l [75]. A similar chemiluminescence method for the determination of AM has also been proposed. It is based on the reaction between luminol and diperiodatoagentate $\{K_2[Ag(H_2IO_6)(OH_2)]\}$ in the alkaline medium along with flow injection technology. It has been observed that chemiluminescence intensity could be greatly increased by AM. The possible mechanism of the chemiluminescence reaction was proposed by the investigation of chemiluminescence kinetics characteristics. A good linearity in the concentration range of

5.1×10^{-8} to 5.1×10^{-6} mol/l with LOD of 1.9×10^{-8} mol/l has been observed. The method showed good reproducibility with RSD of 2.8% for 5.1×10^{-7} mol/l of AM [76].

On-chip micro flow approach has also been used for the estimation of AGs (neomycin, streptomycin, AM) by chemiluminescence detection. The method is based on the inhibition of Cu (II)-catalyzed CL reaction of luminol and H_2O_2 by AGs which leads to the formation of a complex between Cu (II) and AGs. The method was found to be linear in the concentration range of 0.3–3.3, 0.9–13.7 and 0.8–8.5 µmol/l for neomycin, streptomycin and AM whereas the LOD of these compounds were found to be 0.09, 0.28 and 0.24 µmol/l respectively [77]. A novel luminol-based chemiluminescence method has also been used for the determination of AM in serum by using trivalent Cu-periodate complex ($K_5[Cu(HIO_6)_2$ DPC) in alkaline medium. The intensity of chemiluminescence was improved by AM concentration. The method employed flow injection (FI) technology that produced a distinctive oxidative effect of DPC with luminol-based chemiluminescence reaction at a low concentration of 10^{-7} M. The chemiluminescence intensity was proportional to the concentration of AM (4.0×10^{-9} to 4.0×10^{-6} g/ml) with LOD of 1.2×10^{-9} g/ml. The RSD was found to be 2.1% for 8.0×10^{-9} g/ml and recovery was 97–106.5% [78].

6. IMMUNOASSAY METHODS

A specific, rapid and sensitive radioimmunoassay method has been developed for the determination of AM. By immunizing rabbits with AM, conjugated to porcine thyroglobulin, the antisera was produced and screened for AM antibodies with ^3H labeled AM. It has been found that using this method, 5 ng of antibiotic could be measured in serum, cerebrospinal fluid, and urine. The calibration curve was not affected by pH (5–11), ionic strength (0.01–0.3 M), $MgCl_2$ or $CaCl_2$ (1–8 mM/l) and urea (10–300 mg/100 ml) [79]. Another method has been designed to determine the concentration of different AGs such as gentamicin, tobramycin, AM, etc. in plasma and serum. The tracers of AGs were prepared by reacting the parent component with 5-[(4,6-dichlorotriazin-2-yl)-amino] fluorescein using conventional procedures to prepare antisera for a specific component in rabbits. The tracer, sample and diluted antiserum were combined at ambient temperature and the fluorescence polarization of the prepared tracer was determined. The assay results showed accurate concentration values of AGs and the method was found unaltered by the effect of matrices [80].

An enzyme multiplied immunoassay technique (EMIT) has been used for the determination of caffeine, AM, and methotrexate on the Dade Behring Dimension RxL Max chemical system. The study has been carried out to improve the precision of previous EMIT. The method included new parameters such as larger sample volumes and longer observation of optical density. The changes made in the method showed improved sensitivity of methotrexate and precision for all analytes. The intra- and inter-day precision of the method was less than 6% for AM and caffeine and less than 7.5% for methotrexate at a concentration of 0.3 µmol/l and 12.5% at 0.06 µmol/l. The correlation coefficients for caffeine, AM, and methotrexate were found to be 0.973, 0.986 and 0.992, respectively [81]. A long-wavelength fluorescence polarization immunoassay (FPIA) has been used for the determination of AM as an analyte in serum samples. Nile blue was used as a wavelength label to evaluate the potential of FPIA on a solid surface while ruthenium (II) chelate as a wavelength label for the determination of macromolecules by using gliadins as a model. The method involved a low amount of anti-AM antibodies and AM-Nile tracer was immobilized on to nitrocellulose membranes for AM determination. It has been found that by minor changes there was less consumption of reagents as compared to that of the conventional FPIA method. Since the method was based on the displacement of tracer from tracer-antibody immunocomplex, it resulted in a decrease of fluorescence polarization which is proportional to the analyte concentration. It has also been found that gliadins Ru (II) chelate tracer has a long lifetime that helps in the observation of differences in fluorescence polarization values between the tracer complex and tracer alone. The method showed a good linearity in the concentration range of 0.5–10 µg/ml for AM and gliadins with a LOD of 0.1–0.09 µg/ml for both the analytes [82].

A homogeneous enzyme immunoassay has been used for the determination of AM in water samples by using malic dehydrogenase enzyme and oxazine cresyl violet (a long wavelength fluorophore). The covalent binding of AM to malic dehydrogenase using carbodiimide derivative resulted in the formation of an enzymatic tracer. It was found that in between cresyl violet and malic acid the free tracer catalyzes the reaction which results in the decrease of fluorescence intensity of the fluorophore. The reaction kinetic curves were monitored at λ_{em} = 624 nm using λ_{ex} = 585 via a stopped-flow mixing technique and initial rate was found to be 2.3 s. The method showed a linearity range of 1–1.5 ng/ml with LOD of 0.3 ng/ml [83].

7. ELECTROCHEMICAL TECHNIQUES

7.1. Polarographic Method

The analysis of AM in trace amounts in injection, urine and blood samples has been done by a new single scan oscillopolarographic method. The method is based on the study of the polarographic behavior of formaldehyde derivative of AM in Britton Robinson (B–R) buffer solution (pH 8.0). The study also demonstrated that the peak current increases with an increase in the AM concentration. The method has been found linear in the concentration range of 6.0×10^{-7} to 5.0×10^{-5} mol/l with the LOD of 2.4×10^{-7} mol/l. The recovery of AM through the method was found to be 98–100% with RSD of 4.1% [84]. Another new polarographic method has been proposed for the determination of AM using amaranth. It has also been found that amaranth has a sensitive second-order derivative polarographic reduction peak in (B–R) buffer solution (pH 4.0). However, when AM was added to buffer solution the reduction peak current for amaranth decreased linearly with the concentration of AM in the range of 0–40 mg/l. The LOD of the method was found to be 1.0 mg/l [85].

7. 2. Cyclic Voltammetric and Amperometric Method

A cyclic voltammetric (CV) and amperometric (AM) method have been used to determine the concentration of AM by preparing nano-sized Cu oxide modified carbon paste electrode. It has been found that the oxidation current of AM on prepared nano-sized CuO modified carbon paste electrode was 40 times higher than that of the bulk CuO modified carbon paste electrode. The calibration curve was found to be linear in the concentration range of 2–200 µM for AM with a correlation coefficient of 0.9998. The LOD of 1 µM for AM with the signal-to-noise ratio was 3 [86].

8. RAYLEIGH SCATTERING METHOD

The determination of AM by Rayleigh scattering (RS) along with the conventional fluorescence spectrometry has been carried out by a method in which pontamine sky blue (PSB) forms an ion-association complex with AM in acidic medium. The maximum signal of RRS for AM was found at 362 nm. The method is linear in the concentration range of 0–1.7 µg/ml with a LOD of 3.0 ng/ml [87]. Rayleigh scattering method has also been developed for the determination of AM based on covalent bonding of AM with a water-soluble polymer. It involved the carbodiimide cross-linking technique that leads to the formation of an amide bond between the copolymer of 4-styrenesulfonic acid and malic acid. The conditions set to run the reaction to achieve maximum intensity was the pH of 7.0 and 0.02 M ionic strength. The method showed good linearity in the concentration range of 0.25–35mg/l with a correlation coefficient of 0.993. The LOD for the method was 0.08 mg/l [88].

9. MICROBIAL ASSAY METHOD

According to USP, the turbidimetric method has been used for the microbial determination of AM. Similarly, AM concentration has also been determined using Taq-Man quantitative PCR method. These methods showed good linearity in the concentration range of 8.0–30.47 and 5.12–38.08 ng/ml for turbidimetric and RT–PCR methods, respectively. The LOQ of the methods was found to be 8.00 and 5.12 ng/ml for turbidimetric and RT–PCR methods, respectively [89].

10. CONCLUSION

Several analytical methods have been used for the determination of AM in pharmaceutical preparations and biological samples. These methods include chromatographic (TLC, HPLC, HILC, UHPLC, LC, ion chromatography), spectrometric (UV-visible spectrometry, spectrofluorimetry, NMR, MS), chemiluminescence, electrochemical (i.e. polarography, cyclic voltammetry, amperometry), immunoassay, microbial assay, and Rayleigh scattering methods. These methods are selected according to the nature and composition of active components of the sample. Chromatographic methods are particularly more precise, sensitive, and stability-indicating for the determination of AM in pharmaceutical and biological systems.

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