Pharm Methods, 2016; 7(2): 99-103.
A multifaceted peer reviewed journal in the field of Pharm Analysis and Pharmaceutics
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Original Article

Analysis of Amikacin in Human Serum By UHPLC with Fluorescence Detector Using Chloro-Formate Reagent With Glycine

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ABSTRACT

Background: Amikacin belongs to aminoglycosides family, commonly administered in the treatment of systemic infections due to gram negative bacteria. Its narrow therapeutic index results in adverse effects like nephrotoxicity and ototoxicity. **Objective:** Optimize an ultra-high performance liquid chromatography (UHPLC) based analytical method for the determination of amikacin sulfate in human serum using derivatizaon with FMOC-Cl and glycine. **Methods:** Pre-column derivatization reaction of amikacin performed using fluorescence reagent 9-fluorenylmethyl chloroformate (FMOC-Cl) at ambient temperature in the presence of borate buffer (0.2 M). Stabilizing reagent glycine (0.1 M) added into the reaction mixture solution after completion of the derivatization reaction for stabilization of fluorescent complex product. Fluorimetric detection of amikacin was performed at excitation and emission wavelength of 265 nm and 315 nm respectively, using C18 UHPLC column. The reported method was validated by performing linearity, precision, recovery and ruggedness. **Results:** The optimum mobile phase composition was found to be Acetonitrile:water in the ratio of 70:30 (v/v) at flow rate of 0.4 ml/min. A linear response of amikacin in serum samples ranging from 0.5-10 µg/ml was obtained, with correlation co-efficient of 1.00. The limit of detection (LOD) was found to be 50 ng/ml. Both inter- and intra-day analysis co-efficient values were found to be less than 10%. **Conclusion:** The developed UHPLC method will be useful for pre-clinical and pharmacokinetic study of amikacin in human serum.

Key words: Amikacin Sulphate, Ultra High Performance Liquid Chromatography (UHPLC), 9-Fluorenylmethyl chloroformate (FMOC-Cl) reagent, Borate buffer, Glycine, Fluorescence detector.

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DOI : 10.5530/phm.2016.7.15

INTRODUCTION

Aminoglycosides (AG) are highly potent and ample spectrum antibiotics used in the treatment of aerobic gram negative bacterial infections. AG antibiotics are commonly administered parenterally. Major concerns of aminoglycosides therapy are their toxicities.[1] They are frequently associated with side effects and toxicity like nephrotoxicity and ototoxicity, because of their narrow therapeutic index along with the post antibiotic effect. Their major toxicities may lead to death. Clinical monitoring of AG is recommended due to their narrow therapeutic index and undefined clinical end point.[2] An AG drug concentration above the therapeutic window leads to toxicity and below the therapeutic index produce no clinical effects.[1,2]

To ensure competent and therapeutically effective treatment of AG, therapeutic drug monitoring (TDM) is globally recommended. TDM benefits the patient by minimizing side effects and prevent spanning toxic level by maintaining the drug concentration level within the therapeutic range.[1] In AG, amikacin is commonly administered drug for gram negative bacterial infections using intravenous and intramuscular route. The molecular formula and molecular weight of amikacin sulfate is $C_{22}H_{43}N_5O_{13}\cdot H_2SO_4$ and 762.15 g/mol, respectively. It is a broad spectrum aminoglycoside, water soluble and semi synthetic antibiotic derived from kanamycin.[3] The chemical structure of amikacin sulfate is as shown in Figure 1. Similar to other aminoglycosides, it has a narrow therapeutic index with major adverse effects like nephrotoxicity and ototoxicity. The possibility of acquiring resistantby micro-organisms cannot be ruled out which designates potential human health risk.[2]

Consequently, there is a great need to develop sensitive and reliable analytical methods for monitoring the trace level of amikacin in biological matrices. Normally serum fluid is selected for quantitative analysis of amikacin as it provides an accurate estimation of amikacin in body fluids due to its low protein binding property[1] At present, various methods for the determination of amikacin and other aminoglycosides have been developed using microbiological assay, high performance liquid chromatography (HPLC) with PDA.[4-10] and fluorescence,[11,12] Fluorimetric,[13] catalytic derivatization with HPLC,[14] spectrofluorimetery,[15] liquid chromatography—mass spectroscopy (LC-MS).[16] Amikacin sulfate molecule lacks structural properties necessary for production of signals compatible with photo diode array detector (PDA) (ultra violet and visible) for qualitative and quantitative analysis of drug molecule. Amikacin molecule contains amine functional groups, which can be utilized for pre- and post derivatization process to incorporate properties and produce signals for detection in PDA and fluorescence detector. In the reported study, protein precipitation method was used for the analysis of amikacin in human serum. The method is based on the pre-column derivatization of amikacin with FMOC-Cl in acetonitrile in the presence of borate buffer and glycine was used to eliminate excess FMOC-Cl after completion of derivatization process. FMOC-Cl reagent has been selected for the derivatization reaction because it reacts quantitatively with both primary and secondary amine groups of amikacin molecule. It produced stable fluorescent derivatives at ambient temperature.[17] Formed fluorescent derivative complex precisely quantifies amikacin trace level in serum by improving specificity, sensitivity and reactivity of chromatographic characteristic of amikacin. The reported method may be used for qualitative and quantitative determination of other aminoglycosides during TDM and pharmacokinetic studies.

MATERIALS AND METHODS

Chemicals and reagents

Amikacin sulphate (European Pharmacopoeia (EP) Reference Standard, A0365900 FLUKA) and 9-fluoromethyl chloroformate (FMOC-Cl, ≥ 99%) (HPLC Derivatization, 23186 FLUKA) were procured from Sigma Aldrich. All the solvents used during study were of HPLC grade (RANKEM). Boric acid, potassium chloride and potassium hydroxide were procured from Hi-Media. Water used during the study was obtained by using reverse osmosis purification followed by filtration utilizing a Millipore system.

Apparatus and chromatographic conditions

UHPLC system used for development and validation of method was comprised of Shimadzu pump A and B as a separation module (Model LC-30AD), a Shimadzu online degasser (Model DGU 20-A 5R) coupled with a Shimadzu auto-sampler (SIL-30AC) with a column oven (CTO—20AC). Shimadzu fluorescence detector (RF 20A) was used for detection of the sample. Reverse phase (150 X 2.1 mm), 2.1 µm particle size, UHPLC C18 column was used (Shim-Pack XR-ODS III, Shimadzu). The guard column used for the study was Phenomenax C18 with cartridge. The mobile phase comprised of Acetonitrile (ACN):Water (70:30 v/v). Mobile phase was degassed by sonication and filtered through 0.22 µm membrane filter before being used. The flow rate was maintained at 0.4 ml minute^{-1}. Amikacin detected using excitation and emission wavelength at 265 nm and 315 nm, respectively. The experiment was performed with temperature controlled column oven at 30°C.

Preparation of standard solution

The stock solution of amikacin was prepared with water (1 mg mL^{-1}) and stored at 5°C until use. Eight calibration standards were prepared by diluting stock solutions down with blank human serum to a concentration range of 0.5-10 µg/ml. Derivatizing reagent consist of 2.5 mM FMOC-Cl prepared in acetonitrile. Borate buffer (0.2 M, pH 7.3) was prepared by dissolving 0.65 g boric acid and 0.75 g potassium chloride in 50 ml milli-pore filtered water and pH of borate buffer was adjusted by using 0.2 M potassium hydroxide (0.56 g in 50 ml water). Glycine solution (0.1 M) prepared in Milli-Q water. All prepared solvents were stored at 5°C before use.

Preparation of derivatized sample solution

In 2 ml centrifuge tube, 50 µl aliquot of blank human serum were mixed with 50 µl of drug solution, 150 µl of Acetonitrile and vortexed for 30 sec. After centrifugation at 12000 X g for 15 mins, the supernatant was separated. 50 µl of supernatant solution was mixed with 50 µl borate buffer (0.2 M, pH 7.3) to adjust the pH at 7.3 (pH 7.3 is optimum for reaction between derivatizing reagent and amikacin). 50 µl derivatizing reagent (FMOC-Cl, 2.5 mM) was added and vortex-mixed for 60 seconds. The solution was kept atambient temperature for 20 mins to complete the derivatization reaction. After derivatization 50 µl glycine solution was added, vortexed and kept for five minutes at ambient temperature. From resulting solution, 5 µl solutionwere analyzed using the UHPLC system. Once derivatized, samples were stored at 5°C until their analysis.

Different parameters were assessed for their influence on the derivatization reaction to achieve maximum sensitivity and accuracy of the assay. The parameters assessed were pH of borate buffer, solvent selection. Its concentration, volume of FMOC-Cl reagent and reaction time.

Acetonitrile was found to be the most efficient in extracting amikacin from human serum and had a small variation in extraction recoveries of amikacin over the concentration range.

The precision study of the reported method performed using high quality control samples of amikacin at three different concentrations of linearity range. Lower, middle and higher concentration of amikacin as 0.5, 2.5 and 10 µg/ml. Intra-day (repeatability) precision study carried out by analyzing the three different concentrations five times within the same day at different time interval. Inter-day (reproducibility) precision study was performed by analyzing the three different concentrations five times at different days. The results are given in the Table 1.

Recovery study performed by analyzing amikacin spiked sample with and without serum at low, middle and upper concentration range of the linearity curve (i.e. 0.5, 2.5 and 10 µg/ml). The obtained results are shown in the Table 2.

RESULTS AND DISCUSSION

Amikacin molecule lacks chromophore and fluorescence property for ultra violet and fluorescence detection. 9-fluoromethyl chloroformate (FMOC-Cl) reagent had been used earlier for pre-column derivatization reaction to improve specificity and sensitivity for fluorescent detection of many of such compounds which lack chromophore. FMOC-Cl reagent reacts actively with both primary and secondary amine groups of amikacin. It reacts less readily with the hydroxyl groups of amikacin under alkaline conditions[17] as shown in Figure 2.

In pre-column derivatization reaction, pH of the reaction mixture was maintained alkaline throughout the experiment within the range of 7 to 9. FMOC-Cl reacts with amikacin under the controlled alkaline conditions and formed a maximal derivatized complex with higher sensitivity and stability. The complex formed between amikacin and FMOC-Cl was found to be more stable and sensitive as compared to other reported fluorescent reagent such as o-phthalaldehyde. After completion of derivatization reaction, stabilizing reagent glycine solution (0.1 M) was added to stabilize fluorescent complex product. It avoids deformation of fluorescent complex and the formation of interferences which may affect accurate quantification of amikacin.

Experiments had been performed with glycine, as it plays an important role in stabilization of fluorescent complex. Pre-column derivatization reaction performed without the addition of glycine and analysed. Obtained results indicates that amikacin peak not eluted (Figure 3 (a)). Alternatively, in the pre-column derivatization reaction after 20 minutes addition of FMOC-Cl, glycine addition results in elution of amikacin peak(Figure 3 (b)). Glycine is a simple and smallest of all the amino acids. It acts to react with the excess quantity of FMOC-Cl reagent and stablized the amikacin-FMOC-Cl to elute wihtout interferneces. Due to its property, at aqueous solution or near neutral pH, it exists predominatedly as the zwitterionic ions. And reacts with the unbound FMOC-Cl reagent. The chromatographic parameters of amikacin were optimized using UHPLC with fluorescence detector.

a. Represents the obtained chromatogram where glycine not added to the pre-coulmn derivatization reaction, run time upto 15 mins.
b. Represents the obtained chromatogram where glycine added after 20 mins of FMOC-Cl reagent, in pre-coulmn derivatization reaction, run time otpimised to 7 mins.

Optimized Derivatization Conditions

Effect of borate buffer pH

The borate buffer solutions having pH in the range 6 to 9 were studied. The peak was found to be broad below pH 7 while above pH 7.5 the tailing effect was observed. The peak shape was found to be sharp and symmetrical at pH 7.3, so this pH was selected for further studies (Figure 4).

Effect of derivatizing reagent, FMOC-Cl

With respect to the analysis of amikacin, the FMOC-Cl was superior to the o-phthalaldehyde (OPA) as it reacts with both amikacin primary and secondary amine groups whereas OPA reacts only with primary amines[17]

Therefore, FMOC-Cl was used in different concentration to obtain sharp peak and stabilized derivatized complex. Optimum concentration of

Table 1: Intra- and inter-day precision, percentage coefficient variation and percentage accuracy studies performed at three different concentrations of quality control amikacin samples

QC Sample Concentration (µg/ml)	Intra-day Precision and Accuracy			Inter-day Precision and Accuracy		
	Mean(µg/ml)	SD (µg/ml)	% CV	Mean(µg/ml)	SD (µg/ml)	% CV
0.5	0.44	0.014	3.25	0.45	0.02	3.59
2.5	2.51	0.05	2.12	2.58	0.07	2.64
6	6.02	0.13	2.26	5.90	0.34	5.80
10	10.26	0.52	5.07	10.25	0.35	3.39

Table 2: Shows the obtained recovery at different quality control concentration samples

QC Sample Concentration (µg/ml)	% Recovery	SD	% CV
0.5	88.02	2.87	3.26
2.5	100.59	2.14	2.12
6	100.39	2.27	2.26
10	102.56	5.20	5.07

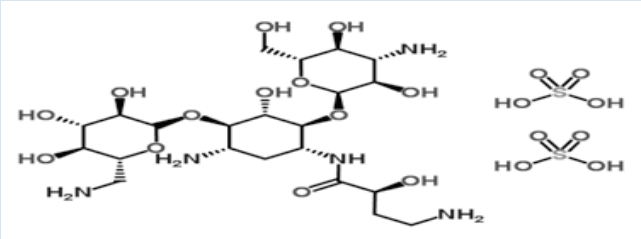

Figure 1: Structure of Amikacin Sulphate.

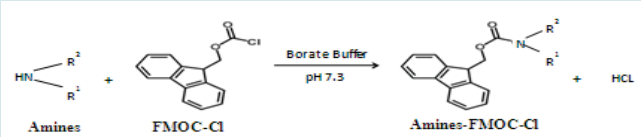

Figure 2: The reaction of Amikacin amines group with derivatizing reagent FMOC-Cl (9-fluorenyl methyl-chloro formate) to form fluorescent complex of amines—FMOC-Cl.

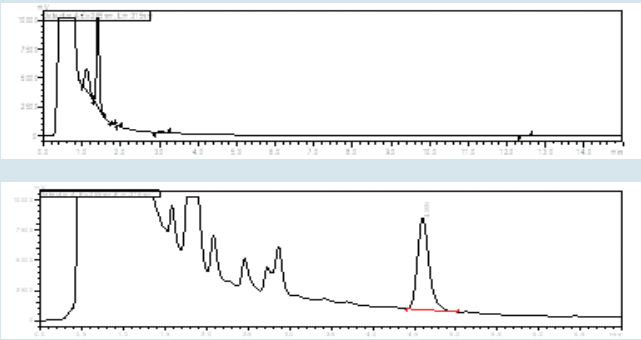

Figure 3: Ultra performance liquid chromatograms of extracts of human serum containing 6 µg/ml amikacin, peak eluted around 4.6 minute and human serum with spiked amikacin. UPLC C18, 150 X 2.1 mm, mobile phase were acetonitrile and water (70:30) at 0.4 ml/min, 5 µl, using fluorescence detector excitation and emission wavelength at 265 nm 315 nm respectively. a) Represents the obtained chromatogram where glycine not added to the pre-coulmn derivatization reaction, run time upto 15 mintues. b) Represents the obtained chromatogram where glycine added after 20 mintutes of FMOC-Cl reagent, in pre-coulmn derivatization reaction, run time optimised to 7 mintues.

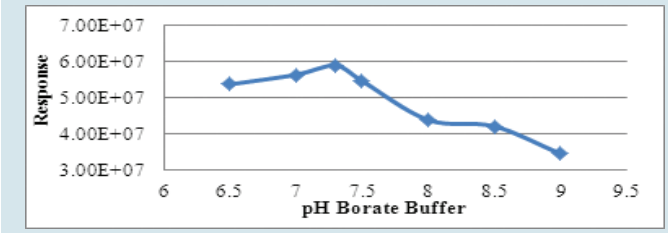

Figure 4: Effect on amikacin serum response studied at different pH of the borate buffer solution (0.2 M).

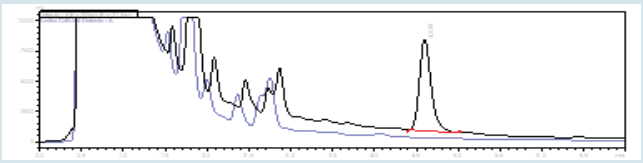

Figure 5: Ultra performance liquid chromatograms of extracts of human serum containing 6 µg/ml amikacin, peak eluted around 4.6 minute and human serum without added amikacin. UPLC C18, 150 X 2.1 mm, mobile phase were acetonitrile and water (70:30) at 0.4 ml/min, 5 µl, using fluorescence detector excitation and emission wavelength at 265 nm 315 nm respectively.

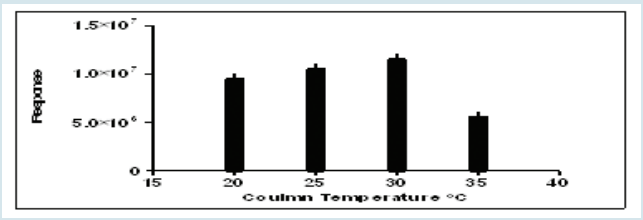

Figure 6: Study of different column temperature effect on the amikacin serum sample obtained response at optimised chromatographic conditions using UHPLC C18 coulmn.

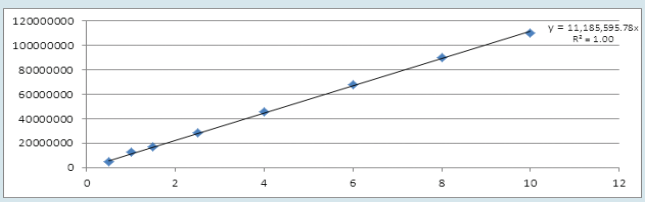

Figure 7: The calibration curve obtained for spiked serum samples of amikacin in the concentration range of 0.5—10 µg/ml representing linearity and R2 were found to be 1.

FMOC-Cl was found to be 2.5 mM. The concentration of derivatizing agent played very influential role on response. Increase in concentration above 2.5 mM decreased the response.

Effect of derivatization reaction time

The optimum reaction time determination is necessary to get stable fluorescent complex with higher sensitivity and reproducibility. The reaction was performed for different time intervals ranging from 10 to 35 mins. Based on the peak shape and area, the optimum time required for completion of derivatization reaction between amikacin and FMOC-Cl was found to be 20 mins. Above this time the deterioration of the complex occurred and bi-products formed which was observed through peaks.

System optimization

Effect of ACN on amikacin retention:

The mobile phase composition is a predominant factor which affects on drug retention time and its elution. The effect of ACN percentage in mobile phase on amikacin retention time was investigated. It was observed that as the percentage of ACN was decreased below 70% the peak became asymmetric and broad. This is an indication of poor elution and separation of drug. Based on observations the optimized mobile phase ratio was found to be ACN : Water (70: 30) with the flow rate of 0.4 ml/min (Figure 5).

Fluorescence detector

Fluorescence detector is highly specific and less susceptible to interferences because of its specific excitation and emission wavelength mechanisms. Its high sensitivity and specificity eliminates interferences from samples prior to analysis. FMOC-Cl reagent was selected because it forms stable and specific derivatized complex with amikacin. This complex excites and emits at specific wavelength at 265 and 315 nm respectively and expedites amikacin analysis(Figure 5).

Effect of column oven temperature

The effect of column oven temperature on drug elution was studied from 20°C -35°C. The peak shape was found to be symmetrical and sharp below 30°C. Above this temperature broad peak with tailing effect was observed. Based on observations optimized column temperature was found to be 30°C. Figure 6 depicts the various peaks obtaind at various temperatures.

Validation parameter study

Linearity

The linearity of the method was studied by spiking blank serum with amikacin solution (0.5—10 µg/ml). The representative linearity equation was found to be y = 10979318x + 1897528 where the slope is 1097931, y is intercept +1897528, and correlation coefficient R^2 calculated to be 1.00. The results indicate that the method is linear over the studied concentration range, as shown in the Figure 7.

Precision and accuracy

The precision of the method was demonstrated by the percent coefficient of variation over the concentration range of low, middle and high quality control sample of amikacin as 0.5, 2.5 and 10 µg/ml. The intra- and inter-day precision study performed and obtained results had been shown in Table 1.

Specificity: UHPLC fluorescence analysis of the blank human serum with and without amikacin drug solution performed and showed no interferences with Amikacin. As no peak elution observed in blank sample when compared with the amikacin sample. The standard chromatogram of amikacin is shown in the Figure 5 indicating no interference in the sample at the retention time of around 4.6 mins.

Sensitivity

The limit of detection and limit of quantification calculated based on the signal to the noise ratio. Limit of detection was found to be 50 ng/ml and limit of quantification was found to be 350 ng/ml.

Extraction recovery

The percentage recovery of amikacin by different solvents from serum sample was determined by comparing the peak areas of plain amikacin sample and serum spiked with amikacin. The obtained recovery range from 88 to 103% (Table 2).

Stability and carry over study

The stability of the amikacin-FMOC-Cl formed fluorescent complex at different amikacin concentration had been performed as per US FDA bio-analytical guidelines.[18] Derivatives were continuously analyzed over a period of 72 hrs. Decomposition in fluorescent complex was not observed and found to be stable at room temperature for 3 hrs. Carry over study had been performed by running a blank serum sample after analyzing amikacin serum sample (4 µg/ml). The result indicates that amikacin carryover was not found after an analysis of blank serum samples.

Robustness and Ruggedness

The robustness of the developed method were determined by performing minor changes in chromatographicconditions like flow rate ($\pm$ 0.01), column temperature ($\pm$ 0.2) and ratio of mobile phase (Acetonirtirle 70 $\pm$ 2). It was observed that no marked changes in the amikacin chromatogram found. Demonstrates the UHPLC developed method robust and rugged.

The reported method had been applied for the quantitative estimation of amikacin in neonates for therapeutic drug monitoring study. This study had been performed in further part of the project. The results obtained are clearly indicative that the method can be effectively used for quantification of drug in biological fluid, i.e. serum which can be used for kinetic and TDM study.

CONCLUSION

In the present study a simple, rapid and sensitive bio-analytical method for the analysis of amikacin in serum was developed and validated. The protein precipitation extraction method was used where pre-column derivatization was carried out by FMOC-Cl reagent. Derivatized complex formed with amikacin is more stable and sensitive. The validation parameter study results assured the linearity, accuracy, preciseness and sensitivity of amikacin in developed method, which allows the satisfactory implication of amikacin trace level analysis of biological samples.

Furthermore, reported method can be utilized in routine analysis in clinical laboratories and pharmacokinetics department. Beneficial in routine analysis of amikacin large number of samples, it provides short analysis time with simple chromatographic conditions and accuracy with high sensitivity.

Validation parameters study results show the developed method is simple, sensitive, linear, accurate, precise and rugged. The developed method can be efficiently used for the kinetic and TDM study of amikacin and other aminoglycosides.

ACKNOWLEDGEMENT

The authors are grateful to Dr Srinivas Patnala, Dr Manisha Bhandankar and Dr Alka D Kale for help and support.

CONFLICT OF INTEREST

The authors declared no competing financial interest.

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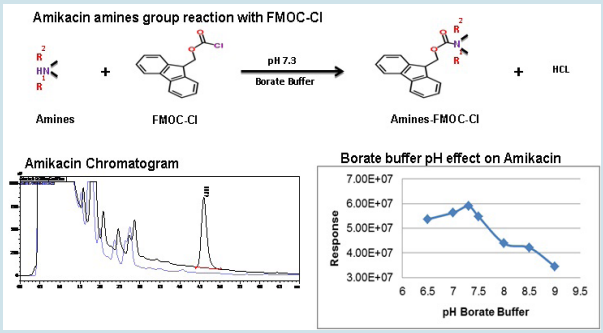
PICTORIAL ABSTRACT



SUMMARY

- A simple, sensitive and rapid method optimized for amikacin in serum utilizing pre-column derivatization reaction. FMOC-CL reagent has been selected for amikacin to form stable fluorescent derivatized complex as react with both primary and secondary amines group. Protein precipitation extraction procedure has been utilized for precipitation procedural using acetontirile. Different parameters in precolumn derivatization reaction were well optimized as borate buffer pH, different volume and concentration of FMOC-Cl reagent and derivatization reaction time and effect of glycine on derivatization reaction.This is a significant method, because serum utilized for sample preparation is only 50 µl and limit of quantification has been improved with reduced analysis time (7 mins) compared to earlier reported methods. The reagent and solvent required are less and selective. Due to its utilization of ultra performance chromatographic analysis technique, it is a cost effective, less-time consuming, precise and efficient study.

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