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Original Article

Development and Validation of a HPLC-PDA Method to Quantification of Ketobemidone in Rat Plasma and its' Application in Pharmacokinetic Study

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ABSTRACT

Aim: Ketobemidone is an opioid drug that have been using against pain in various circumstances. The current research was aimed to develop and validate a HPLC-PDA method to estimate the ketobemidone in rat plasma. **Materials and Methods:** The ketobemidone in the biological matrices (rat plasma) was separated using solvent extraction method using methanol as extracting solvent. An isocratic mobile phase consisting of methanol-water-phosphate buffer at 40:60:0.01 % v/v/v and Kromasil C_{18} column were used for separation in Liquid Chromatography (LC). **Results:** The selected linearity range i.e., 0.10-25µg/mL was acceptable with r^2 value 0.9998 as least square method. The determined Lower Limit of Quantification (LLOQ) is 0.10 µg/mL. The intermediate precision studies were performed within and between days; and the data obtained are within a significant range, i.e., %RSD <9.2 and the accuracy was within 10.0% of the relative error. **Conclusion:** The developed method to detect the ketobemidone was successfully validated and it was successfully applied on rat pharmacokinetic study.

Keywords: Hematocrit, HPLC-PDA bioassay, Ketobemidone, Rat plasma, Validation.

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INTRODUCTION

Ketobemidone is a potent opioid analgesic drug and has been used in the management of severe pain when other treatment options have proven ineffective. Ketobemidone is known for its strong pain-relieving properties, making it a valuable medication in situations where other pain medications such are non-opioid drugs like acetaminophen, gabapentin, acetylsalicylic acid etc., are inefficient to reduce the pain.[1,2] Ketobemidone is first reported in 1960 as synthetic drug, it works on opioid receptors of the central nervous system and controlling the transmission of signal to inhibit the different pains (analgesic effect). Ketobemidone is considered a schedule II-controlled substance due to its high potential for abuse and the risk of physical and psychological dependence. It is available in various formulations, including tablets and injectable solutions and is typically administered under medical supervision.[3]

The effective controlling nature of severe pains ketobemidone has been widely used in different circumstances. As its extensive usage is associated with certain side effects includes dizziness, nausea, constipation and respiratory depression.[4,5] The usage of ketobemidone is strictly controlled as prescribed by a healthcare professional. Due to its potency and potential for misuse such as overdoses may cause minor to major side effects,[6] ketobemidone is tightly regulated in many countries. It is typically prescribed for short-term use in acute pain situations or for chronic pain management in carefully monitored cases. The narcotic analgesic opiate ketobemidone (4-(3-hydroxyphenyl)-1-me thyl-4-propionylpiperidine) is structurally related to morphine and pethidine (Figure 1).[3,7] It is a potent opioid analgesic used to treat cancer pain (chronic and acute) that is unresponsive to other opioids. Additionally, it has some NMDA-antagonist qualities and works by interacting with the receptors to block the transmission of pain signals to the brain.[8,9] However, when combined with a spasmolytic drug (A29), ketobemidone is more effective at NMDA receptors.[10] Ketobemidone is often used as

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analgesic drug, an alternative to morphine.[11] When used to treat postoperative pain in children, ketobemidone has analgesic potency comparable to morphine and meperidine as well as adverse effects (such as body shivering, nausea, or vomiting).[11] The opioid ketobemidone, however, can effectively treat neurogenic pain in contrast to pethidine and morphine.[12] This has led us to hypothesize that ketobemidone's analgesic efficacy in neurogenic pain may be influenced by both opioid and non-opioid effects.[12] The standard dosage recommendation for oral dosing in patients with severe pain may be too low,[13] as seen by the fast elimination of ketobemidone when delivered intravenously, orally, or rectally and the limited oral bioavailability of the drug. Numerous analytical techniques, like Gas Chromatography-Mass Spectrometry (GC/MS) and Liquid Chromatography-Mass Spectrometry (LC/MS)[14] have been reported for the detection of the drug ketobemidone alone or in conjunction with its metabolites in plasma and urine.[15]

In vivo research is a good fit for evaluating micro-dialysis methodology since it has several benefits over traditional approaches (i.e., drug samples present in plasma or serum). A variety of chemical medications, including those having macromolecules as active components, can be sampled using micro dialysis. This reverse micro-dialysis technique allows for simultaneous sampling of the medicine's localized metabolites in the tissue and local delivery of the medication.

It's important to note that the analytical application of ketobemidone is mainly limited to its detection and quantification in biological samples for clinical, forensic and toxicological purposes. Its use as an analytical standard allows for accurate measurements and assessments related to its pharmacokinetics, toxicology and therapeutic monitoring.[11,13]

The sample size ranges from a few microliters to several and the target analytes are often present at low concentrations. As a result, extremely high sensitivity and selectivity are required for analyzing micro-dialysate samples, which place enormous demands on the analytical apparatus. To achieve certain analytical requirements, including the use of several columns, miniaturization is necessary.

The High-Performance Liquid Chromatography with Photodiode Array Detector (HPLC-PDA) method was utilized in the current investigation to measure and verify ketobemidone in rat plasma. The novel chromatographic approach and the process for preparing the rat plasma sample were both optimized. With reliable and precise drug detection, we were able to attain lower limit of quantitation is 0.1µg/mL in less than 10 min. Additionally, we investigated the *in vivo* pharmacokinetics of ketobemidone (10 mg/kg) in rate samples.

MATERIALS AND METHODS

Chemicals and Reagents

The solvent used in current research was analytical grade. Diethyl amine and ethanol were purchased from Sisco Research Laboratories Pvt. Ltd., (SRL), India. N-hexane and methanol were acquired from Rankem and SD Fine Chemicals, India, respectively. Ketobemidone and pethidine (IS) were acquired as gift samples with 99.6% purity from Vivan labs Pvt. Ltd., India (Thanks to Vivan labs).

Blood samples from Wistar rats were taken, used as a control and kept at 20°C. A centrifuge (model Sigma 216P), heparin-coated capillaries (blood collection tubes) and a multi-pipette were used to collect plasma samples. To get sealing plastic bags and

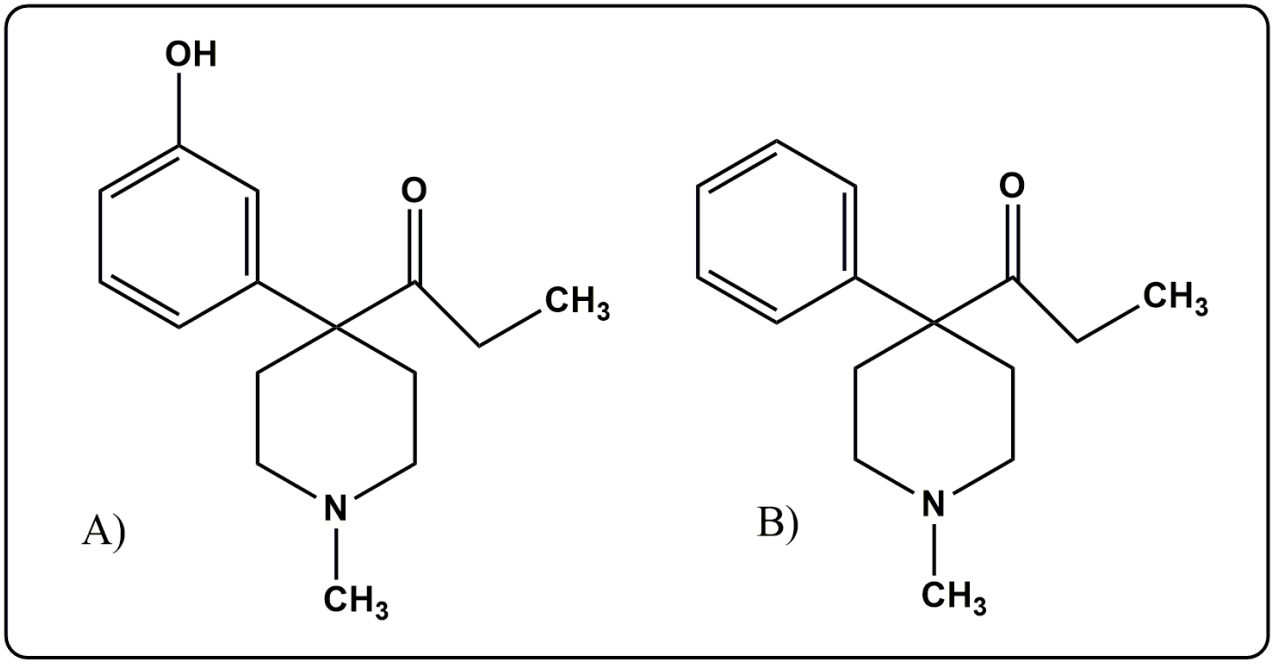

Figure 1: A) Ketobemidone; B) Pethidine.

Table 1: The precision and accuracy details of ketobemidone.

Nominal Concentration (µg/mL)	Mean, SD			Accuracy[b] (%)
		Intra day Precision	Inter day Precision	
0.1	0.13±0.05	7.9	9.2	10.4
0.25	0.25±0.02	6.7	8.1	-3.2
2.5	2.71±0.22	7	7.6	5.2
20	20.4±0.31	5.1	7.2	6.1

[a] %RSD, [b]Calculated as %RE, i.e. (mean determined concentration-nominal concentration)/nominal concentration×100%.

silica gel sachets for keeping the collected samples, a trip to the neighborhood store was made (Eppendorf tubes).

Albino Wistar rats (180 g to 220 g) were received from laboratory animal center (Aragen Life Sciences, Hyderabad, India) as complementary and the ethical approval as 2003/PO/Re/S/18/CCSEA. The rats were kept at a 12-hr light-dark cycle (lights on 600 to 1800; 300 lx; 123 µW/cm^2) and fed on standard sterilized rat chow and water. All experimental protocols complied with the recommendations of the laboratory animal center's animal care committee.

Apparatus and Chromatographic conditions

The detection and validation of samples were carried out using an Agilent liquid chromatographic system (1100 series, software: Chemstation) having different components such as degasser, pumps, column compartment and detector. The kromasil C18 column having features 250 mM×4.6 mM, 5µM was used for separation. The mobile phase consists of methanol:water:phosphate buffer (40:60:0.01 v/v/v) at flow rate 1.0 mL/min. The auto sampler temperature was 4ºC and column oven temperature 25ºC. The wavelength of the PDA detector was adjusted to 232 nm.

Stock Solution Preparation

The stock solutions (analyte and ISTD) were prepared as 1.0 mg/mL using methanol. Then further working standard dilution (1, 5, 10, 50, 100 and 500 µg/mL) with the range to get the effective range in rat plasma at 0.10-25.0 µg/mL were prepared with diluent methanol:water (1:1 V/V).

Calibration Standards

A Calibration (standard) curve is the relationship between instrument response and reliable analyte concentrations. The relationship between the response and the focus should be obvious and foreseeable. Rat blood samples were spiked with various amounts of stock samples to form Calibration Standard (CS) solutions with concentrations. The spiked rat plasma at concentrations 0.10, 0.25, 0.75, 2.5, 5, 10 and 25 µg/mL were stored at 4ºC for further usage.

Quality Control Samples

The creation of samples for Quality Control (QC) involves the use of several stock solutions. If the consistency of the sample and the accuracy of the stock solution have been established, QCs can be carried out using the same spiking stock solution. After independently weighing the reference standard, the QC samples were prepared at concentrations of 0.25, 2.5 and 20 µg/mL. After producing a pethidine (IS) stock solution at a concentration of 1 mg/mL using methanol, a working solution of 25 mg/mL of ketobemidone was made by using diluent (methanol: water (1:1 V/V)).

Plasma sample extraction and preparation

The extraction procedure was done as per flow cart given below:

20 µL of the working solution (IS, 25 µg/mL).

↓

100 µL of plasma sample.

↓

Vortex for Iminute.

↓

Add 3mL of Ethyl acetate to sample.

↓

vortexed for 15 min.

↓

Centrifuge the samples at 4000 rpm for 20 min.

↓

Separate the organic layer and evaporate at 40°C.

↓

Reconstitute the samples with 0.300mL mobile phase and votex for 1 min.

↓

Centrifuge the samples at 13000 rpm for 10 min.

↓

Load the supernatant sample into autosampler vials and inject into HPLC analysis (Figure 2).

Method Validation

The developed bioanalytical method validation approach was utilized to establish the different parameters such as Limit of Quantitation (LOQ), Limit of Detection (LOD), linearity, accuracy, precision and different stabilities in biological matrices.

Specificity, linearity and sensitivity

Six separate plasm batches were used for specificity assessment. The assessed plasmas' peak should be less than 20% of LLOQ standard. The assessed six batches of rat plasma were less than 20% of LLOQ nominal concentration. The calibration standards were tested in duplicate on three different days to confirm linearity. Except for LLOQ, the accuracy of each standard and QCs were 100±15% for their acceptance criteria to nominal values. Peak area ratios and analyte concentrations were used to create the calibration curve. The lowest quantifiable concentration of CCs was to be the LLOQ, which had a signal-to-noise ratio of 5, an accuracy of 20%, a precision of 20% and a 20% precision. Six copies were used over the course of three days to determine the LLOQ.

Precision and accuracy

Three different QC concentration levels, in 6 replicates over three different validation days, with each having a different standard curve for quantification used to evaluate precision and accuracy. Accuracy was calculated using below formula:

$$\text{Accuracy} = \times 100$$

The Relative Standard Deviation (RSD) was necessary to demonstrate the experiment's accuracy.

Recovery

The ratio of the peak responses of the extracted and reference solutions was used to calculate the extraction recovery of rate plasma. During the recovery trials, the analysis was repeated six times for each concentration (0.25, 2.5 and 20 µg/mL). Calculations like those used to determine the extraction recovery of the IS at a concentration of 10.0 µg/mL were performed.

Stability data

The stability of ketobemidone in rat plasma was assessed by using QCs as three replicates. Rat plasma was exposed to room temperature for 2 hr to evaluate its stability. Additionally, three complete cycles of freezing and thawing (-20° to 25°C) were employed to test the stability over a period of consecutive days.

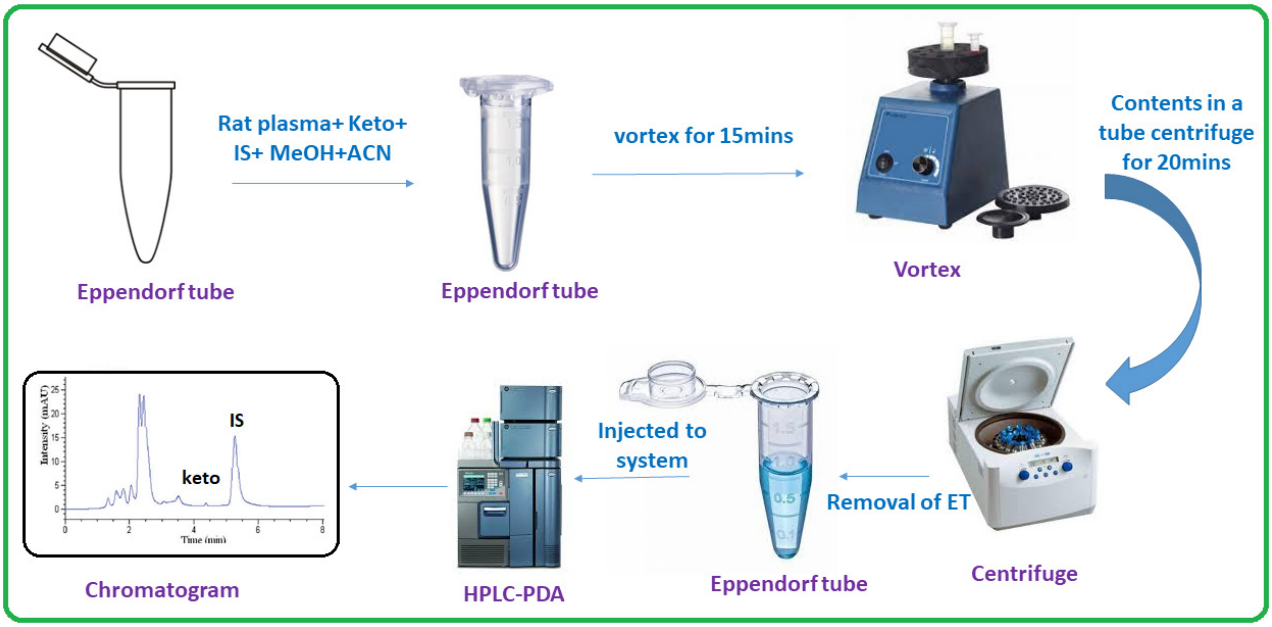

Figure 2: The systematic extraction procedure for Ketobemidone in rat plasma.

Table 2: Ketobemidone stability data in processed and unprocessed samples.

Concentration[a]	[25°C, 2 hr]	[-20°C, 1 month]	Remained [%][b]	Auto sampler [4°C, 24 hr]
0.25	86.7	91.7	91.6	99.3
2.5	91.5	93.6	93.5	100.7
20	93.8	95.4	95.9	102.5

[a]Expressed as µg/mL; [b]Calculated for three freeze-thaw cycles; *n*=3.

The sample (standard spiked plasma sample) was stored at -20°C for a month to study the stability of rate plasma in addition to confirming the long-term stability.

Both freshly made QC samples and stability samples at each level were double-checked. For a month, stock solutions were kept at 4°C while the stability of the ketobemidone stock solution was evaluated. Rat plasma levels for the analyte range from 85% to 115% of the initial value, showing that the samples are stable.

Pharmacokinetic Study

Six albino male rats received intravenous injections of the medication ketobemidone (10 mg/kg) to evaluate the pharmacokinetics. Centrifuging the blood samples (250 mL) at 4000 rpm for 10 min and at various times (0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 9, 12 and 24 hr following drug treatment), separated the plasma and were stored at -20°C until analysis. Ketobemidone samples are administered, extensive statistics are acquired and software based on the Ramkin method can be used to assess all relevant data. The final four points and slope of the plasma concentration vs. time logarithm were used to construct the terminal elimination rate constant (Ke). The elimination half-life ($t_{1/2}$) is anticipated to be 0.693 Ke. The linear-trapezoidal method was used to measure the area under the plasma concentration-time curve from zero to the last identifiable sample (AUC_{0-t}). The $AUC_{0-\infty}$ (plasma concentration-time from zero to infinity) was exposed using integrating AUC_{0-t} and extrapolated area. The extrapolated area was obtained by dividing the final measurable concentration with Ke. Total body clearance was calculated using the formula X_0/AUC.

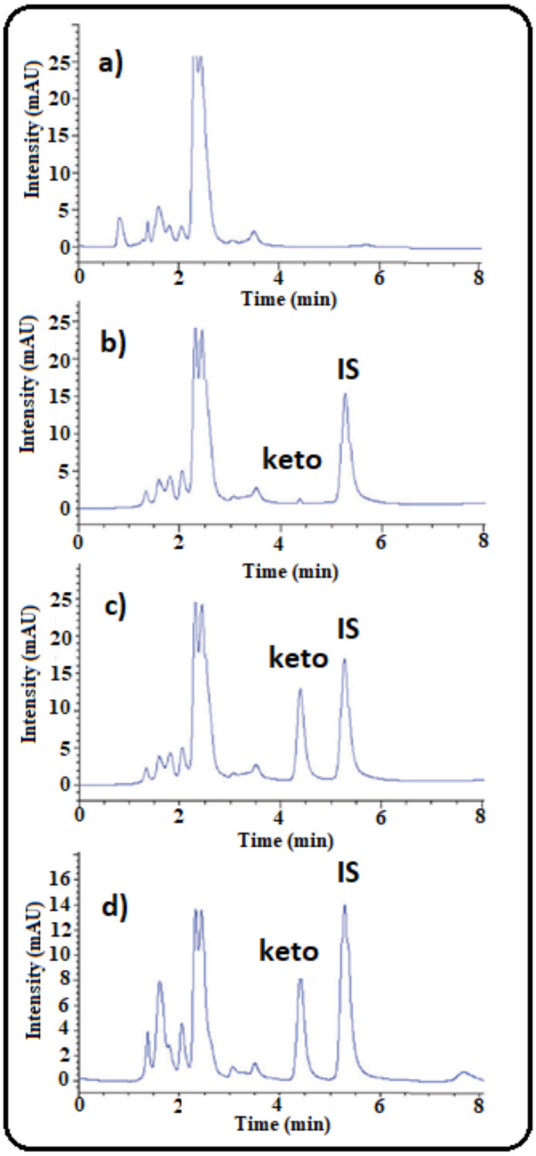

Figure 3: a) Chromatogram of Blank plasma sample; b) Chromatogram of LLOQ sample; c) Chromatogram of ULOQ sample; d) Chromatogram of processed rat plasma sample after 1 hr of dosage.

RESULTS AND DISCUSSION

Chromatographic Optimization

For optimal peak separation and resolution of the ketobemidone and IS peaks, the chromatographic conditions must be combined with an appropriate mobile phase. To achieve the best results, the mobile phase's concentration was altered using acetonitrile and methanol in water. Ketobemidone and IS were successfully separated with good resolution using the methanol-water (45:55 v/v) mobile phase condition and triethylamine was added at a rate of 0.1-3%. In this case, ketobemidone's peak form featured a small tail.

Even so, more endogenous compounds were eluted, and the tailing factor remained constant. The study used phosphoric acid as pH modifiers to examine the peak form and resolution of ketobemidone. Using mobile phase containing 0.01% phosphoric acid, an excellent peak shape (peak resolution) for ketobemidone was observed without any endogenous interference. The finally optimized mobile phase was water: methanol: phosphoric acid (60:40:0.01 V/V/V).

Sample Preparation

Initially, acetonitrile and methanol were used as solvents in the usual protein precipitation procedure. Ketobemidone was extracted along with numerous other endogenous compounds, although the extraction efficiency was quite poor. Attempted liquid-liquid extraction utilizing a variety of organic solvents, such as acetone, dichloromethane, ethyl acetate and ethyl ether, as well as chloroform. It was discovered that the ketobemidone can be successfully extracted using ethyl acetate as solvent with little interference. Ethyl acetate, methanol and acetonitrile were used as extraction solvents and the use of ethyl acetate produces the highest extraction efficiency.

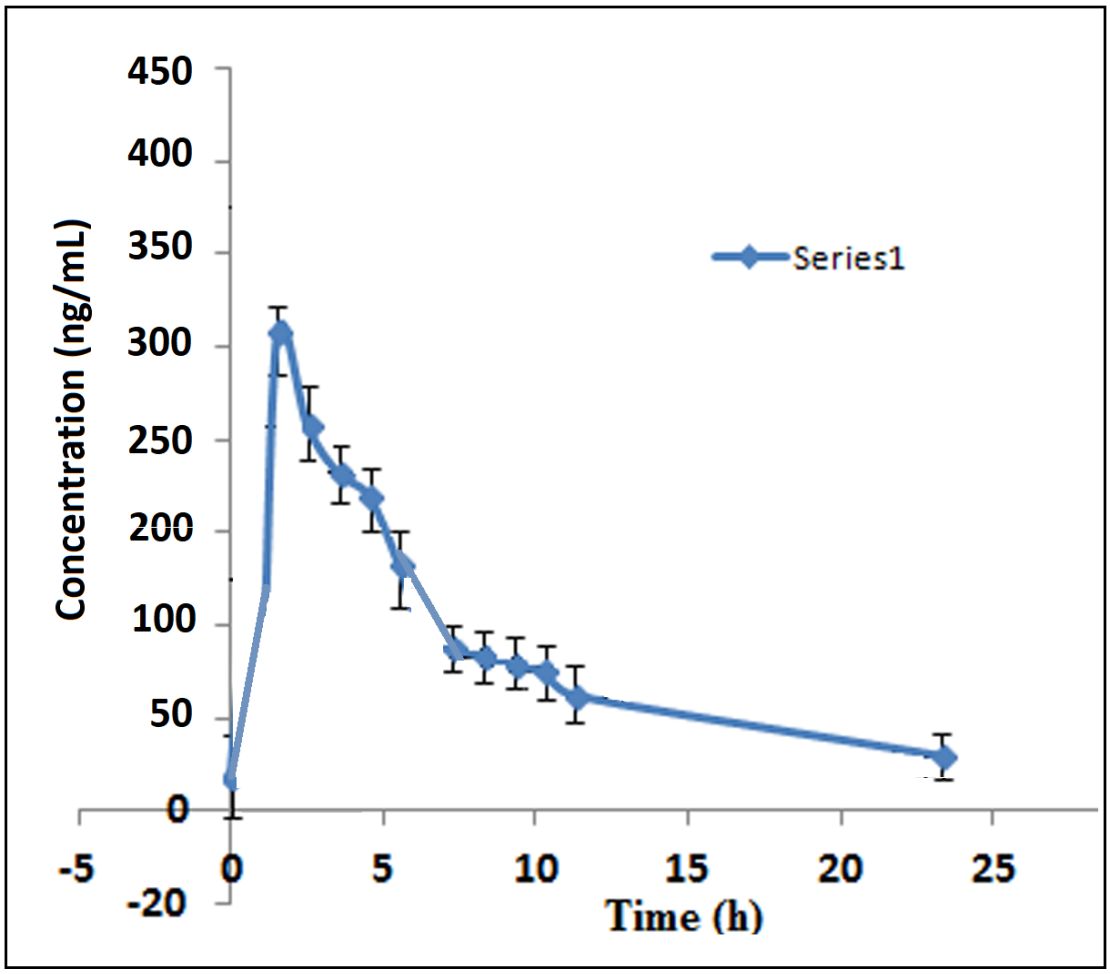

Figure 4: The concentration-time profile of ketobemidone.

Selection of Internal Standard

Among other drugs, oridonin, isopsoralen, paracetamol and phenacetin were assessed as internal standards. The structural relative of ketobemidone, oridonin, showed notable endogenous chemical interference. When compared to ketobemidone, isopsoralen and paracetamol revealed different retention patterns. Pethidine was ultimately advised because there was no proof of endogenous interference and because it had retention qualities comparable to those of ketobemidone. Its retention qualities resemble those of ketobemidone and there is no sign of endogenous interference. It improves the accuracy and precision of quantitation procedures more than other factors.

Validation

Specificity, Linearity and sensitivity

There was no interference was observed (Figure 3) as blank plasma, blank plasma+IS (5 µg/mL), blank plasma+ ketobemidone (10 µg/mL) + IS (5 µg/mL) and rat plasma collected after 1 hr of the intravenous dosage of ketobemidone (5 µg/mL). The chromatographic results are shown in Figure 3. There were no detectable interfering endogenous substances present during the analyte and IS retention times. The correlation value for the calibration curve was more than 0.999 and it was linear for concentrations between 0.1 and 25 µg/mL. The obtained regression was 0.9995 with respect to area ration of ketobemidone to IS. Table 1 shows the precision and accuracy for detecting ketobemidone in rat plasma with LLOQ 0.1 µg/mL.

Accuracy and precision

Table 1 provides a summary of the precision and accuracy for the intra-day and inter-day analysis of ketobemidone was done on 3 consecutive days (n=6). The precision of intra and inter day were 8.2 and 9.1% respectively, while the RE accuracy was found to be within 10.0%. The technique was precise and accurate, and the results were acceptable.

Extraction recovery

It has been demonstrated how to extract liquid using a straightforward procedure. At dosages of 0.25, 2.5 and 20 µg/mL, ketobemidone achieved mean extraction recoveries of 84.1, 6.0, 76.7 and 4.5% (n=6). The recovery of analyte and IS are lower to

Table 3: The pharmacokinetic data of ketobemidone at 10mg/Kg dose in rat plasma.

Parameter	Ketobemidone±SD
C_{max}	39±4.2 ng/mL
t_{max}	5.1±0.8 hr
AUC_{0-24}	305.1±27.3
$AUC_{0-\infty}$	305.8±23.8
$t_{1/2}$	2.05±0.41

AUC [ng/mL*hr]: area under concentration-time curve; $t_{1/2}$: half-life [hr].

higher QC level is consistent and with overall average 92.3 and CV is 5.7%.

Stabilities

To take into account, the expected handling conditions for plasma samples, the stability test was developed. According to the results of the stability test, ketobemidone was stable even after III freeze thaw cycles which were stored at -20ºC for 30 days. The processed samples showed stability at 4ºC for 24 hr in autosampler. The stock solutions showed stability for 30 days at 4ºC. The results are shown in Table 2.

Pharmacokinetic Study

A very few ketobemidone's pharmacokinetics studies were available[16-18] and they have some limitations such as sample volume for analysis, low recovery, high runtime, expensive extraction procedure like Solid Phase Extraction (SPE). 6 rats were given 10 mg/kg of ketobemidone intravenously (Figure 3) and the developed and validated method was successfully applied to assess the plasma levels and pharmacokinetics of the drug. The crucial pharmacokinetic parameters were identified, and Figure 4 depicts the mean plasma concentration vs. time curve. The $t_{1/2}$ of 2.05 hr, which showed a decrease in plasma concentration, showed that ketobemidone was released rather slowly. Table 3 shows the outcomes for area under the plasma concentration-time curve (AUC_{0-t}) and total drug exposure across time ($AUC_{0-\infty}$) as 305.1 ng\mL and 305.8ng\mL, respectively.

CONCLUSION

A simple, precise and reliable method was developed on HPLC-PDA successfully to measure and validate the presence of ketobemidone in rat plasma. Ketobemidone, a medicine, was directly recovered from rat plasma by simple solvent extraction method. According to USFDA regulations, the enhanced HPLC-PDA method was verified using the indicated validation parameters. The results of the validation investigation demonstrated the developed method's sensitivity, accuracy and precision throughout a wide linear range of ketobemidone concentrations in rat plasma. The method's suitability was established after oral ketobemidone dosing in order to discover the pharmacokinetic characteristics.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HPLC: High performance liquid chromatography; **IS:** Internal standard; **LLOQ:** Limit of Quantification; **QC:** Quality control; **LOD:** Limit of Detection; **AUC:** Area under curve; **USFDA:** United States Food and Drug Administration.

SUMMARY

The current research was aimed to develop simple and robust method to quantify the ketobemidone in biological matrices. The developed method was successfully validated as per the regulatory guidelines with the calibration range 0.10-25.0 µg/mL and was applied to pharmacokinetic study in rats.

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