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Efficacy of Liquid-Liquid Extraction and Protein Precipitation Methods in Serum Sample Preparation for Quantification of Fexofenadine in Human Serum

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ABSTRACT

Biological samples contain many intrinsic and extrinsic compounds in different concentrations which makes it very challenging to analyze it and cannot normally be injected directly into the analyzing system without sample preparation. Two different sample extraction procedures liquid-liquid extraction and protein precipitation have been employed in this study to quantify fexofenadine in human serum and the recovery rates have been compared. Protein precipitation by methanol has an advantage over liquid-liquid extraction with recovery rates of more than 90% but has limitations due to rise of column back pressure. Due to better recovery rate and quick sample preparation technique the protein precipitation method has been chosen for extraction of drug from serum sample. The developed HPLC method was validated and found to be accurate, precise and specific within the linearity range of 0.8- 4.0 μg/mL. Lower limits of detection and quantification were established as 0.6 and 0.8 μg/mL respectively.

Keywords*:* Liquid-liquid extraction, protein precipitation, bioanalysis, sensitivity

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INTRODUCTION

In the pharmaceutical industry pharmacokinetics, toxicokinetics and bioequivalence studies are immensely facilitated by bioanalysis as it provides quantitative measure of the active drug and its metabolite. Sample preparation is an important aspect of bioanalytical estimation because biological samples are extremely complex matrices composed of many components like proteins, which can lead to protein binding of the analyte that can interfere with good separation and detection. Thus biological samples cannot normally be injected directly into the analyzing system without sample preparation[1]. Numerous sample preparation techniques have been developed for bioanalytical purposes. Solid phase extraction, liquid – liquid extraction and protein precipitation are a few of them.

Fexofenadine, a selective peripheral H1 blocker is a second generation antihistamine. Chemically it is (±)-4[1 hydroxy-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-butyl]-α, α-dimethyl benzeneacetic acid hydrochloride (Figure 1).

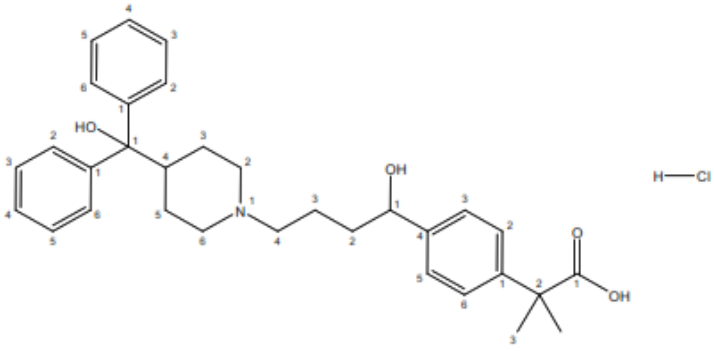

Figure 1. Fexofenadine hydrochloride

Unlike the first generation antihistamines it does not cause sedation. It can exist in zwitter ionic form that prevents it to pass the blood-brain barrier and therefore it is non- sedative[2]. Fexofenadine is widely prescribed for seasonal allergic rhinitis, hay-fever, sneezing, rhinorrhea, itchy nose/palate/throat, itchy/watery/red eyes etc.[3]. After oral application it takes about one hour for its action and two to three hours to reach maximum plasma concentration[4]. Steady state plasma concentration of fexofenadine remains in the range of 0.058-4.677μg/mL[5].

It has been a subject of interest among researchers worldwide for quite a few years now and several chromatographic methods have been reported for determination of Fexofenadine in human plasma. Nevertheless it can be quite

challenging to determine Fexofenadine in biological samples such as serum, plasma or whole blood owing to a number of interferences that may affect the analysis. So prior to analysis biological samples need purification to remove unwanted components and also sometimes the compound of interest needs to be concentrated. Different kinds of extraction techniques like solid phase extraction, liquid- liquid extraction, protein precipitation have been employed to purify these samples[6]. Previous work on fexofenadine quantification in biological samples using different extraction techniques are presented here in a tabular format [7-20] (Table 1).

Table 1. Previous work done on biological quantification of fexofenadine.

References	Extraction techniques	Solvents/ Cartridge	Instrumentation	Recovery
Miura et al., 2007	Solid phase extraction	Methanol, Oasis HLB cartridge	HPLC coupled with UV	Around 67-71.5%
Nigori et al., 2007	Solid phase extraction	Methanol, Oasis HLB cartridge	LC/MS	58.2±1.5%
Yamane et al., 2007	Solid phase extraction	Methanol, ammonium acetate, acetic acid, Oasis HLB cartridge	LC-MS/MS	92.5%
Bharathiet al., 2008	Solid phase extraction	Methanol, ammonia, Oasis HLB cartridge	LC-MS/MS	91.5%
Fu et al., 2004	Solid phase extraction	Methanol, acetate buffer 96-well plate	LC-MS/MS	>70%
Uno et al., 2004	Solid phase extraction	SPE C_{18} minicolumn	HPLC with fluorescence	72.8-76.7%
Hofmann etal.,2002	Solid phase extraction	Methanol, acetate buffer, triethylamine. C_{18} SPE cartridge	LC-MS/MS	88.96±2.9%
Coutant et al., 1991	Solid phase extraction	Analytichem C_{18} minicolumn	HPLC with fluorescence	59.6-66.5%
Isleyen et al., 2007	Liquid-Liquid extraction	Formic acid, DCM:EA:DEE(30:40:30)	LC/MS	52-55%
Stanton et al., 2010	Protein precipitation	Acetonitrile	LC-MS/MS	95.3±10.3%
Pathak et al., 2008	Protein precipitation	Acetonitrile, trichloroacetic acid	HPLC with fluorescence	81.79%-85.23%
Guo et al., 2010	Protein precipitation	Methanol	LC-MS/MS	87.6-93.6%
Flyne et al., 2011	Protein precipitation	Ammonium formate, methanol, acetonitrile	LC-MS/MS	87.6-93.6%
Helmy et al., 2015	Liquid-liquid extraction	Diethylether	HPLC coupled with UV	95.4%

Most of the reported methods used solid phase extraction techniques for sample preparation. Though solid phase extraction is a very selective method and yields high recoveries and is highly reproducible it is a very complicated, lengthy and costly method[21].

Compared to solid phase extraction, relatively few methods are reported where liquid-liquid extraction or protein precipitation has been used to determine fexofenadine in biological samples. Isleyen et al., 2007 and Helmy et al., 2015; both used liquid-liquid extraction (LLE) technique in their attempt to eliminate the need of solid phase extraction. These methods were sensitive, precise and accurate with the absolute recoveries 52-55% and 95.4% respectively. This sample preparation procedure is efficient and cost-effective but it is labor intensive, difficult to automate and require a large amount of organic solvent[22].

Protein precipitation (PP) with miscible organic solvents (usually acetonitrile or methanol) is the simplest approach that requires minimal method development and removes the majority of the protein from the sample[23]. Pathak et al., 2008; Guo et al., 2010; Flyne et al., 2011; all used protein precipitation techniques for sample preparation in their attempt to quantify fexofenadine in biological matrices. PP is the most commonly used sample preparation method because of its ability to remove the unwanted plasma proteins from samples prior to analysis with minimal method development requirements and low cost[24]. The only drawback it has is it may increase the back pressure of the HPLC system and may affect the column performance[25].

In bioanalysis the recovery rate is directly related to the extraction procedure. Moreover, proper sample pretreatment procedure should be developed to ensure sufficient sensitivity and selectivity, whereas the run time should be kept to a minimum in order to obtain adequate speed.

The aim of this study was to find out the optimum extraction method for determining fexofenadine in human serum by RP-HPLC with UV detector from liquid- liquid extraction and protein precipitation. The recovery rates were compared to see which better fits routine laboratory applications. We also validated the developed HPLC method using the protein precipitation extraction procedure with greater recovery rate.

METHODOLOGY

Drugs, Chemicals and study products

Fexofenadine and Cetirizine (Internal standard, IS) were obtained as a gift sample from Beximco pharmaceutical limited, Bangladesh. High purity deionized water was obtained from Millipore, Milli-Q (Merck KGaA, Darmstadt, Germany) water purification system and used throughout the process, HPLC grade acetonitrile, methanol was purchased from active fine chemicals, Bangladesh. Ammonium acetate, trifluoroacetic acid and acetic acid were purchased from Merck, Germany. Dichloromethane, ethyl acetate, diethyl ether were analytical grade and also purchased from active fine chemicals.

Instrumentation and chromatographic system

A high performance liquid chromatographic system was used from Hitachi High-tech Science Corporation, Tokyo, Japan comprising of Hitachi Chromaster 5110 quaternary pump for constant flow and constant pressure delivery, a column oven (Chromaster 5310 Column Oven), Chromaster 5210 auto sampler and Photodiode array detector (Chromaster 5430 detector). Data was integrated using Agilent Open Lab control panel CDS software running on a personal computer. The chromatographic analysis was performed on a C18 column (250 mm x 4.6 mm i.d., 5µm particle size), LaChrom, Hitachi, Japan with C18 guard column (23mm × 4 mm; 3 µm), LaChrom, Hitachi, Japan. Different mobile phase compositions were considered for successful separation of the analyte from different matrix interferences.

Mobile phase optimization

The separation efficiency along with the system suitability parameters like retention time (RT), Tailing factor, and number of theoretical plates were checked for the mobile phase as in Table 2.

Table 2. Mobile phase choosen for Fexofenadine bioanalysis

Mobile Phase Composition	Buffer	Buffer: Methanol: Acetonitrile
MP1	5mM ammonium acetate, pH adjusted to 4.0 with 0.03% trifluoroacetic acid	50:30:20
MP2	5mM ammonium acetate, pH adjusted to 4.3 with acetic acid	55:10:35
MP3		55:15:30
MP4		57:10:33

Mobile phases were filtered through 0.2µm Nylon 66 membrane filters and degassed before use to remove particulate matter. The mobile phase was pumped isocratically at a flow rate of 1.0 mL/min during analysis at ambient temperature. The volume of injection was 20 µL. Eluent was detected at 220 nm.

Collection of serum sample

The human serum was collected from healthy volunteers after obtaining approval from the National Research Ethics Committee. After collection it was allowed to stand for 30 min and then centrifuged at 3000 rpm for 10 minutes and serum was collected. Then the serum sample was stored in the freezer at -20˚C for further use.

Preparation of standard stock solution

10 mg of Fexofenadine working standard was accurately weighed and transferred into 50 mL clean dry volumetric flask and about 20 mL of diluent (methanol: water 70:30, v/v) was added to dissolve it completely by shaking. Finally the volume was made up to 50 mL with same solvent to make a solution of 200 µg/mL. The stock solution of IS was prepared by dissolving 10.0 mg of Cetirizine in 50 mL of diluents and further diluted to make the final concentration of 100 µg/mL. Working solutions of Fexofenadine and Cetirizine were stored in 4°C.

Preparation of serum sample

To prepare a serum sample, three samples at a concentration of 8, 16, 24 and 32 µg/mL were prepared from stock solution. Serum samples were prepared by spiking 30 µL of Fexofenadine solution from each dilution and 30 µL of cetirizine solution in to 270 µL of blank serum to produce serum samples with the concentration of 0.8, 1.6, 2.4 and 3.2 µg/mL.

Extraction technique

Liquid-liquid extraction (LLE)

For liquid-liquid extraction an extraction solution was prepared using HPLC grade dichloromethane: ethyl acetate: diethyl ether in the ratio of 30:40:30 (% v/v/v). In this procedure 270 µL of serum was taken into a centrifuge tube where 30 µL of fexofenadine solution and 30 µL cetirizine solution was added and vortexed for 5 second for adequate mixing. After vortexing, 150 µL of formic acid solution was added and again vortexed for 5 seconds. 5 mL of extraction mixture was then added into the centrifuge tube and vortexed again for 40 seconds. This mixture was then centrifuged at 5500 rpm for 5 min. The organic layer was separated and evaporated to dryness. The solution was reconstituted into 500 µL diluent and vortexed. Finally it was poured into clean and dried HPLC vial for injection.

Protein precipitation (PPT)

Methanol extraction

Samples of spiked serum with fexofenadine (300 µL) were transferred to 2 mL eppendorf tubes where 30 µL of cetirizine solution was added and vortexed for 30 seconds. Then 870 µL of methanol was added to precipitate the protein. The eppendorf tubes were vortexed for 30 seconds and kept static for settling down. After 15-20 minutes samples were centrifuged for 12 min at 12,000 rpm.

The supernatant was collected, transferred to autosampler vials through a 0.22 syringe filter and directly injected into HPLC.

Acetonitrile extraction

Samples of spiked serum with fexofenadine (300 µL) were transferred to 2 mL eppendorf tubes where 30 µL of cetirizine solution was added and vortexed for 30 seconds. The protein was precipitated with 870 µL of Acetonitrile. The eppendorf tubes were vortexed again for 30 seconds, left aside for approximately 15- 20 minutes and then centrifuged for 12 min at 12,000 rpm. The supernatant was collected, syringe filtered to autosampler vials and directly injected into HPLC. This whole procedure is summarized in Figure 1.

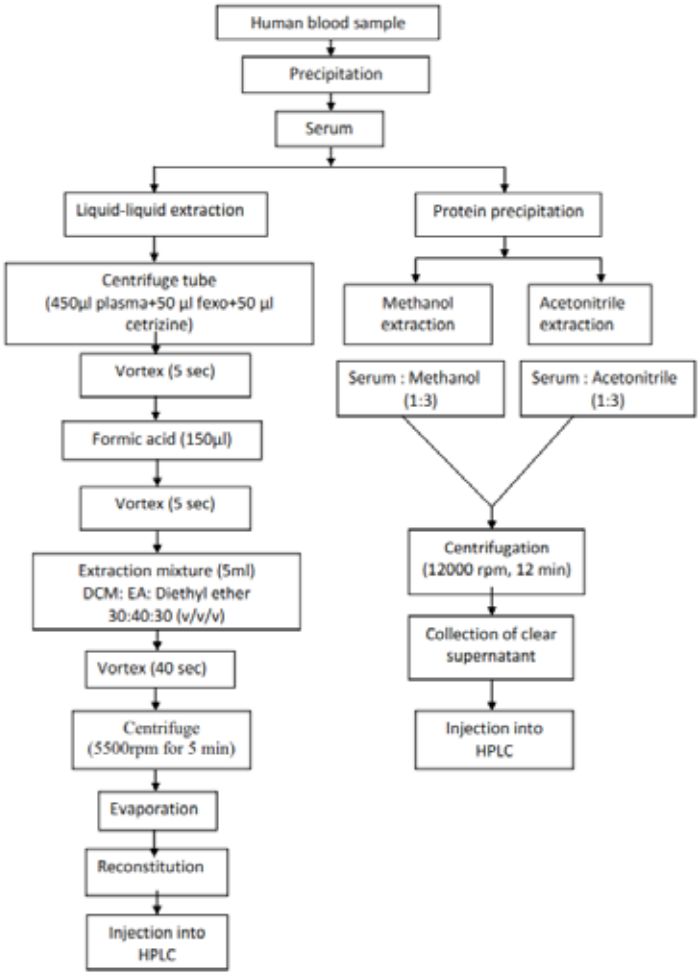

Figure 2. Sample preparation for bioanalysis

Method validation

After evaluating the recovery rates of fexofenadine from serum samples by liquid-liquid extraction and protein precipitation the bioanalytical method was validated using the extraction procedure that gave greater recovery rates. The method was validated according to ICH guidelines for validation of bioanalytical procedures in terms of linearity, specificity, limit of detection (LOD), lower limit of quantitation (LLOQ), recovery, accuracy and precision[26].

Linearity, LOD and LLOQ

Linearity was tested for the concentration range of 0.8–4.0 μg/mL. For the determination of linearity, standard calibration curves of six points (0.8, 1.0, 1.5, 2.0, 3.0 and 4.0) were used along with zero concentration blank serum sample to confirm the absence of interferences. The acceptance criterion for correlation coefficient is 0.99 or more, otherwise the calibration curve would be rejected. Three replicate analyses were performed for each concentration.

The LLOQ is the lowest amount of analyte in a sample that can be quantitatively determined with suitable accuracy (percent error <20%) and precision (coefficient of variation <20%). The analyte response at the LLOQ level should be at least five times greater than the analyte response of the zero calibrator. The lower limit of detection (LOD) was the minimum concentration that can be detected by detector response with analyte response, which should be equal to or greater than three times of the analyte response of the zero calibrator. LLOQ was measured by five replicate analyses of the analyte.

Specificity

The specificity was evaluated by analyzing the chromatogram of the human drug-free serum from different volunteers (n=6) in triplicate to check for the matrix interference. The retention time of fexofenadine in the HPLC chromatogram of spiked serum and blank samples were compared to define any endogenous materials and/or degradation peaks appearing at the same retention time as fexofenadine or cetirizine.

Recovery and matrix effect

The recovery rate was determined by comparing the peak area ratios (fexofenadine/IS) of serum samples that have gone through the sample preparation and extraction procedures with the serum free samples directly injected in the mobile phase. This procedure was repeated for the four different serum concentrations of fexofenadine such as 0.8, 1.6, 2.4 and 3.2 μg/mL with three replicate analyses for every concentration.

Precision and accuracy

To determine the precision of the assay, replicate analysis of four concentration levels of fexofenadine were used (0.8, 1.6, 2.4 and 3.2 µg/mL). Intra-day precision and accuracy were determined by repeated analysis of the group of standards on one day. Inter-day precision and accuracy were determined by repeated analysis on three consecutive days. The concentration of each sample was determined using a standard curve prepared and analyzed on the same day.

RESULTS and DISCUSSION

The sample preparation step before the HPLC analysis is intended to facilitate the determination of components of the drug candidate that involve pharmacokinetics and metabolic stability. Two extraction procedures, liquid-liquid extraction and protein precipitation, have been employed to extract fexofenadine from human serum and the recovery rates for both procedures have been considered. Chromatogram of fexofenadine and cetirizine has been observed at the wavelength of 220 nm (Figure 2).

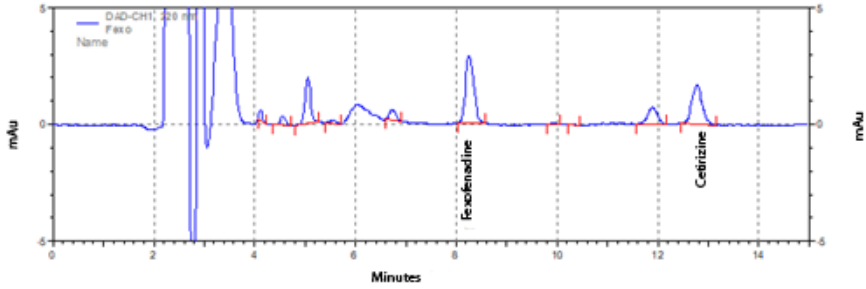

Figure 3. Chromatogram of serum sample spiked with fexofenadine and cetirizine at 220.0 nm

Method optimization

Fexofenadine is amphoteric in nature as it has both a carboxylic acid end and a tertiary amine end in its structure. Therefore it would remain in an ionized form regardless of acidic or basic pH conditions. Peak tailing, worsening of peak shapes, long retention time, proper chromatographic separation to avoid overlapping of sample peak with the serum's interfering peaks- all these factors need to be closely monitored while developing the method. Moreover aging of analytical column makes the optimization of the mobile phase with the right balance of pH and organic content more crucial. A solution of 5mM ammonium acetate has been chosen as the buffer solution. The pH of the buffer was first adjusted with 0.03% trifluoroacetic acid (TFA) considering TFA's effect in improving peak shape and resolution by solubilizing interfering proteins in

the serum sample[27]. Also it has an impression of facilitating chromatographic separation of zwitterionic compounds like fexofenadine. But with time it was observed that the pH of the buffer is not stable and the baseline keeps fluctuating. So we replaced the mobile phase additive and instead of TFA we adjusted the buffer's pH at 4.3 with acetic acid. This gave us a stable pH condition as well as steady baseline. Another challenge during method development was figuring out the right combination of organics and buffer. Higher portion of the buffer resulted in longer retention time. But more organic compounds can coagulate proteins that are left in the serum sample and block the column. Considering all these factors different ratios of buffer, methanol and acetonitrile have been tested and the observation has been listed in table no 3. A mobile phase consisting of 5mM ammonium acetate (pH~4.3), methanol and acetonitrile in the ratio of 57:10:33 has been finalized for further method validation.

Table 3. Mobile phase optimization

Mobile Phase Composition	Buffer: Methanol: Acetonitrile	Retention time		Remark
		Fexofenadine	Cetirizine	
MP1	50:30:20	8.32 min	14.26 min	Fluctuation of baseline makes this system unstable
MP2	55:10:35	7.69 min	13.28 min	Peaks overlapping with serum's interfering peaks
MP3	55:15:30	12 min	25 min	Too long retention time for IS
MP4	57:10:33	11.63 min	18.62 min	Well separated peaks

Recovery rates after liquid-liquid extraction

The absolute recoveries and extraction efficiency were determined by the HPLC analysis of fexofenadine for three different concentrations 0.06 µg/mL, 1 µg/mL and 2 µg/mL in serum and compared with the peak areas ratios with those obtained from direct injection of the same amount of fexofenadine dissolved in the diluent. Recovery rates are given in table no 4. The value of recovery rates was approximately 33- 42%, which is really low.

A representative chromatogram (Figure 3) was generated to show that other components, which could be present in the sample matrix, are resolved from the parent analyte. No significant changes in retention times of the drugs clearly indicated the specificity of the method.

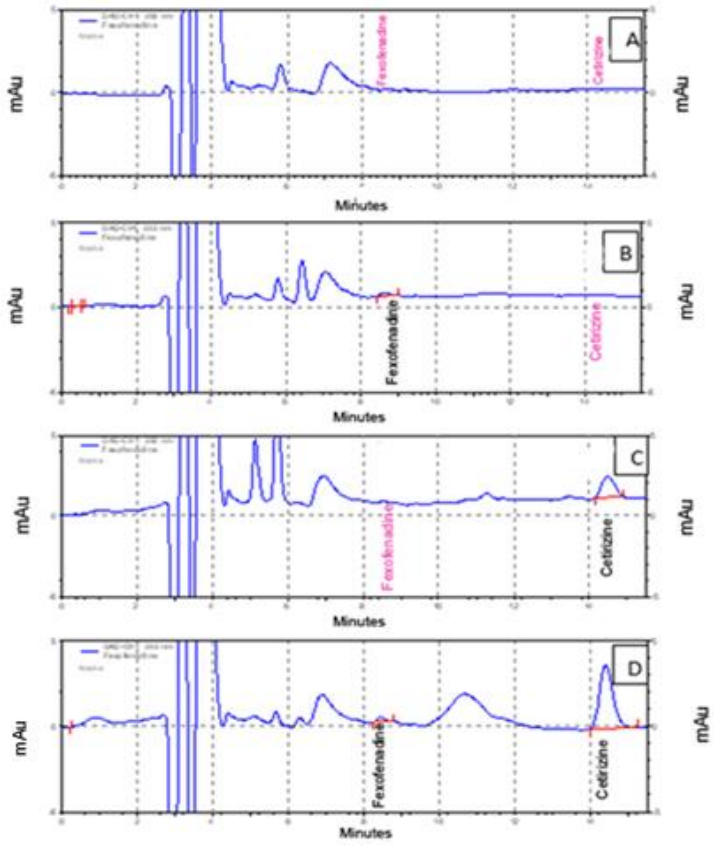

Figure 4. Representative chromatogram of blank plasma (A), fexofenadine pure drug (B), cetirizine (C) and both in human plasma (D) after liquid- liquid extraction.

Recovery rates for protein precipitation

In case of acetonitrile precipitation the peaks were all splitted. It may be due to sample solvent incompatibility with mobile phase. The solubility of investigational compounds is an important factor in method selection and in this study the investigational compounds are methanol soluble hence methanol was selected as the most suitable serum precipitation method. (Figure 4).

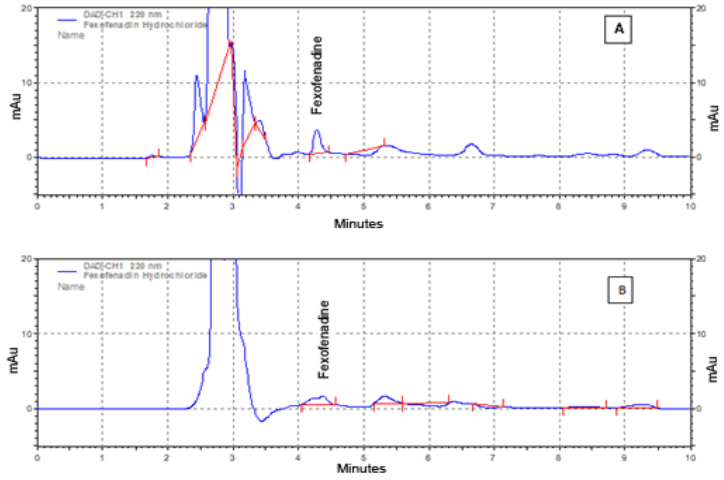

Figure 5. Representative chromatograms of fexofenadine after protein precipitation with methanol (A) and Acetonitrile (B).

Mean drug recovery was calculated by comparing the peak area ratios of extracted serum samples with those obtained from non extracted calibrators with the same amount of drug. The recovery rate for 0.06 µg/mL concentration was 74.26%, for 1 µg/mL 93.20% and 95.20% for 2 µg/mL concentration. A representative chromatogram (Figure 5) was generated to show that other components, which could be present in the sample matrix, are resolved from the parent analyte that ensures specificity of the method.

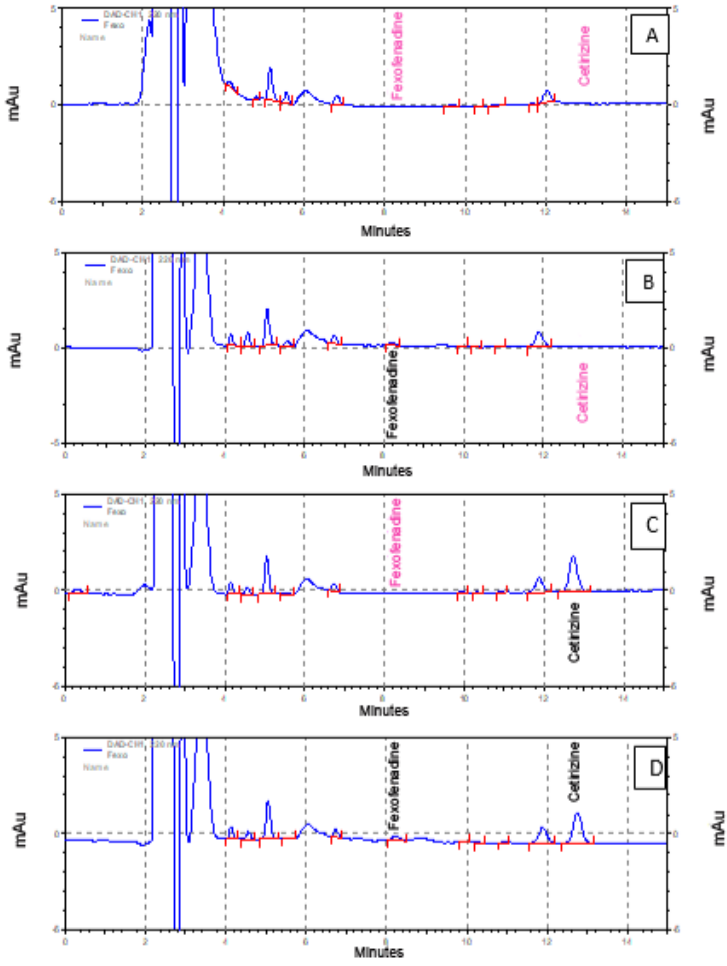

Figure 6. Representative chromatogram of blank plasma (A), fexofenadine pure drug (B), cetirizine (C) and both in human plasma (D) after protein precipitation.

It was found that the recovery rates after protein precipitation was higher than the recovery rates after liquid- liquid extraction (Table 4).

Table 4. Recovery of Fexofenadine from Serum by liquid-liquid extraction and protein precipitation

Added concentration (µg/mL)	% Recovery	
	Liquid-liquid extraction	Protein precipitation
0.06 µg/mL	33.87	74.26
1 µg/mL	40.23	93.20
2 µg/mL	42.22	95.20

The recovery rate for 0.06 µg/mL concentration is very low, which is why a higher concentration of 0.8 µg/mL has been chosen as the lower limit of quantification.

Method validation

Compared to liquid-liquid extraction, protein precipitation gave higher recovery rates. A method has been validated using protein precipitation for sample preparation to quantify fexofenadine in human serum.

System suitability

To ensure the instrument performance, system suitability parameters such as peak asymmetry, peak capacity factor, peak purity, and theoretical plate number were monitored. The retention time for fexofenadine and cetirizine was 11.13 minute and 19.51 minute respectively. Peak asymmetry was less than 2 and the peak purity value was greater than 0.9 with an RSD value 1.36%. The value of peak theoretical plates is more than 2000 for both fexofenadine and cetirizine, which is in acceptable range.

Selectivity

The selectivity of the method was investigated by comparing the chromatograms of blank serum, serum sample spiked with only fexofenadine, serum sample spiked with only internal standard cetirizine and serum sample spiked with both fexofenadine and cetirizine. The method was found to be specific and selective. The retention times were 11–12 min and 18–19 min for fexofenadine and cetirizine respectively. There were no interfering peaks from endogenous substances at the elution time of fexofenadine and cetirizine as shown in Figure 6.

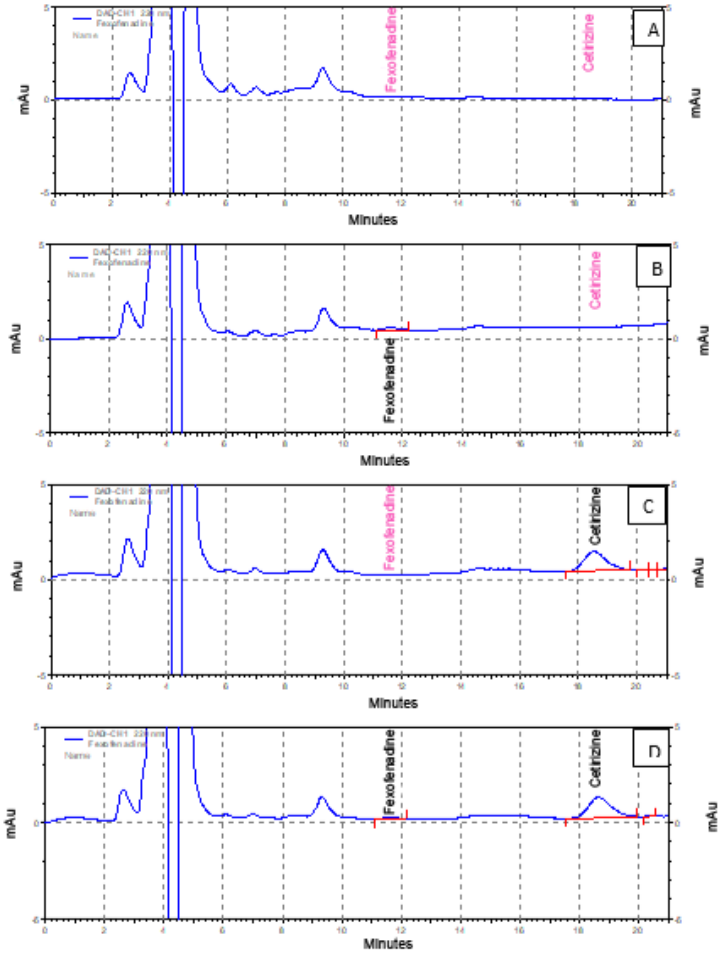

Figure 7. Representative chromatograms of blank plasma (A), fexofenadine pure drug (B), cetirizine (C) and both in human plasma (D)

Linearity, LOD and LLOQ

The method shows linearity over the concentration range of 0.8– 4.0 μg/mL, with a coefficient of correlation (R^2) 0.9986 (Figure 7).

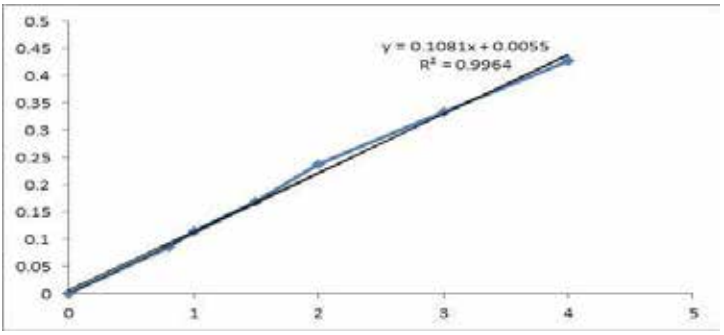

Figure 8. Calibration curve

The LLOD and LLOQ was found to be 0.6 µg/mL and 0.8 µg/mL respectively with accepted accuracy (percent error <20%) and precision (coefficient of variation <20%). Results are presented in Table 5.

Table 5. Linearity, lower limit of detection and quantification.

Parameters	Values
Linearity range (µg/mL)	0.8-4.0
Correlation coefficient(R^2)	0.9964
Regression equation	y = 0.1081x + 0.0055
LOD (µg/mL)	0.6
LLOQ (µg/mL)	0.8

Recovery and matrix effect

The extraction recoveries of fexofenadine were determined at four different concentrations (0.8, 1.6, 2.4, and 3.2 µg/mL). The method showed good efficiency in terms of recovery as the average recovery for fexofenadine ranges from 97.89 to 102.93% (Table 6).

Table 6. Recovery rates of fexofenadine.

Added concentration	% Recovery (mean±SD)	%RSD
0.8	102.93±1.94	1.88
1.6	99.678±0.43	0.43
2.4	97.897±1.32	1.35
3.2	100.32±1.54	1.46

The recovery of cetirizine was consistent and precise. Extraction method was simple and faster as there was no drying step present in the sample preparation process.

Accuracy and precision

The intra-day precision of 0.08, 0.24, 2.40, and 4.80 μg/mL was in the range of 0.49–1.80 % and inter-day precision was 0.41– 1.98%. The accuracy was in the range of 108.21– 92.75% and 93.45%- 104.67% for intra- day and inter- day respectively. Results are presented in table 7.

Table 7. Intra- day and inter- day accuracy and precision.

Within day (intra-day)				Between day (inter-day)		
Nominal conc. (μg/mL)	Conc. Found (μg/mL; mean±SD)	Precision (%)	Accuracy (%)	Conc. Found (μg/mL; mean±SD)	Precision (%)	Accuracy (%)
0.8	0.75± 1.9	1.80	93.23%	0.81± 0.87	1.56	93.45%
1.6	1.4± 0.4	0.49	92.75%	1.56 ± 1.9	0.41	95.56%
2.4	2.6± 1.3	1.30	108.99%	2.8 ± 1.7	1.87	104.67%
3.2	3.4± 1.5	1.47	106.21%	3.6 ± 1.4	1.98	102.55%

Sample preparation prior to chromatographic separation is a crucial part of the bioanalytical method development process. It is performed to dissolve or dilute the analyte in a suitable solvent, removing the interfering compounds and pre-concentrating the analyte. Sensitivity and selectivity of a method is highly dependent on it. In this study of fexofenadine liquid-liquid extraction provided low recovery rates compared to protein precipitation technique, which gave protein precipitation an edge over liquid-liquid extraction for quantification of fexofenadine in human serum. The low recovery rate can be a result of solvent-mobile phase incompatibility or selection of an extraction mixture. As fexofenadine is methanol soluble, methanol worked just fine as a protein precipitating agent. Besides these protein precipitation technique is a very quick process for sample preparation as there is no drying step and it does not need any extra instrumental set up, but it causes increased column back pressure which is harmful for analytical columns. Every day after running the serum samples the column needs to be washed properly for around 35 to 45 minutes

to maintain column quality and peak shape. The developed method is simple, reproducible, accurate and precise.

In most of the bioanalytical procedures sample preparation takes about half to three quarter of the total time of analysis yet most technical innovations of the recent years are related to separation and detection rather than sample preparation or extraction. It is a work of great importance but has not been enough emphasized. There is great scope for further innovative sample preparation techniques to quantify drugs in biological samples.

STATEMENT OF ETHICS

An approval from the National Research Ethics Committee of Bangladesh Medical Research Council has been obtained (registration number 06924082017).

CONFLICT OF INTEREST

There are no conflicts of interest.

AUTHOR CONTRIBUTION

All authors have contributed significantly in the work presented here. Author MHS formulated the presented research project and supervised all the work along with SMM. Author MHS and FM designed the research work. The data acquisition, data analysis and statistical analysis was done by authors FA [1,2] and FM. Author FA [1,2] performed the experimental work. Drafting of the manuscript was done by FA[1,2] and SRR which was corrected by FM, SS and FA[1]. Authors FM, FA[1], and MHS finalized the final drafting of the manuscript. All authors read and approved the final manuscript.

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