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Development and validation of an HPLC method for pentazocine quantification: Insights into pharmacokinetics using biodegradable polymeric nanoparticles

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Abstract: **Background**: High-Performance Liquid Chromatography (HPLC) has been employed as an analytical procedure for the quantitative analysis of drugs in pharmaceutical and biological samples, owing to its higher sensitivity, accuracy, and reproducibility. In pharmacokinetic analysis, accurate analytical procedures are needed to quantify the concentration of drugs, especially when new delivery systems are used. Biodegradable polymeric nanoparticles have been used as carriers for drugs because they can increase the stability, control the release and improve the bioavailability of drugs. **Objectives**: The objectives of the study was the development/modification of a performance liquid chromatographic method and its validation before it was applied to profiling the pharmacokinetic behavior of the developed biodegradable polymeric nanoparticles. **Methods**: Pentazocine-loaded biodegradable polymeric nanoparticles were prepared using chitosan-STPP (CP-2) via ionic gelation and Poly (lactic-co-glycolic acid) (JB5) via emulsion solvent evaporation. The method, adapted from reported protocols, was optimized for laboratory conditions and validated according to ICH Q2(R1) guidelines. Validation parameters included linearity, precision, accuracy, working range, limit of detection (LOD) and limit of quantification (LOQ). **Results**: The method demonstrated excellent linearity (10-1000ng/mL, R^2 = 0.9983), precision (%RSD <2%), accuracy (98-102%), LOD of 3.3ng/mL and LOQ of 10ng/ml. *In vivo* pharmacokinetic studies were performed in 12 healthy male rabbits divided into control and two test groups (n=4 per group). The test groups received nanoparticle formulations, while the control received pentazocine solution. Both nanoparticle formulations showed higher C_{max} (185.26 and 193.71 ng/mL) and prolonged half-life (9.50 and 8.58 h) compared to control (101.49 ng/mL; 1.69 h). Area under the curve AUC_0-∞ values were markedly increased for CP-2 and JB5, indicating enhanced bioavailability. **Conclusion**: The validated method is suitable for pharmacokinetic profiling and results demonstrate the potential of biodegradable nanoparticles to improve pentazocine delivery.

Keywords: Bioavailability; Biodegradable nanoparticles; HPLC; Pentazocine; Pharmacokinetics

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INTRODUCTION

Pentazocine is a synthetic analgesic having both agonist and antagonist activity. The antagonist activity is weak and present at the receptors (opioid). Pentazocine has one-third analgesic effect as of morphine. It is mainly indicated to treat and manage severe pain such as cancer pain, osteoarthritis (OA), biliary colic, menstrual pain, surgical and labor pain and postoperative pain (Madni *et al.*, 2018). The drug has a low molecular mass (285.4) and suitable pKa (8.5 and 10) (Verma and Chandak, 2009a). It is a benzomorphan derivative and is an opioid painkiller for the treatment of pain (moderate to severe) (Mahapatra *et al.*, 2019). It has a short half-life of 2-4 hours. After oral administration of pentazocine, the peak plasma level of the drug is achieved within 1 hour. It is significantly metabolized in the liver and the parent compound and metabolites of the drug are detected in urine or plasma. The intake of pentazocine is associated with anxiety, dysphoria and hallucinations (YIP *et al.*, 2007). The bioavailability of pentazocine is less than 20%. The limited bioavailability of the drug is due to protracted hepatic breakdown and low solubility (Eleje *et al.*, 2015). After being formulated into a novel drug delivery system, a drug exhibits a change in pharmacokinetic profile, which needs to be evaluated, analyzed and represented scientifically. Considering it necessary, the pharmacokinetic profiles of developed nanoparticle formulations of pentazocine with biodegradable polymers (Chitosan and Poly (lactic-co-glycolic acid) (PLGA) were assessed and analyzed. To analyze/ quantify drugs and analytes of interest in certain biological fluids, various analytical techniques are employed. These techniques may include Gas Chromatography with Mass Spectrometry (GCMS), Liquid Chromatography with Mass Spectrometry (LCMS), Liquid Chromatography with Refractive Index detector (LCRI), Liquid Chromatography with fluorescence detector (LCFLR) and High-Performance Liquid Chromatography (HPLC). Various techniques and

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methods are reported in the literature for quantifying pentazocine in biological samples (urine & plasma). This method involves but is not limited to HPLC equipped with mass spectrometry (MS), UV-visible detector and fluorescence detector, (Ehrnebo *et al.*, 1977, Imamura *et al.*, 2000, Moeller *et al.*, 1990, Murata *et al.*, 1992, Pond *et al.*, 1980, Prasad Verma and Chandak, 2009, Venishetty *et al.*, 2011).

Some of these methods use the derivatization technique. These methods have advantages and disadvantages and based on these characteristics; a suitable method may be selected based on a given condition or setting. HPLC is a highly employed method for pharmacokinetic profiling of analytes of interest in biological samples. The prime reason for its use arises from its selective nature towards a specific analyte and the specificity of its action, sensitivity for accurate quantification and simplicity and ease of operation compared with other analytical techniques (López *et al.*, 2006) in combination with a fluorescence detector (Moeller *et al.*, 1990).

The purpose of the study was the development/ modification of a performance liquid chromatographic method and its validation before it was applied to profiling the pharmacokinetic behavior of the developed biodegradable polymeric nanoparticles.

MATERIAL AND METHODS

Materials

Pentazocine was a kind gift by M/S Global Pharmaceuticals (Pvt) Ltd., Islamabad-Pakistan. Methanol and Acetonitrile of high-quality HPLC grade were purchased from Merck, Germany. Phosphate Buffer was purchased and manufactured by Daejung, Korea. As per the compendial requirements, water was used at a conductivity of < 1.3 µS/cm, which was prepared freshly in the Drugs Testing Laboratory (DTL) Punjab, Bahawalpur. Different high-quality and HPLC-grade solvents were obtained from Merck, Germany. HPLC manufactured by Waters, USA, having separation module e2695 and fluorescence detector (FLR detector 2475 equipped with Empower 3 software). Other ancillary equipment used include Sonicator (Elmasonic E30 H, Germany), Analytical balance (Shimadzu, AUX220, Japan), Filtration assembly (Pyrex, France), pH meter (WTW pH 300, Germany), and Vortex mixer (Seouline Bioscience Model, Korea). Nylon filters of 0.45 µm were used for filtration of the mobile phase and were of Merck Millipore origin. For the purpose of filtration of samples, standards, and plasma samples, syringe filters (Agilent Technologies, USA) of Teflon nature/ Polytetrafluoroethylene (PTFE) having a pore size of 0.22µm were used. The centrifuge machine (Sigma, Germany) was employed for the separation of different components. Rabbits were procured from the animal house of Department of Pharmacology, Faculty of Pharmacy, The Islamia University of Bahawalpur.

Development of pentazocine biodegradable nanoparticles

PLGA based nanoparticles were developed by using Oil in water (O/W) emulsion solvent evaporation method. A set of seven formulations (JB1 to JB7) with different PLGA and PVA concentrations was prepared while the concentration of other constituents was kept constant. PLGA was weighed and dissolved in 2.5 mL of acetonitrile. The pentazocine (10mg) was taken and dissolved in 2.5mL of methanol and then added to PLGA solution to make organic phase. Similarly, a specific amount of surfactant (PVA) was dissolved in 20 mL of distilled water, and aqueous phase was made. After this, the organic phase was added drop wise slowly into the aqueous phase followed by high-speed homogenization for one hour (Homogenizer, Heidolph Germany), which led to the formation of a clear transparent dispersion. For the evaporation of the organic phase, the dispersion was subjected to stirring using a magnetic stirrer at 700 rpm for four hours at room temperature. The obtained formulation was washed with distilled water to remove any entrapped unreacted content on the surface of the formulation and then dried for 3 to 4 hours in a lyophilizer (CHRIST alpha 1-4 LD, UK) to obtain dried nanoparticles.

Pentazocine loaded chitosan nanoparticles was fabricated by the ionic gelation method (Bruinsmann *et al.*, 2019) with slight modifications. In brief, the chitosan was dissolved in 1.5% acetic acid solution in distilled water by overnight stirring. A fixed amount of the pentazocine was dissolved in dichloromethane (DCM) and varied concentrations of Sodium tripolyphosphate (STPP) were made in deionized water. The resultant solution was then mixed drop by drop to the chitosan solution. The mixture was then allowed to be stirred for 120 minutes and homogenized for 60 minutes at 10000 rpm. The obtained nanoparticles were then further coated with Eudragit S100 using the solvent evaporation method and were used for further characterization after lyophilization by freeze dryer (Christalpha1-4LD, UK. With this method, five formulations (CP-1 to CP-5) were prepared. All the developed formulations were assessed for their size, zeta potential, surface morphology, percentage yield, entrapment efficiency and loading capacity, FTIR and DSC analysis and *in-vitro* drug release characteristics. Based on the results of abovementioned physicochemical attributes, one formulation from each set was selected (JB5 and CP-2) for further *in-vivo* evaluation.

Optimized chromatographic conditions and mobile phase

A non-polar stationary phase was used, which means reverse phase chromatographic (RP Chromatography) separation method. Column of ACE Generix® was used. The column's dimensions were 4.6 (internal diameter), 250 mm (length), and had a particle size of 5µm. After trials, an

optimized composition was accessed, which remained constant throughout the process, leading to isocratic elution. Before the initiation of the experimentation, extensive literature reviews and trials were performed. Isocratic elution was achieved through a mobile phase composition of 70:30 phosphate buffer (10mM Monopotassium phosphate of pH 4.0 with phosphoric acid) and acetonitrile, which was found to be the most suitable. 10mM Monopotassium phosphate was prepared by dissolving 680.43 mg of Monopotassium phosphate in 350 mL of water, and then, the final 500 mL volume was made with low-conductivity water. The target pH of the solution was 4.0, which was achieved with the help of phosphoric acid and potassium hydroxide (KOH). The mobile phase prepared as described was filtered using a Merck Millipore-made 0.45 µm nylon filter followed by 05 min sonication for degassing. Degassing is an important step, as it diminishes the air bubbles. It helps prevent air entry into HPLC cartridges and keeps the pressure stable. The mobile phase was pumped at a flow rate of 1mL/min. The fluorescence detector was set at 280 nm for excitation and 320 nm for emission, respectively after slight modification of the previous reports (Imamura *et al.*, 2000, Kelly *et al.*, 1994, Verma and Chandak, 2009a). The system was kept at an ambient temperature i.e. 25±2°C. The mobile phase always prepared fresh. Before placing a command on the flow of the mobile phase, the line was purged with the mobile phase. This leads to achieving a constant flow throughout the analysis.

System suitability testing (SST) parameters

System suitability testing (SST) parameters i.e. tailing factor, capacity factor and theoretical plates were calculated using Empower®3 software. Acceptance criteria was as kept as per FDA guidelines i.e. tailing factor should be less than 2, capacity factor should be higher than 1 and number of theoretical plates should be more than 2000 (Tarawneh *et al.*, 2025).

Plasma spiked samples and standard curve

Fresh rabbit plasma (blank) was needed for method development and validation. To obtain plasma, the jugular vein of the rabbits was pricked, and a small amount of blood (1.5-2 mL) was withdrawn. The obtained blood was centrifuged at 5000 rpm for 15 minutes and a separate plasma (supernatant) was collected. This clear supernatant/plasma was carefully stored at -70°C. Different amounts of drug PTZ were spiked in the plasma to obtain serial dilution and prepare the standard curve.

Method validation

Linearity

Standard solutions of different concentrations (10 ng/ml to 1000 ng/ml) were prepared and injected in a replicate. The mean response (peak area) of duplicate injections of a concentration was plotted against each concentration, and the value of the linearity coefficient was calculated. A value higher than 0.9 indicates acceptable linear behavior.

Precision

Precision needs to be done at two levels, i.e., Repeatability and Intermediate Precision. Repeatability is usually performed by six injections of a single concentration and then by calculating the standard deviation (SD) and relative standard deviation (RSD). For intermediate precision, one factor (analyst, equipment, day) needs to be changed to evaluate the consistency of the results. It will be more convenient to evaluate the consistency of the method on two different days. The percent relative standard deviation (%RSD) values of less than 2% indicate the precise result of the method in either case. Considering the linearity/calibration curve, three quality control concentrations (Low quality control concentration (LQC) i.e. 10 ng/ml, medium quality control concentration (MQC) i.e. 250ng/ml and High-quality control concentration (HQC) i.e. 1000ng/ml) were selected to assess the precision of developed method.

Accuracy

Three different concentrations (lowest, medium, or center concentration and highest) from the calibration curve were selected, and their response was quantified using the calibration curve. Using six replicates was useful in the evaluation of precision as well as accuracy. Considering the linearity/calibration curve, three quality control concentrations (i.e. 10ng/ml, 250ng/ml and 1000ng/ml) were selected to assess the accuracy/bias of developed method.

Working range

The working range is the range of concentration over which the calibration curve is made, and the concentration of the analyte of interest can be determined reliably and efficiently (Mir *et al.*, 2023). The range over which the value of linearity coefficient R^2 remains more than 0.9, was declared as the working range of developed method.

Limit of quantification (LOQ)

LOQ can be measured using the residual method. If a satisfactory level of precision and accuracy is achieved at the lowest concentration of the calibration curve, then it can also be regarded as LOQ. The limit of quantification was calculated using the lowest plasma spiked concentration on the calibration curve that provided a suitable level of accuracy and precision (Garg and Ackland, 2000b, Krier *et al.*, 2011a, Sánchez-Sellero *et al.*, 2025).

Limit of detection (LOD)

LOD can be measured from signal-to-noise ratio (S/N), usually one-third of the LOD. It can be calculated simply by dividing the LOQ by 3 i.e. LOD=LOQ/3 (Yi *et al.*, 2010).

Pharmacokinetic assessment

Study design

A parallel study design with a single dose was adopted wherein a total number of 12 healthy male albino rabbits

was selected and divided into three equal groups (four rabbits in each group). Formulations were administered orally by suspending them in normal saline and then administering them with the help of a Nasogastric (NG) tube (Mir *et al.*, 2023). The mean weight of the rabbits was approximately 2 kilograms. The first study group was used as a control group, which received pentazocine, while two other groups were treated as test groups. From the two test groups, one group received a novel biodegradable formulation of pentazocine developed with an ionic gelation technique using chitosan with STPP (CP-2). In contrast, the second test group received a novel biodegradable formulation of pentazocine developed with PLGA (JB5). Before the start of the study, all the rabbits were kept in an animal house for 15 days. The temperature and humidity of the animal house were controlled at 25±5°C and 60%, respectively. All the rabbits were privileged with free access to water and excess food. Plasma for the method development and subsequent verification was obtained from the animals at a certain amount of blood (1.5-2mL). To avoid any mix-up and confusion, rabbits were properly labeled.

Experimental groupings

The study included three groups for pharmacokinetic evaluation. Group I (Control Group) received orally administered pentazocine. Group II (Test Group A - TA) was administered an oral formulation of pentazocine-loaded biodegradable nanoparticles developed using chitosan with STPP (CP-2). Group III (Test Group B - TB) received an oral formulation of pentazocine-loaded biodegradable nanoparticles formulated with PLGA (JB5) are given in table 1.

Blood sampling of rabbits

Commercially available Ethylenediaminetetraacetic Acid (EDTA) tubes were purchased from the market and used for blood sample collections. The blood sample (after obtaining plasma) was mixed with an equal volume of perchloric acid. This was vortexed for 3-5 minutes to maximize the reaction of protein precipitation so that all the proteins are precipitated, and a clear supernatant free from plasma proteins may be collected. It would help to avoid any problem during further analysis, as if protein remains in the plasma, it may choke the system. A sterile syringe of 3 mL was used for the purpose of sampling. Every time, a blood sample of 1.5-2mL was withdrawn at predetermined intervals. The obtained blood samples were centrifuged at 5000 rpm for 0.25 hours, resulting in the collection of supernatants. This clear supernatant/plasma was carefully stored at -70°C in an Ultra-Low Freezer (Sanyo, Japan) until processed. After sampling, animals were removed from metal cages and returned to the animal house. During experimentation, the plasma sample was removed from the ultra-low freezer and placed at room temperature for some time to allow it to thaw. After thawing, it was processed to make dilutions. Plasma proteins were separated using protein precipitating agents. Literature reports different chemicals for the purpose, including acetone, acetonitrile (Mir *et al.*, 2023, Mohamed *et al.*, 2019, Zhao *et al.*, 2009), perchloric acid, and trichloroacetic acid (Sedgwick *et al.*, 1991). Perchloric acid was chosen for the purpose of the current study project. Plasma mixed with protein precipitating agent was then vortexed for 3 to 5 minutes, followed by centrifugation for 10 minutes at 12000 rpm. The clear supernatant was separated in a fresh clean centrifuge tube and labeled accordingly. The injection volume was 50 µL. 0.22 µm syringe filters (PTFE, Agilent Technologies) were used to filter injection. All the samples were run at HPLC and equilibrated with the mobile phase.

Pharmacokinetic profiling of pentazocine by quantification in the plasma

The pharmacokinetic analysis was performed using non-compartmental analysis (NCA) after extravascular input in PK Solver 2.0 to calculate different important pharmacokinetic parameters of pentazocine in test formulations and control groups (Haripriyaa and Suthindhiran, 2023, Singh *et al.*, 2020). Plasma concentration versus time curves of different groups were used to calculate the important pharmacokinetic parameters like area under the first moment curve ($AUMC_{0-o}$), clearance, maximum plasma concentration (C_{max}), time to reach maximum plasma concentration (T_{max}), half-life ($T_{1/2}$) and area under the curve from zero to infinity ($AUC_{0-\infty}$).

Statement on statistical tests

In our study, statistical significance testing was not applicable (due to the study design / descriptive nature of the pharmacokinetic assessment). The aim was to provide a descriptive comparison of pharmacokinetic parameters rather than to perform hypothesis testing. Applying Analysis of Variance (ANOVA) or t-tests in this context could lead to misleading interpretations, as the study was not powered for such analysis. We have clarified this rationale in the Methods and Discussion sections and have ensured that our conclusions remain appropriately cautious and supported by the descriptive data presented.

RESULTS

Method development and optimization

The mean responses of each concentration replicate injections (10ng/mL to 1000ng/mL) were plotted. The precision (repeatability and intermediate precision) was evaluated on two different days by using three different quality control concentrations (low, medium and high) from the calibration curve. The results/values for all the pharmacokinetic parameters were calculated using pK Solver (an add-in of Microsoft Excel).

Method validation

From 10ng/mL to 1000ng/mL (1 µg/mL), pentazocine in the plasma solution was made using the serial dilution method. Plasma proteins were separated using the protein

precipitating agent Perchloric acid. Plasma mixed with perchloric acid was then vortexed for 3 to 5 minutes, followed by centrifugation for 10 minutes at 12000 rpm. The clear supernatant was separated in a fresh, clean centrifuge tube and labeled accordingly. The injection volume was 50 µL. 0.22 µm syringe filters (PTFE, Agilent Technologies) were used to filter injection. All the samples were run at HPLC previously equilibrated with the mobile phase. Representative chromatograms of pentazocine from standard alone and plasma samples are given hereunder.

Validation parameters

The parameters discussed in this section are linearity, precision, working range, limit of detection (LOD), accuracy/bias, and limit of quantification (LOQ). ICH (International Council for Harmonisation) Q2R1 guidelines were followed for validation criteria (ICH, 2005) along with US-FDA Analytical Procedures and Methods Validation for Drugs and Biologics-Guidance for Industry (USFDA, 2018).

Linearity

The standard curve/calibration curve establishes the linearity of a given method (Guirguis *et al.*, 2023, Shah *et al.*, 1992). Each concentration with the respective area is given in Table 2.

Accuracy and precision of the HPLC method

Repeatability is done by keeping all the factors the same. Intermediate precision is done by altering one of the aspects e.g. day, analyst, equipment (ICH, 2005, Shah *et al.*, 1992). The average of the observed/experimental values is compared with the true value and results are expressed as accuracy (Guirguis *et al.*, 2023, Shah *et al.*, 1992). Results are given in Table 3.

Working range

Low and higher concentrations at which the response of an analytical method remains accurate, precise and linear are termed the range of the analytical method (Chen *et al.*, 2012). Results suggest that the method remained linear over a 10 ng/mL concentration range to 1 µg/mL of pentazocine in rabbit plasma.

Limit of detection (LOD) and limit of quantification (LOQ)

The lowest concentration of an analyte that a given analytical method can quantify is termed its Limit of Quantification (LOQ) for a specific analyte of interest in given circumstances. If a satisfactory level of accuracy and precision is achieved at the lowest point of the calibration curve, then it will be termed the Limit of Quantification (Garg and Ackland, 2000a, Krier *et al.*, 2011a, Xu *et al.*, 2010). Additionally, the Limit of Quantification (LOQ) is usually three times higher than the LOD and LOD can be achieved by splitting LOQ into three equal parts. LOD can also be measured by the signal-to-noise ratio (S/N Ratio).

Pharmacokinetic assessment

Plasma level time curve of pentazocine (Control Group) and test groups (TA & TB)

Plasma levels of pentazocine, after administration in each rabbit of control group, test formulation (chitosan-STPP) in test group A (TA) and test formulation (PLGA) in test group B (TB), were quantified by HPLC method and results of mean plasma levels (ng/mL) of all rabbits of each group with standard deviation are given hereunder in Table 4.

A representative peak of standard pentazocine is given in Fig. 1 (a), wherein elution was observed at a retention time of 8.277 minutes. Figs. 1 (b) and 1 (c) represents the pentazocine chromatogram in plasma and the peak of interest appeared at 8.481 and 8.457, respectively. The plasma level time curve for the pentazocine from control group is given hereunder in Fig. 2. The plasma level time curve for the pentazocine from test group A (chitosan-STPP) is given hereunder in Fig. 3. The plasma level time curve for the pentazocine from test group B (PLGA) is given hereunder in Fig. 4.

Pharmacokinetic parameters profiling

After the plasma concentrations were assessed, pK solver (an excel add-in program) was applied to calculate values of different important pharmacokinetic parameters. Pharmacokinetic parameters were calculated from plasma levels of pentazocine after administration of control solution and test formulations to respective groups. These parameters include half-life, C_{max}, T_{max}, area under the curve and Mean Residence Time (MRT) etc. Comparative values of these parameters are given hereunder in Table 5. The comparative plasma level time curve for test groups (both A/TA and B/TB with control group C is given in Fig. 5.

DISCUSSION

Initially, the method was developed after trials of previously reported methods, the method found suitable was adopted and modified to make it applicable in the present laboratory settings (representative chromatograms of pentazocine with and without plasma). In the later part of the study design, important parameters of method verification are discussed, and the results/values are calculated. The parameters discussed in this section are linearity, precision, working range, limit of detection (LOD), accuracy/bias, and limit of quantification (LOQ). ICH Q2R1 guidelines were followed for validation criteria (Bhavyasri *et al.*, 2019).

A representative peak of standard pentazocine showed consistent elution of the pentazocine from the matrix as the retention time remained constant. In HPLC, analytes are separated on the basis of retention time and this is the major reason behind the identification of analyte of interest (Ali, 2022). System suitability testing (SST) parameters i.e. tailing factor, capacity factor and theoretical plates.

Table 1: Labelling and grouping of the animals

Group	Group name	Tested	Labeling to avoid mix-up
Group 1	Control group	Pentazocine	C1, C2, C3, C4
Group 2	Test group 1/Test group A/ TA	Oral administration of pentazocine containing biodegradable nanoparticle formulation developed using chitosan with STPP (CP-2)	TA1, TA2, TA3, TA4
Group 3	Test group 2/ Test group B/ TB	Oral administration of pentazocine containing biodegradable nanoparticle formulation developed using PLGA (JB5)	TB1, TB2, TB3, TB4

Rabbits were fasted half a day before dosing with *ad libitum* access to the water. Before 2 hours of doing this, rabbits were shifted to metal cages. The dose administered to every rabbit was equivalent to 10 mg. This dose was given orally using a flexible catheter. For the control solution, 100mg was diluted to a concentration of 10 mg with normal saline immediately before administering to avoid any solution instability.

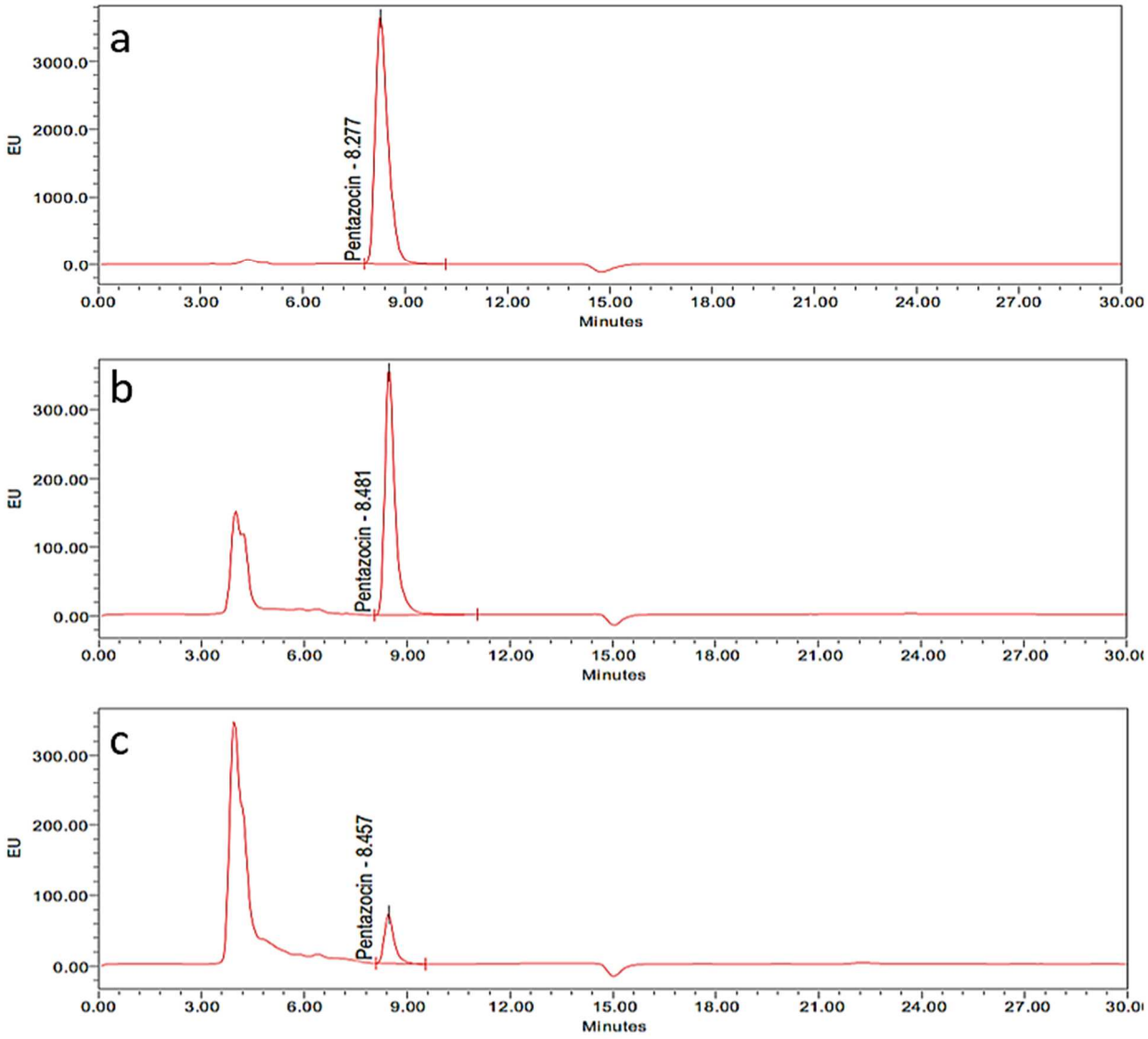

Fig. 1: a) Chromatogram of pentazocine standard (Retention time 8.277 minutes), tailing factor 1.68, capacity factor 3.8 and theoretical plate count 3488. b) Chromatogram of pentazocine standard with plasma (Retention time 8.481 minutes), tailing factor 1.63, capacity factor 3.91 and theoretical plate count 3564 c) Chromatogram of pentazocine standard with plasma (Retention time 8.457 minutes), tailing factor 1.58, capacity factor 3.94, theoretical plate count 3696.

Table 2: Standard concentration and response as the area under the curve

Sr. No	Concentration (ng/mL)	Response of injection 1	Response of injection 2	Average
1	10	94456.67	94241.47	94349.07
2	20	148772.58	149987.49	149380.035
3	50	1748767.52	1748441.36	1748604.44
4	100	2314876.55	2314475.72	2314676.135
5	200	4556589.97	4556578.32	4556584.145
6	250	6167827.33	6168845.43	6168336.38
7	500	11732465.73	11732777.89	11732621.81
8	1000	24745170.29	24745475.92	24745323.11

Table 3: Combined results of accuracy and precision i.e. intra-day precision (Repeatability) on 1st and 2nd day and inter-day precision (Intermediate Precision)

Parameters/QC Concentrations	Precision (Repeatability & Intermediate Precision) and accuracy results								
	Results for six replicates on day 1			Results for six replicates on day 2			Combined results for day 1 and day 2 (for intermediate precision)		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Mean	10.01	251.7367	1002.783	9.935	254.555	1010.147	9.9725	253.1458	1006.465
RSD (%)	0.788726	1.643241	0.567792	1.67799	3.854085	1.029198	1.360889	3.025707	0.9095786
Accuracy	100.1	100.6947	100.2783	99.35	101.822	101.0147	99.725	101.2583	100.6465

Table 4: Comparative average plasma concentrations with standard deviation (ng/mL) of four rabbits each from three groups (Control, Chitosan-STPP and PLGA).

Sampling time (hrs)	Average plasma concentration ± Standard Deviation (ng/mL), n=4		
	Control group (C)	Chitosan nanoparticles with STPP in test group A (TA)	PLGA nanoparticles in test group B (TB)
0.25	16.943 ± 2.324	30.368 ± 2.574	30.023 ± 1.574
0.5	44.578 ± 2.211	42.515 ± 2.353	43.038 ± 2.072
1	69.995 ± 1.749	63.358 ± 1.480	65.633 ± 2.142
2	101.490 ± 2.234	86.858 ± 1.750	90.743 ± 2.806
3	71.775 ± 3.405	116.350 ± 1.929	122.848 ± 4.350
4	46.705 ± 2.561	144.400 ± 3.469	152.733 ± 4.866
5	33.453 ± 2.034	171.565 ± 3.350	177.698 ± 3.119
6	18.925 ± 1.734	185.258 ± 2.959	193.703 ± 2.450
8	---	157.593 ± 3.175	148.813 ± 2.696
12	---	125.763 ± 3.483	109.995 ± 2.416
24	---	49.633 ± 2.544	41.120 ± 1.346

Table 5: Comparative mean values of pharmacokinetic parameters obtained from plasma concentrations of three groups (Control, Chitosan-STPP and PLGA).

Pharmacokinetic parameters	Pharmacokinetic values from Mean plasma level of four rabbits		
	Control group (C)	Chitosan nanoparticles with STPP in test group A (TA)	PLGA nanoparticles in test group B (TB)
$T_{1/2}$ (hrs)	1.68143184	9.50172113	8.58300435
T_{max} (hrs)	2	6	6
C_{max} (ng/mL)	101.49	185.2575	193.7025
$AUC_{0-\infty}$ (ng/mL*h)	382.241577	3325.15095	2989.7352
$AUMC_{0-\infty}$ (ng /mL* h^2)	1277.88844	52340.7165	42429.3736
MRT (hrs)	3.34314348	15.7408543	14.1916828

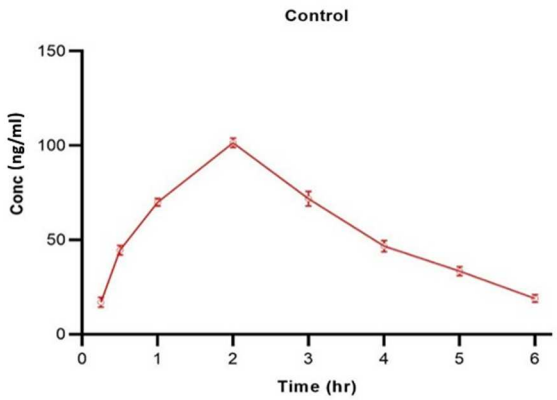

Fig. 2: Plasma concentration profile for control group

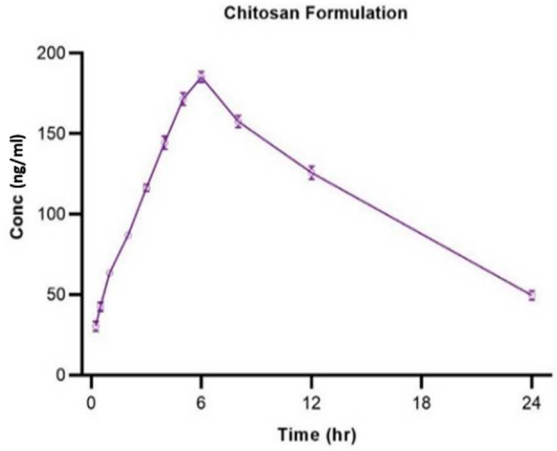

Fig. 3: Plasma concentration profile for chitosan formulation in test group A.

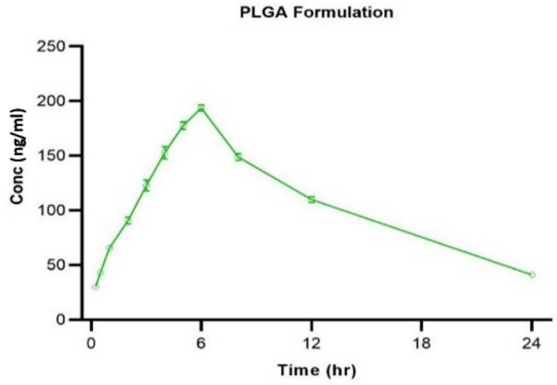

Fig. 4: Plasma concentration profile for PLGA formulation in test group B.

Tailing factor for all the peaks was less than 2 and number of theoretical plates was higher than 2000 (Epshtein, 2020), this reflects that perfect Gaussian peaks were obtained. The mean responses of each concentration replicate injections (10ng/mL to 1000ng/mL) were plotted. The method used in the current study was linear over a concentration range of 10ng/mL to 1000ng/mL (1 µg/mL) of pentazocine. The response was calculated using Empower®3 software. A good linear behavior over the mentioned range was observed, which was depicted by the value of linearity coefficient R^2, i.e. 0.9983. This value of linearity coefficient R^2 clearly depicts the reliable linearity response of the developed method which is a prerequisite for an analytical method (Araujo, 2009).

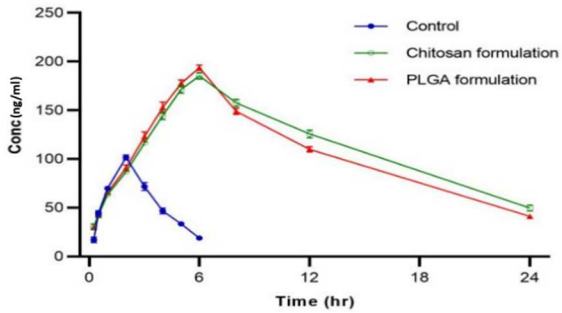

Fig. 5: Comparative analysis of plasma concentration profile of control group with test group A and test group B.

An analytical method should be precise to be suitable for its application in a research study. Precision means a method always yields close results for a given analyte concentration. Percent relative standard deviation (%RSD) is an indicator of precision, and a lower value reflects a high degree of precision. The precision was evaluated on two different levels i.e. repeatability and intermediate precision (Betz *et al.*, 2011). This attribute of an analytical method was assessed on two different days by using three different quality control concentrations (low, medium, and high) from the calibration curve. High-quality control concentration (1µg/mL), medium-quality control concentration (0.250µg/mL), and low-quality control concentration (10ng/mL) were decided. The closeness of the results with true value is known as accuracy or bias (Betz *et al.*, 2011). The results of precision and accuracy/bias show that the method is accurate as the values for accuracy ranged from 99.35 to 101.82, showing results (98 - 102%) are not in deviation of more than ±2%. Results also suggest that the method is suitable for analytical application as it is precise at both levels, i.e., repeatability and intermediate precision. Percent relative standard deviation found on 1st day for low, medium, and high-quality control concentrations are 0.79, 1.65, and 0.57, respectively. The percent relative standard deviation found on 2nd day for low, medium, and high-quality control concentrations is 1.68, 3.83, and 1.02, respectively. Results suggest that the method of analysis of pentazocine is precise. These results provide sufficient evidence that the developed method for the analysis of pentazocine is precise and accurate and is suitable for analysis in biological samples and further pharmacokinetic profiling of the drug. Low and higher concentrations at which the response of an analytical method remains accurate, precise, and linear are termed the range of the analytical method. From the results of the experiments, it was concluded that the concentration range from 10-1000ng/mL can be termed as the working

range for pentazocine quantification in rabbit plasma for the method currently being employed in the given conditions.

If a satisfactory level of accuracy and precision is achieved at the lowest point of the calibration curve, then it will be termed the Limit of Quantification (Garg *et al.*, 2000, Krier *et al.*, 2011b). The current method has 10ng/mL as LOQ, as the value of %RSD for six replicates of day 1 was found to be 0.788 with an accuracy/recovery value of 100.1%. On day 2, the value of %RSD for six replicates was found to be 1.677 with an accuracy/recovery value of 99.35%. These values of %RSD less than 2 shows acceptable results while recovery is within acceptable range of 98-102%. These values suggest that 10ng/mL is regarded as LOQ of the current method so the LOD is 3.3. It can be predicted from the said result that the method is of good sensitivity with appreciable accuracy.

*In-vivo p*harmacokinetic study revealed that the developed formulations (test formulations of chitosan and PLGA nanoparticles designated as TA and TB, respectively) showed a better *in-vivo* pharmacokinetic profile and higher bioavailability. Plasma half-life, mean residence time and area under the curve were higher for the test formulations than the control pentazocine. The mean values of C_{max} for the control, TA and TB are 101.49 ng/mL, 185.26 ng/mL and 193.71 ng/mL, respectively. It is evident from the relatively higher values of C_{max} for TA and TB that these formulations may enhance the drug's bioavailability. C_{max} values of all three test items were in the following order: C_{max} TB >C_{max} TA >C_{max} Control. Pentazocine control achieved C_{max} in 2 hours, while T_{max} for TA and TB is 6 hours each. This may be attributed to the slow release of pentazocine from the developed nanoparticulate formulations.

In this study, mean values for the $AUC_{0-\infty}$ are 382.24 ng/mL*h, 3325.15 ng/mL*h and 2989.74 ng/mL*h for control, TA and TB, respectively. Higher values of the $AUC_{0-\infty}$ for TB and TA compared to Control show higher bioavailability associated with these developed formulations. As the name indicates, MRT is the body's average stay of drug molecules. MRT values for the control, TA and TB are 3.34 hrs, 15.74 hrs and 14.19 hrs, respectively. MRT for TA and TB are more than thrice higher as compared to pentazocine Control (Verma and Chandak, 2009b). The plasma half-life is a significant pharmacokinetic attribute and represents the time the drug needs in which it is decreased to 50% of its original plasma concentration. This parameter reflects the circulation time and stability of the delivery system inside the physiological environment. This study's calculated half-life values are 1.69 hours, 9.50 hours and 8.58 hours for control pentazocine, TA and TB, respectively. Calculated values state that the observed half-life for TA and TB is more than threefold that of the half-life of control Pentazocine, which will ultimately contribute towards increased bioavailability.

CONCLUSION

The study developed and validated a reliable HPLC technique for quantifying Pentazocine in plasma. It demonstrated excellent linearity (R^2 = 0.9983), precision (%RSD < 2%), accuracy (99.35-101.82%) and sensitivity (LOD = 3.3 ng/mL; LOQ = 10 ng/mL) throughout the 10-1000 ng/mL range. This approach was used to evaluate the pharmacokinetics of Pentazocine-loaded biodegradable polymeric nanoparticles and it indicated significant improvements in drug exposure and sustained release when compared to the control formulation. The C_{max} increased from 101.49 ng/mL (control) to 185.26 ng/mL (chitosan-STPP, TA) and 193.71 ng/mL (PLGA, TB), while the $AUC_{0-\infty}$ increased from 382.24 ng·h/mL to 3325.15 ng·h/mL (TA) and 2989.74 ng·h/mL (TB). The half-life increased from 1.69 hours (control) to 9.50 hours (TA) and 8.58 hours (TB), while the MRT increased from 3.34 to 15.74 hours (TA) and 14.19 hours (TB). These findings quantitatively confirm a large increase in systemic exposure, longer circulation time and a possible reduction in dose frequency. Future study should focus on the long-term safety, stability and efficacy of these nanoparticle formulations in larger animal models, as well as their translational potential in human clinical trials. Furthermore, evaluating production scalability and cost-effectiveness is critical for prospective commercialization.

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Authors' contributions
Abdul Jabar contributed to the experimental design, data collection and analysis, and participated in the drafting of the manuscript. Arshad Khan, Muhammad Ahmad Mahmood, Faizan Akram and Syed Ahmed Shah edited and refined the manuscript with a focus on critical intellectual contributions and made significant contributions to date interpretation and manuscript preparation. Asadullah Madni and Sajid Bashir for research supervision, manuscript revision, and final approval. All authors have read and approved the final manuscript.

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Data availability statement
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

The approval to conduct the animal study was granted by the Pharmacy Animal Ethics Committee (PAEC), Department of Pharmacy, the Islamia University Bahawalpur under reference No. 17-2020/PAEC, dated 12-08-2020.

Conflict of interest

The authors declare no conflict of interest.

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