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ORIGINAL PAPER

A rapid HPLC–DAD method for quantification of amikacin in pharmaceuticals and biological samples using pre-column derivatization with Hantzsch reagent

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Abstract
Amikacin (AMK) is an important member of aminoglycoside class, and its determination has therapeutic importance due to its matchless potency against gram –ve pathogens. Due to narrow therapeutic window, its monitoring in clinical samples is inevitable. Direct determination of AMK using HPLC with UV–visible detection is not possible because of its limited absorbance. Herein, Hantzsch reagent (mixture of acetylacetone, formaldehyde and acetate buffer) was used as pre-column derivatization for AMK. UV–visible detection was performed at 340 nm. Separation and identification of derivatized drug (amikacin) were carried out using C-18 column Kromasil 100 (15 cm × 0.46 mm, 5 µm) with isocratic mobile phase elution of pH 5 (acetate buffer 0.01 M):acetonitrile (30:70 v/v) with flow rate of 1 ml/min. The procedure was able to resolve AMK from endogenous compounds and from cephalosporin drug (most prescribed combination) with run time of 10 min. Under optimized conditions; calibration curve was linear in the range 0.10–25.0 µg/mL with LOD and LOQ values of 0.024 and 0.071 µg/mL. Method was also validated for reproducibility, ruggedness and accuracy. The procedure was found sensitive, robust and precise for the comprehensive analysis (qualitative and quantitative) that was applied for determination of AMK in pharmaceuticals, urine and blood samples.

Keywords Therapeutic monitoring · Pre-column derivatization · Amikacin · Hantzsch reagent

Introduction

Amikacin (AMK) is a semisynthetic form of kanamycin with wide spectrum activity against *anaerobic bacilli*. It has a more susceptibility to resist enzymatic attack. AMK binds to the bacterial ribosomal subunits that causes misreading to messenger RNA by making it inactive, which forms the basis of its mode of action. Aminoglycosides are proposed recently as a double-edge sword, viz., against respiratory infection as well as immunity enhancer (along with anti-virals) for COVID-19 regimen [5]. AMK can be used in combination with other β lactamase inhibitors such as ceftriaxone [13] and other cephalosporin for broader antimicrobial coverage. The combination reduces therapeutic time and cost [14]. AMK can also be added to blood to prevent chances of pseudo-thrombocytopenia with ethylenediaminetetraacetic acid as anticoagulant [30]. However, its prolong use may cause ototoxicity and nephrotoxicity. Its therapeutic plasma concentration is in the range of 8–16 µg/mL [7]. To assure therapeutic concentration level and to minimize the side effects, it is essential to monitor plasma levels of AMK frequently [25, 6]. Several analytical methods have been developed using liquid chromatography and other techniques. For example, assay of AMK in skin samples was carried out after derivatization by 1-flouro-2,4-dinitrobenzene reagent using HPLC–UV [16]. Pulsed electrochemical detector with Au electrode is reported for determination of amikacin where interfering components

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were separated in two steps where separation was carried out with poly(styrene–divinylbenzene) packed column [1]. An high-performance capillary electrophoresis (HPCE) procedure was employed for AMK analysis in human plasma, by derivative preparation with l-methoxycarbonylindolizine-3,5-dicarbaldehyde. PLC with evaporative light scattering detection system was also applied for AMK estimation using ion-pairing acidic reagents which enhanced the sensitivity of procedure. An LC method for determination of amikacin with pulsed electrochemical in cerebrospinal fluids is also reported [4]. Amikacin in biological tissues is determined through HPLC using O-phthaldialdehyde [2]. Serum level monitoring in newborn babies for amikacin using ion-pair liquid chromatography with tandem mass spectrometry (IPLC-TMS) [3] which is very complex and hard to adopt for routine analysis. Other methods are UV/Vis spectrophotometer [21,27], immuno-assay for the determination of AMK in serum using gliadins analyte model [23, 26] are also reported. Chemiluminescence method using naturally occurring catalyzing agent [17] is also reported. Determination of AMK in human plasma by molecular imprinted SPR nano-sensor was also carried out by Isaksson et al. [12, 29]. Further, due to complexity of biological samples and inherent drawback of single component analysis, methods other than chromatography are not suitable for accurate quantification of AMK. Liquid chromatographic methods mentioned above are reported for determination of AMK only and there is need to develop a reliable method for determination of AMK drug along with most prescribed drug 'cephalosporins.' AMK as described earlier is used in combination with other, antibacterial especially β lactamase synthesis inhibitors [8]. Therefore, determination methods of AMK in the presence of co-administered drugs are also needed. AMK lacks chromophore, therefore requires derivatization prior to LC–UV analysis. Herein, Hantzsch reagent (mixture of acetylacetone, formaldehyde and acetate buffer) was used to derivatize amine of amikacin [10], which imparts chromophore in structure hence may be suitable as pre-column derivatization reagent for determination of AMK. Method was optimized and validated for analytical figures of merit. The developed procedure was also applied to determine amikacin in real biological samples.

Experimental

Materials

Chemical and reagents

Chemicals and reagents were of analytical purity. Pure amikacin (AMK) was kindly provided by Sami Pharmaceuticals a (Pvt) LTD, Karachi, Pakistan. Standard of cephalosporin's (cefixime, ceftazidime) was collected from Bosch pharmaceutical a (Pvt) LTD, Barrette & Hodgson's Pharmaceuticals. Chemicals for Hantzsch reagent (acetylacetone and formaldehyde) were purchased from Merck Germany. Sodium acetate and acetic acid were acquired from Sigma-Aldrich (St. Louis, Missouri, USA); methanol and acetonitrile (HPLC grade) from Fisher Scientific (Hampton, New Hampshire, USA). Low conductivity water from Milli Q system was used throughout the analysis.

Instrumentation

The separation with spectral identification of AMK, Hantzsch reagent and their derivative was carried out with Spectra system SCM 1000 (Thermo Finnigan, California, USA) liquid chromatography equipped with degasser and diode array detector. Chromquest, Version 4.2 Software was used for recording chromatograms. UV–visible spectra were recorded using double beam spectrophotometer from Agilent (Cary 100), USA. Eppendorf centrifuge 5430 R was used for serum separation. Orion model 420 a pH meter with glass electrode (Orion Research Inc Boston USA) was used for buffer preparation.

Standards preparation

Stock solution of 1000 mg/L for AMK was prepared by taking appropriate amount of pure compound and dissolved in distilled water. The prepared stock solution was further diluted in the range of 1.0 to 100 mg/L using deionized water. For derivatization, Hantzsch reagent was freshly prepared each time by using 2.0 mL acetylacetone, 1.0 mL formaldehyde, 1.0 mL of 0.1 M acetate buffer pH 5 and 6 mL Milli Q water in 10 mL of volumetric flask.

Procedure for derivatization

Derivatization of AMK drug with Hantzsch reagent was carried out by following a reported method [14, 15] with some modification. Briefly, derivatization was carried by mixing 1.0 mL of AMK drug with 1.0 mL of freshly prepared Hantzsch reagent in to a 5.0 mL screw cap test tube then heated in water bath adjusted at 60 ºC for 15 min. After complete derivatization, mixture was kept under cold conditions (ice box) till analysis by HPLC–DAD. For blank, the same procedure was followed except inclusion of AMK drug.

Samples collection and preparation for analysis

Pharmaceuticals

The hermitically sealed ampoule of AMK (Amkay 25 mg/mL) was purchased from pharmacy. The collected sample

was further diluted to bring the concentration of AMK about 125.0 µg/mL with Milli Q water. Further dilutions were performed similarly if needed. The same procedure of derivatization was applied on different volume ratios (i.e., 0.1, 0.5 and 1.0 mL) of prepared stock solution of AMK drug.

Urine

AMK clearance mostly takes place via urine and to some extent from feces as well [9]. AMK dose to patients was started on using 500 mg TDS and collection of urine sample was done after 48 h from the start of therapy. The collected samples were filtered through 0.2 µm filters. Different volumes (i.e., 0.5, 1.0 and 1.5 mL) of filtered urine sample were taken in 5.0 mL flask and procedure of derivatization was followed.

Blood (serum)

A 5.0 mL of blood was collected from patients under treatment and stored in anticoagulant containing heparin tube (written consent was obtained from patients for collection of samples). Tube was centrifuged for 10–15 min at 3000 RPM, to separate serum from blood. The blood specimen was withdrawn after achieving therapeutic concentration of drug. Approximately 0.1, 0.5 and 1.0 mL of supernatant solution were taken and followed the same derivatization procedure.

Chromatographic separation

The acetonitrile (A) and aqueous 0.01 M acetate buffer pH 5 (B) were used as mobile phase. The analysis was performed using isocratic system for 10 min using 70:30 (A:B *v/v*) at flow rate of 1.0 mL/min. A column of Teknokroma KROMASIL 100 C-18 (15 cm × 4.6 mm, 5 µm, Spain) was used for separation of Hantzsch reagent and derivative. Injection volume of sample was 20 µL. Recognition of Hantzsch reagent and new derivative was performed by diode array detection at two different wavelengths, i.e., 285 and 340 nm, respectively. During monitoring of reaction, 340 nm was used for identification and quantification of AMK derivative in real samples. Peaks of Hantzsch reagent and derivative were confirmed using, peak height, retention time along with UV spectra of respective peak using diode array detector. Calibration curve of AMK derivative was observed by diluting the solution in the range of 0.10–25.0 µg/mL. Ten blanks were run under same conditions for measurement limit of detection and limit of quantification.

Method validation

The validation of HPLC method for synthesized derivative was carried out by following the rules of ICH international guidelines for linearity parameters, i.e., % recovery, accuracy, sensitivity (limit of detection and quantification), precision (repeatability and intermediate) and stability of new method.

The calibration and linearity of synthesized derivative were carried out by applying different concentrations in the range of 0.10–25.0 µg/mL. The method was validated by plotting the peak area against each concentration of (AMK-HANZ) derivative. The intercept (b), slope (m), with coefficient (r^2) were calculated using regression coefficient equation.

For recovery (%) measurement, the synthesis of AMK derivative was applied in three different samples such as serum, urine and pharmaceutical formulation.

The percentage recovery was measured by following formula as

$$\text{Recovery}\ (\%) = \left(\left(D_t - D_s\right)/D_a\right) \times 100,$$

where D_t is the drug quantity after standard addition; D_s is the drug quantity (AMK-HANZ) in real sample and D_a is the drug added quantity. Intraday precision was monitored by running sample three times in a same day up to 5 h. Data are reported as average and standard deviation (± SD) and RSD from the results. For interday precision measurements, same concentration of AMK was derivatized on each day for three consecutive days and results were reported as average along with standard deviation. Standard addition method was used to calculate recovery (%) in real samples.

Results and discussion

Derivatization of AMK drug

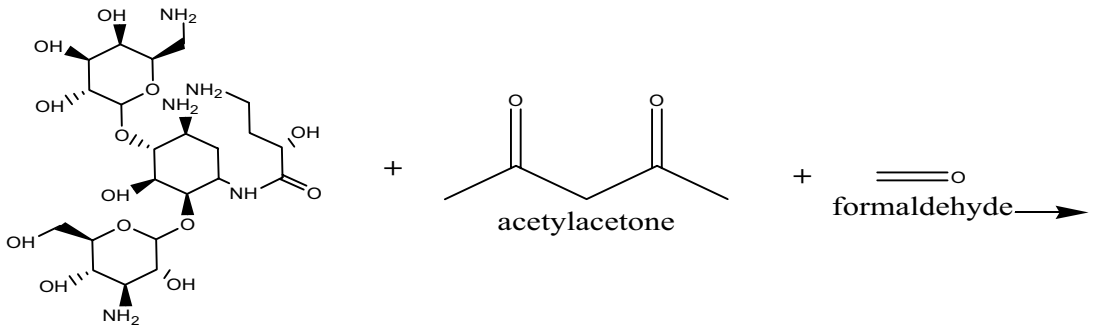
Derivatization plays important role in the determination at trace level of non-chromophoric group bearing drugs. The derivatization reaction enhances the selectivity, sensitivity and stability of drugs by any involved analytical technique. However, after derivatization the modified drug structure known as derivative (has closely or related structure to the analyte) is to be identified, characterized and separated out by any chromatographic methods [18]. AMK drug belongs to non-chromophoric group bearing drugs (Scheme 1) and act as an antibiotic under tag of aminoglycosides. Structure of AMK is shown in Scheme 1; derivatization at free amines can impart color to the AMK derivative. Scheme 1 shows the detailed reaction of Hantzsch reagent **2** with amino group (NH_2) at pyranose ring of AMK structure **1** at 60 ºC heating

for 15 min and derivative **3** of AMK was synthesized. Proposed reaction scheme may be as: [24] the acetylacetone and formaldehyde in the presence of AMK and under optimum medium (pH) are cyclized (cyclization). The cyclization is taking place on the amine group bearing pyranose ring of AMK. With this cyclization, the pyridine ring based derivative formation takes place at pH 5 (acetate buffer) to produce a yellow chromogen (derivative) and was analyzed by HPLC–DAD.

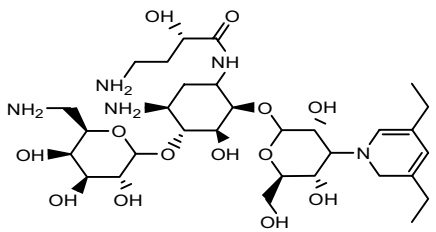
Figure 1 shows UV–Vis spectra of reagent and derivative with AMK, λ_{max} was within UV range of 285 nm, while an additional peak after derivative formation was observed at 340 nm. The yellow-colored (visible) chromogen was also observed visually as well which correspond to wavelength 340 nm. The optimization of pH is very essential for derivatization and completion of reaction. For Hantzsch reagent, the strong acidic medium causes hydrolysis of drug and reagent, whereas in alkaline medium the mixture for derivatization gets precipitated. Therefore, pH study was carried out and pH 5 was found optimum for derivatization reaction and stability of derivative.

Optimization and separation of AMK

To achieve the optimization and separation of derivatized drug from unreacted derivative molecules; AMK, Hantzsch and prepared derivative were injected on a reverse phase liquid chromatographic column. Mobile phase optimization was carried out by using of different solvents. First methanol and water were used with 50:50, 70:30 and 90:10, respectively, but resolution was poor. Moreover, reproducibility was also not satisfactory. After many trials, methanol was replaced by acetonitrile and likewise different ratios were used for obtaining best separation. After few trials, required separation was achieved with 70:30 ratio of mobile phase. The isocratic system (70:30) of mobile phase containing (acetonitrile: acetate buffer at pH 5) at flow rate of 1 mL/min was used for separation of all components. At pH 5.0, the derivative was stable (as mentioned in derivatization section); therefore, no further experiments were conducted to test the pH during chromatographic separation. Figure 2 shows the chromatographic separation of Hantzsch reagent and AMK-HANZ derivative with their retention time of 4.61 and 8.52 min, respectively. After optimization of organic phase composition, the ionic strength of buffer solution was optimized to maintain the separation efficiency of newly



4-Amino-*N*-[5-amino-2-(4-amino-3,5-dihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-4-(6-aminomethyl-3,4,5-trihydroxy-tetrahydro-pyran-2-yloxy)-3-hydroxy-cyclohexyl]-2-hydroxy-butyramide



(*S*)-4-amino-*N*-((2*R*,3*S*,4*R*,5*S*)-5-amino-4-((3*R*,4*S*,5*R*,6*R*)-6-(aminomethyl)-3,4,5-trihydroxy-tetrahydro-2*H*-pyran-2-yloxy)-2-((3*R*,5*S*,6*R*)-4-(3,5-diethylpyridin-1(2*H*)-yl)-3,5-dihydroxy-6-(hydroxymethyl)-tetrahydro-2*H*-pyran-2-yloxy)-3-hydroxycyclohexyl)-2-hydroxybutanamide

Scheme 1 Synthetic mechanism for the derivatization of amikacin drug using Hantzsch reagent as derivatizing reagent

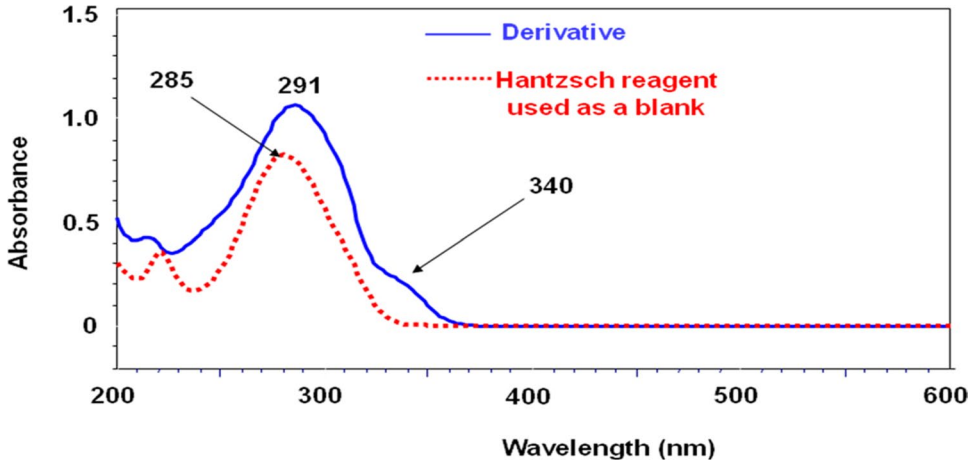


Fig. 1 UV spectra of Hantzsch reagent showing additional peak at 340 nm after derivatization. Amikacin 25.0 µg/mL by reacting with 1.0 mL of Hantzsch reagent by heating the mixture at 60 °C for 15 min at heating water bath

formed derivative at lowest possible salt concentration. Different strengths were tried (i.e., 0.001, 0.005, 0.05, 0.01, 0.1 M), the best chromatographic separation was achieved with 0.01 M buffer used as mobile phase with acetonitrile. The standard addition method was also applied for further confirmation of AMK derivative and Hantzsch reagent in a mixture and increase in peak height corresponding to peak AMK derivative at 8.52 min was found increasing, which confirms the t_r for AMK for quantification.

To ensure completion of reaction for quantitative analysis, reaction was monitored through HPLC by taking an aliquot every 3 min of interval. No change in peak height was observed after 15 min which shows the equilibrium time of reaction. Storage stability of derivative was also studied and found that it was stable at cold temperature (≅4 °C) for 4–5 h. Derivative was kept in ice box during processing for HPLC analysis, while kept in refrigerator when not in use.

HPLC method validation for AMK derivative analysis

Table 2 shows the summary of validation parameters and analytical figures of merit for AMK derivative analysis following the ICH international guidelines (ICH, 2005) for linearity, sensitivity, % recovery, precision, reproducibility (accuracy) and stability of developed HPLC method.

Linearity

The linearity was evaluated for AMK derivative up to six different concentrations in the range of 0.10–25.0 µg/mL. Calibration curve was plotted by getting average peak area ($n = 3$) against the concentration of derivative and the result was analyzed by linear regression method. A coefficient of regression is tabulated in Table 2, i.e., 0.998 which confirms the method was linear for the determination of AMK derivative. Figure 3 shows the calibration of derivative after injection of a series of concentration into HPLC and detected by diode array detector.

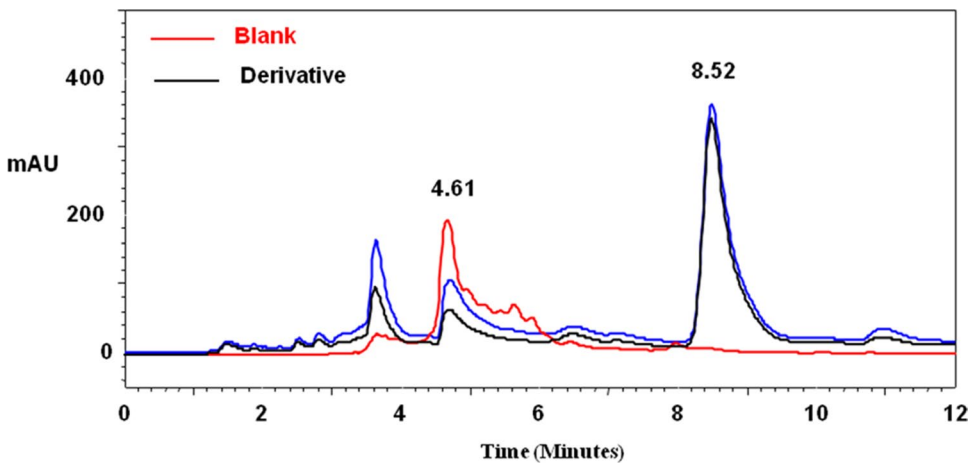


Fig. 2 A representative HPLC–DAD chromatograph shows the best separation of Hantzsch reagent (blank) and amikacin derivative was achieved at following conditions: concentration of amikacin drug was 25 µg mL^{-1}, 1.0 mL of Hantzsch reagent, mobile phase composition was kept 0.01 M acetate buffer with pH 5 with acetonitrile by ratio (30:70), respectively. The flow rate was not more than 1.0 mL/minutes

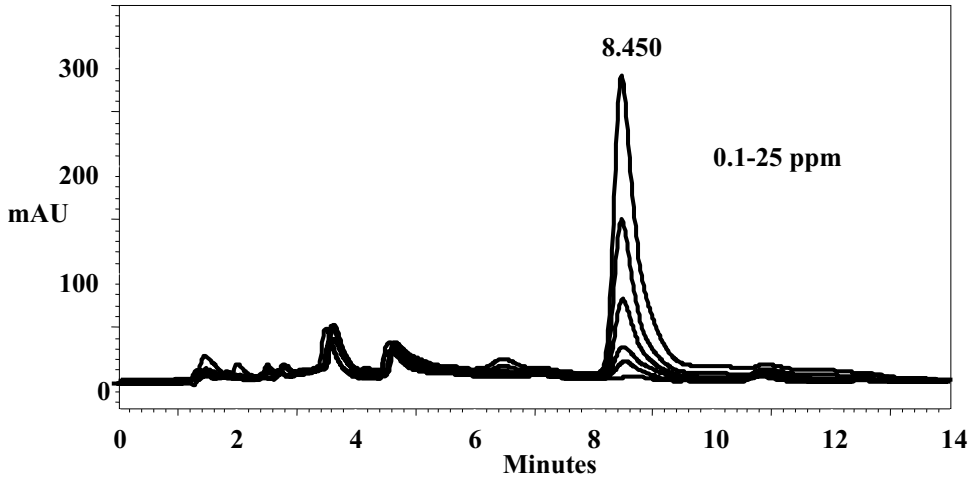


Fig.3 Chromatograms for various amikacin concentrations (0.10–25.0 μg mL^{-1}) showing linearity with chromatographic peak height

Sensitivity

The sensitivity of the proposed method was carried out by calculating the limit of detection and the limit of quantification by preparing serial dilution of derivative mixture in the range of 0.10–25.0 μg/mL until the signal-to noise ratio reached the value of three for LOD and ten for LOQ. The limits of detection and quantification are summarized in Table 2 as 0.024 and 0.071 μg/mL, respectively.

Recovery

The percent recovery of AMK derivative from all three samples (serum, urine and pharmaceutical formulation) was carried out by standard addition method. A fixed amount, i.e., 60.0 μg/mL of AMK was added to each sample during reaction and recovery was obtained within the range 92–97% with lower RSD value which proves derivatization and its analysis is precise and highly accurate (Table 2).

Precision

For precision of analytical procedure, the interday and intraday of synthesized AMK derivative were carried out by injecting the freshly prepared mixture (Hantzsch and AMK drug) into HPLC–DAD. The lowest concentration (1.0 μg/mL) of AMK derivative was injected six times at various intervals, i.e., from 0 to 5 h. The intraday results show that AMK derivative was stable up to 5 h under cold environment, therefore intraday assay was not extended to full day but kept limited to 5 h. For intraday precision, fresh solutions were prepared each day and data were used to calculate interday precision measurements. The achieved data were satisfactory by lower % of RSD and indicate high measurement of reproducibility and robustness in the current

Table 1 Validation parameters and analytical figures of merit for amikacin derivative analyzed by HPLC method

Parameter	Merit
Retention time (min)	8.5
quantification limit (μg mL^{-1})	0.071
Detection limit (μg mL^{-1})	0.024
Linear range (μg mL^{-1})	0.10–25.0
Regression equation	$y = 608.13x + 1080.4$
Regression coefficient (R^2)	0.998
Reproducibility ($n = 6$), 1.0 μg mL^{-1} (% RSD)	4.1
Stability of derivative	Up to 5 h (under cold storage)

Recovery

Sample	Amikacin (μg mL^{-1})	Amount added (μg mL^{-1})	Amount found (μg mL^{-1})	Recovery (%)
Serum (control)	0.00	60	55.20	92 ± 1.2
Urine (control)	0.00	60	57.41	95 ± 1.2
Pharmaceutical Formulation	100	60	155.4	97 ± 1.4

Table 2 Different methods used for the analysis of amikacin drug

Instrumental method	Sample type	Derivatizing reagent	R^2	LOD/LOQ	Linear range	References
FTIR	Pharmaceuticals	N/A	0.9990	2.1 mg/mL	7.7– 77 mg/mL	Ovalles et al. [22]
HPLC /UV	Water samples	4-chloro-3,5-dinitrobenzotrifluoride	–	0.2 /1 µg/L	0 to 500 µg/L	Li et al. [16]
HPLC/Chemiluminescence	Plasma/urine	Cu(II),/luminal/H_2O_2	–	LOD is 55/50 ng/mL	1.5–20 µg/ mL	Serrano et al. [25]
RpHPLC/Fluorescence	–	o-phthaldialdehyde/mercaptoethanol	–	–	0.25–25 mg/mL	Benjamín et al. [2]
RPHPLC–PDA	–	Hantzsch condensation	0.9999	0.15 /0.5 µg/ L	–	Korany et al. [14, 15]
Chemiluminescence/Pharmaceuticals	–	Luminol in alkaline medium by H_2O_2 catalyzed by Cu(II)	–	2.97 mg/L (LOD)	9.89–20 mg/L	Fernández et al. [8]
HPLC/UV	Skin	1-fluoro-2,4-dinitrobenzene	0.9995	–	1.64–49.21 µg/mL	Nicoli et al. [19]
HPLC/PED	Pharmaceuticals	–	0.9993	–	2–12 µg/mL	Adam et al. [1]
HPEC/ FD	–	Methoxy carbonylindolizine-3,5-dicarbaldehyde	0.998	0.5 µg/mL	5–100 µg/mL	Oguri et al. [20]
HPLC/UV	Serum human/ Genie pigs	l-Fluoro-2,4-Dinitrobenzene	0.9996	0.936 µg/mL	2–64 µg/mL	Wong et al. [28]
Spectrofluorometer	–	Indocyanine green/ cerium-IV	0.9980	0.02 µg/mL	0.06–0.4 µg/mL	Sánchez-Martínez et al. [23]
RPLC/ELSD	Pharmaceutical	NA	0.9995	2.2 µg/mL	7 to 77 mg/L	Galanakis et al. [11]
Spectrophotometric	–	2,4,6-trinitrophenol/2,4-dinitrophenol	0.9990	2.5/1.34 µg/mL	10–100 and 5–100 µg/mL	Omar et al. [21]
HPLC–DAD	Biological fluid/ pharma	Hantzsch reagent	0.9980	0.024/0.071 µg/mL	0.1–25.0 µg/mL	Current work

optimized procedure. These data justified the usability of the developed method to be stability indicating assay process (Table 1).

The details of qualitative and quantitative analysis of AMK drug from various samples reported in literature are summarized in Table 2. Closer look into the details of methods reveals that current method has better sensitivity as compared to earlier methods and covers the required therapeutic monitoring levels for AMK (8–16 µg/mL).

Interference

Biological samples contain significant number of endogenous compounds which may interfere in clinical monitoring of drugs. Also, drugs from combination therapy (i.e., ceftriaxone, cefotaxime) may result in erroneous findings. Henceforth, these drugs were spiked to test the possible interference. Figure 4 shows that AMK-derivative is well

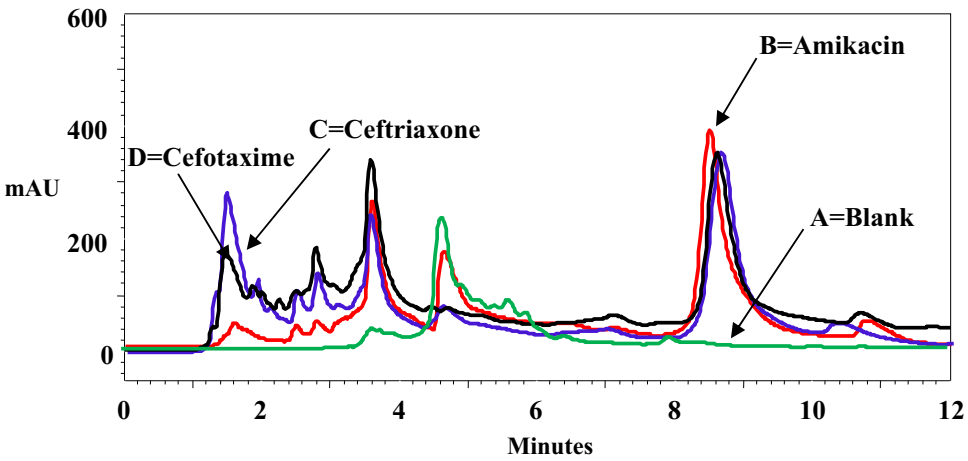


Fig. 4 HPLC–DAD chromatogram for interference study with amikacin, **a** blank, **b** amikacin derivative, **c** cefixime and **d** ceftazidime

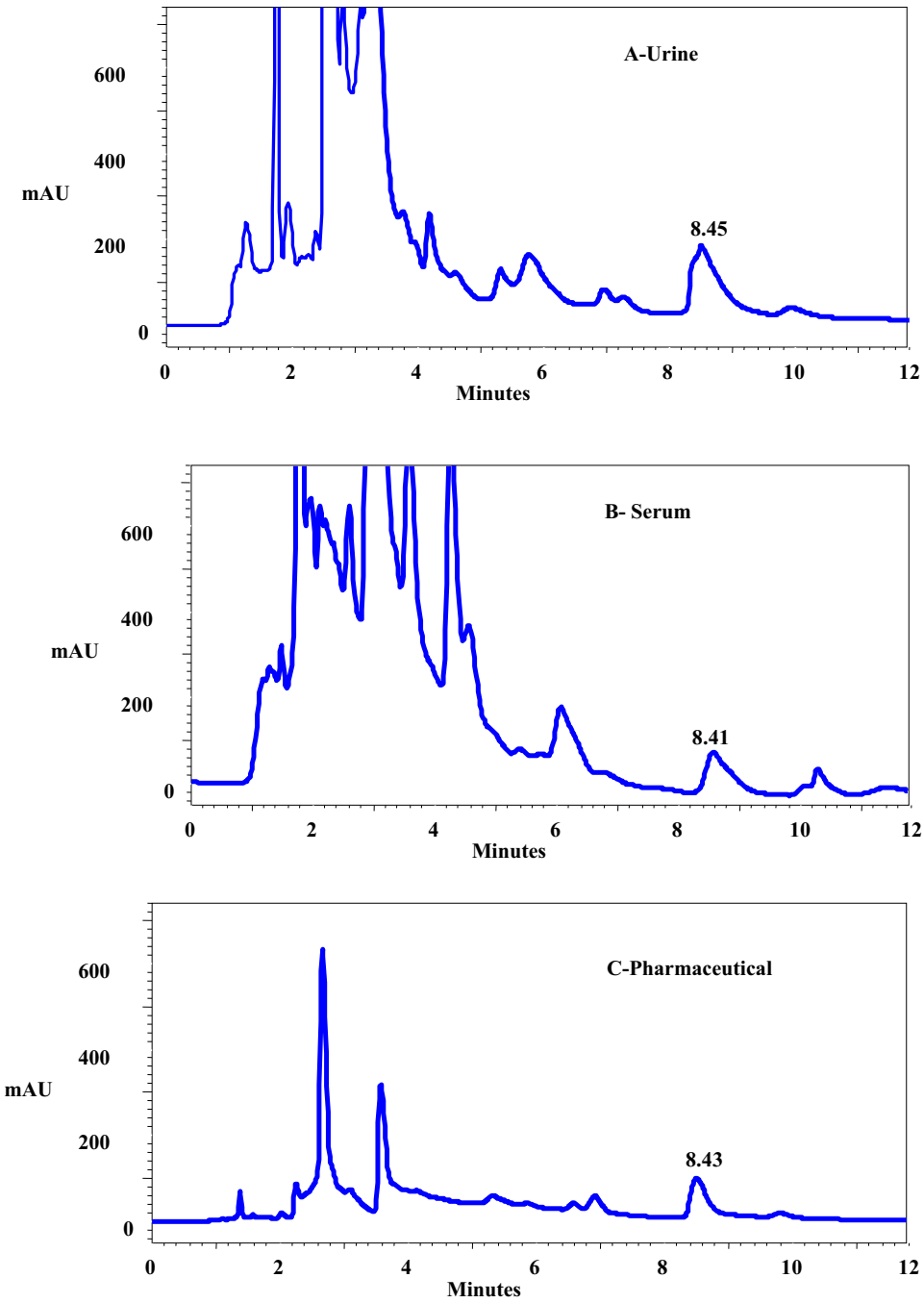


Fig. 5 A representative HPLC–DAD chromatogram for separation of amikacin derivative from three samples: **a** urine, **b** serum and **c** pharmaceutical formulations

separated from endogenous compounds and coexisting drug compounds.

Applications

The method was applied to the analysis of three different samples including serum, urine and pharmaceutical formulations. These samples were subjected to a pre-column derivatization using Hantzsch reagent. AMK was not detected in biological samples as obtained from patients. Therefore, recovery study was carried out adding three different amounts of analyte to all samples so that the final concentration was within the therapeutic range and subtracting the results from similarly prepared unspiked samples. Table 2 lists the recoveries obtained which were in the range 92–97%. Figure 5 shows the separation of AMK derivative from urine (Fig. 5a), serum (Fig. 5b) and pharmaceutical formulations (Fig. 5c) was carried out at respected retention time by applying HPLC–DAD.

Conclusion

In this procedure, a simple way has been designed in which Hantzsch reagent was used to derivatize AMK and analyzed on HPLC–DAD for quantification purpose. LOD and LOQ of derivative were calculated as 0.024 & 0.071 µg/mL consequently; the results show the sensitivity and selectivity of synthesized derivative were enhanced. The method was further assured with addition of standard. Validation of method according to ICH rules proved that method was sensitive, precise and stable. Among all the parameters; the peak size (concentration) was little influenced; any other remarkable effect was not observed. The method can be successfully applied for determination of AMK derivative in pharmaceuticals, urine and blood. There was no interference from endogenous compounds and co-administered drugs. The proposed method is fast and precise for routine monitoring of AMK using commonly available liquid chromatograph with UV–visible or DAD detector.

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Author contributions M.L.M. has carried out all experiments and prepared initial draft; N.M. is principal investigator and she has supported in all chromatographic experiments intellectually and laboratory instrumentation; A.A.M. has supervised all experiments and finalizing draft; M.Y.K. has coined the idea and drafted the proposal and A.H.M. has provided HPLC column.

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Availability of data and material Research data and description of materials have been provided in the manuscript.

Compliance with ethical standards

Competing interests On the behalf of all authors, corresponding author declares no competing interest among authors and any other party.

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