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Forensic Assessment of Tramadol Detection: Comparative Analysis of Drug Persistence in Albino Rat Hair Follicles and Serum Samples

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ABSTRACT

Drug abuse presents significant challenges to public health and safety, necessitating effective methods for detecting and monitoring. Tramadol, a widely used opioid analgesic, has garnered attention due to its abuse potential. Traditional methods of drug detection, such as serum analysis, may not capture long-term exposure patterns effectively. This study aims to assess the feasibility of using hair follicles as an alternative matrix for detecting tramadol exposure and to compare the detection window of tramadol in hair with traditional serum samples. Thirty-two albino rats were divided into low and high dose groups and exposed to tramadol orally for seven days, while control groups received water. After euthanasia at various intervals post-exposure, blood and hair samples were collected and analyzed for tramadol concentration using spectrophotometric method. Results revealed that Serum tramadol levels declined rapidly attaining undetectable concentration after one week post-exposure, while tramadol concentrations in hair persisted for an extended period, up to the study endpoint with significant detectable concentration even after one month. The low dose group exhibited detectable tramadol concentrations across all time points post-exposure, while the high dose group showed higher initial concentrations, gradually decreasing over time. In conclusion, the study highlighted that serum analysis is suitable for detecting recent tramadol ingestion, whereas hair analysis offers a retrospective assessment of drug exposure over longer periods. These findings highlight the complementary nature of serum and hair analyses in tramadol toxicology assessments, providing insights into drug use patterns beyond the acute phase.

Keywords: Tramadol, Forensic Assessment, Drug Detection, Hair Analysis, Serum Analysis.

INTRODUCTION

Drug abuse is a pervasive and complex societal issue with profound implications for public health and safety.[1] Identifying effective methods for detecting and monitoring drug exposure is crucial for addressing this problem.[2] Tramadol, a centrally acting analgesic, has gained popularity and concern due to its potential for abuse.[3]

Traditional methods of drug detection often rely on biofluid samples such as urine and serum; however, these methods may not capture long-term exposure patterns.[4]

Assessing drug concentrations in hair may serve as a promising avenue for understanding prolonged drug exposure due to the unique characteristics of hair, which allows for the accumulation of substances over an extended period.[5]

The objective of this study is to explore the feasibility of utilizing hair follicles as an alternative matrix for detecting tramadol exposure, offering a retrospective perspective as an indicator of drug exposure as well as Comparing the detection window of tramadol in hair with traditional serum samples.

MATERIALS AND METHODS

StudyaArea

The research was carried out within the Faculty of Veterinary Medicine at the University of Maiduguri, situated in Borno state, Nigeria. Specifically, the investigation took place in the Department of Pharmacology. Borno state is positioned in the extreme northeastern part of Nigeria, spanning an extensive area of 61,435 square kilometers and sharing borders with the Republic of Niger to the north, the Republic of Chad to the northeast, and the Cameroon Republic to the east. It also borders Adamawa State to the south, Gombe State to the southwest, and Yobe State to the west, encompassing a total of 27 local government areas.[6]

As per the 2006 census, Borno state had a population of 4,171,104, consisting of 2,163,358 males and 2,007,746 females. The geographical location, coupled with its extensive border connections, renders Borno State a region marked by significant ecological and environmental diversity. [7]

Moreover, the population of the state has been grappling with heightened tramadol drug abuse, particularly in the aftermath of the Boko Haram insurgency.[8]

Study Design

The study animals were categorized into two primary groups, Group 1 and Group 2, for the investigation of tramadol exposure effects. Group 1 received a low dose of tramadol, administered orally in the form of tramadol hydrochloride, while Group 2 received a high dose of the drug. Within each main group, subgroups (a, b, c, d, and e) were established to explore variations in response and concentrations.

Control groups were administered only water as a placebo, ensuring a baseline for comparison, while treatment groups were exposed to low and high doses of tramadol over a seven-day period.

To adhere to safety and ethical considerations, a lethal dose (LD50) determination was conducted before the main experiments, ensuring that the administered tramadol doses were within safe limits. Following the seven-day tramadol exposure period, animals were euthanized at different time intervals, ranging from 24 hours to one month after the last dose, to investigate the temporal aspects of tramadol presence in the system.

Post-euthanasia, blood and hair follicle samples were collected from each animal and subjected to thorough analysis to determine the presence and concentration of tramadol, providing insights into the drug's absorption and retention in biological matrices.

Experimental Animals

For this study, thirty-two (32) apparently healthy mature male albino Wister strain rats, with an estmate age of 12 weeks and a weight approximately 160kg, were employed. Prior to the initiation of the research, the rats underwent a one-week acclimatization period to familiarize them with the environment.

Throughout this period, they were housed in hygienic stainless-steel cages, maintained at a

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constant temperature of 30±2oC with a 12-hour light: 12-hour dark cycle, and provided with a diet comprising rat/mouse pellets. This acclimatization phase aimed to ensure that the rats adapted to their surroundings before the commencement of the actual study, establishing a foundation of stable and reliable baseline conditions for the experimental procedures.

Exclusion Criteria

To ensure a focused association between observed effects and tramadol exposure, rats with pre-existing health conditions such as infections, tumors, or chronic illnesses were excluded. Additionally, those exhibiting abnormal baseline values in essential physiological parameters, including body weight or behavioral assessments, were omitted to uphold homogeneity within the study population.

Female rats were entirely omitted from the study to avert potential pregnancy or lactation during the acclimatization period or initial assessments, as the reproductive state could introduce confounding variables. Moreover, rats with a history of tramadol exposure before the study were omitted to confirm that the observed effects were primarily linked to the administered tramadol doses during the experimental period.

To ensure the dependability of the study outcomes, rats that failed to adequately acclimate to the controlled environment during the one-week acclimatization phase were excluded, minimizing the potential influence of stress or environmental factors. Lack of adherence to experimental procedures or failure to receive the prescribed tramadol doses led to the exclusion of rats from the analysis, maintaining consistency in the study.

In the pursuit of data accuracy and integrity, rats that developed unexpected illnesses or health issues during the study, as well as those facing unexpected mortality before the study's completion, were excluded to forestall confounding variables and ensure a comprehensive dataset.

Animal Welfare Compliance and Ethical Approval

The research adhered to the prevailing guidelines for animal welfare as outlined by the National Academy of Science (NIH, 1985). The institutional Animal Care thoroughly reviewed the protocol. Ethical clearance for the study was duly obtained from the Animal House within the Faculty of Veterinary Medicine, specifically from the Departments of Pharmacology, at the University of Maiduguri in Borno State

Acute Toxicity Studies (Determination LD50) for Tramadol

Acute Toxicity Assessment: The acute toxicity of tramadol was evaluated following the method described by Lorke,[9] with a focus on determining the LD50. The study comprised two phases, both involving the oral route for administration.

Phase I: Nine healthy male albino Wister rat strain rats, with an average weight of 160g, were randomly selected and divided into three groups (A, B, and C), each consisting of three animals. Individual rats within each group were weighed and marked with identification using picric acid on the head, back, or tail. The groups were then treated with incremental doses of tramadol hydrochloride at 10 mg/kg, 100 mg/kg, and 1000 mg/kg, administered orally. The animals were observed for 24 hours to assess signs of toxicity and mortality.

Phase II: Building on the findings of Phase I, three healthy male albino Wister rat strain rats, averaging 160g, were randomly selected and grouped into three categories (A, B, and C).

Based on the results of Phase I, graded doses of tramadol hydrochloride (600 mg/kg, 1000 mg/kg, 1600 mg/kg and 2900 mg/kg) were administered orally. Throughout the experiment, the rats had unrestricted access to food and water. Observations for signs of toxicity and death were conducted for 24 hours. Subsequently, the LD50 was calculated.

Table 1: Oral Acute Toxicity Studies of Tramadol

Phase	Group	No of Rats	Dose (mg/kg)	Clinical Sign	Mortality
I	a	3	10	Dizziness	0/3
I	b	3	100	Weakness	0/3
I	c	3	1000	Weakness	1/3
II	a	1	600	Weakness	0/1
II	b	1	1000	Weakness	0/1
II	c	1	1600	Death	1/1
II	d	1	2900	Death	1/1

In Phase I, escalating doses of tramadol were administered to three groups of rats. The lowest dose (10 mg/kg) resulted in no observed mortality, but dizziness was reported. The mid-dose group (100 mg/kg) also showed no mortality but with weakness. However, in the highest dose group (1000 mg/kg), one out of three rats experienced mortality, while weakness was observed in the rest.

The absence of mortality at the lowest and mid-doses suggests a threshold below which acute toxicity effects are not lethal. These results influenced the selection of doses for Phase II and provided a basis for understanding the range of tramadol doses that induce adverse effects.

In Phase II, Increased doses of tramadol was administered to individual rats. The lower doses (600 mg/kg and 1000 mg/kg) did not result in mortality but exhibited weakness as a clinical sign. However, in the higher dose groups (1600 mg/kg and 2900 mg/kg), each rat experienced mortality within the 24-hour observation period, with weakness noted in conjunction.

The LD50 (lethal dose for 50% of the tested rats) is typically estimated in this study based on the minimum dose of tramadol that leads to rat mortality. In this investigation, the LD50 for tramadol is inferred to be at least 1600 mg/kg, as rat mortality was observed at this dose level. This information guided the selection of low and high tramadol doses for the primary experimental groups. Meanwhile, the precise doses at which 50% of the rats die are calculated using the formula described by Lorke,[9] as outlined below:

$LD\ 50 = \sqrt{a \times b}$

Where: a = lowest dose that kills an animal's, 1/1

b = highest dose that does not kill any animal, 0/1.[9]

$LD\ 50 = \sqrt{a \times b} = 1600 \times 1000$

$= \sqrt{1{,}600{,}000} = 1{,}265$ mg/kg.

$LD\ 50 = 1{,}265$ mg/kg

Tramadol Detection Study

*Animal Allocation:*A total of thirty-two albino rats were utilized and divided into two primary groups, namely Group 1 and Group 2, each comprising 16 animals. Group 1 received a modest oral dose of tramadol through tramadol hydrochloride administration (100mg/kg), while Group 2 received a higher dose (400mg/kg). Within each main group, subgroups (a, b, c, d, and e) were established, with each subgroup consisting of 3 animals.

Control groups, labeled as 1a and 2a, were subjected to water administration only, serving as a placebo for comparison. Conversely, treatment groups, denoted as 1b to 1d and 2b to 2d, underwent exposure to low and high tramadol doses, respectively, over a consecutive seven-day period.

Sacrifice and Sampling: Timeline In accordance with established protocols, all animals utilized in the study were euthanized to obtain blood and hair samples subsequent to the seven-day tramadol exposure period. Euthanasia was performed on the animals at different intervals, ranging from 24 hours to one month after the final dose. Following euthanasia, blood and hair follicle samples were collected from each animal and meticulously analyzed to ascertain the presence and concentration of tramadol.

Sampling Timeline: Twenty-four hours after the final tramadol dose: One animal from each control group (1a and 2a) and all animals from low dose groups (1b and 2b) were euthanized.

Three days after the final tramadol dose: One animal from each control group (1a and 2a) and all animals from groups 1c and 2c were euthanized.

One week after the final tramadol dose: One animal from each control group (1a and 2a) and all animals from groups 1d and 2d were euthanized.

One month after the final tramadol dose: One animal from each control group (1a and 2a) and all animals from groups 1e and 2e were euthanized.

Sample Collection

For the collection of hair samples, a standardized procedure was implemented.[10] Hair samples were obtained by shaving using a razor blade after the rats were slaughtered. This method ensured a consistent and non-invasive approach to gather hair samples for subsequent analysis.

Concurrently, blood samples were collected through slaughtering the rats to minimize stress and ensure humane handling. The blood was collected from major blood vessels at the neck of the rats, to obtain high-quality samples for analysis. Following blood collection, the samples were allowed to clot by leaving it undisturbed at room temperature for approximately 1 hour in the tubes. They were then centrifuged at 5000rpm for 5 minutes. This step separates the serum from the clot and blood cells. Using a plastic Pasteur pipette, the serum was carefully transferred from the collection tube into a clean and labeled sample tube.[11]

Both the hair and serum samples were handled with meticulous care to prevent contamination, appropriately labeled for identification, and stored at 4 degrees Celsius until the analysis phase.

Laboratory Analysis

The laboratory analysis was carried out at the Analysis Laboratory of the National Agency for Food and Drug Administration and Control (NAFDAC) in Maiduguri.

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Hair Sample Preparation

The hair samples were individually weighed to achieve a standardized weight of 100mg each. Subsequently, each batch of hair was placed in 25ml beakers labeled accordingly. To facilitate the extraction of lead content from the hair into a water solution, 10mls of purified water were added to each labeled beaker. Care was taken to arrange the beakers in the tank of an ultrasonic cleaner machine, manufactured by OLenyer Inc. (model number: 164767-5294-1004267781), in a manner that allowed the ultrasonic waves to freely reach all beakers without overcrowding.

The ultrasonic cleaner machine was set to a temperature of 65°C and operated for 15 minutes after being powered on. This machine generates high-frequency sound waves in the cleaning solution, producing microscopic bubbles. The collapse of these bubbles creates energy that induces cavitation in the hair follicle, leading to the release of hair shaft content into the solution.[12] Special attention was given to avoiding overcrowding to ensure optimal exposure of all beakers to the ultrasonic waves.

Tramadol Detection in Hair Follicle and Serum Spectrophotometric Method(using Jenway Spectrophotometer 6305 Gemini BV)

Assay Procedure for Tramadol:

Firstly, 0.5 ml of the solution (hair shaft content and serum sample) was dispensed in a 10 ml clean test tube, then the volume was made upto 10 ml by adding 9.5 mls of 0.1N NaOH. The absorbance of the prepared solution was measured at 271nm wavelength against 0.1N NaOH as blank (using Jenway Spectrophotometer 6305 Gemini BV) and then the values are recorded. Finally the amount of Tramadol present in the sample solution was computed from its calibration curve.[13]

RESULTS

Figure 1 illustrates the creation of the calibration curve through spectrophotometric analysis, where absorbance values are depicted on the y-axis in nanometres, while corresponding tramadol concentrations in milligrams per millilitre are represented on the x-axis. The linear regression equation derived from this curve is expressed as follows:

$y=6.090x+0.023$ Here:

y denotes the absorbance values obtained from the spectrophotometer,

x represents the concentration of tramadol in the serum samples. The slope of the line is approximately 6.090, indicating that with each unit increase in concentration (x), the absorbance (y) rises by approximately 6.090 units. The y-intercept is around 0.023, representing the absorbance when the concentration of tramadol is zero. Moreover, the correlation coefficient (R^2) associated with the calibration curve is documented as 0.999, denoting an exceptionally robust linear relationship between absorbance and concentration. This high correlation coefficient

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underscores the reliability and accuracy of the calibration curve in faithfully representing the association between absorbance and tramadol concentration in serum samples.

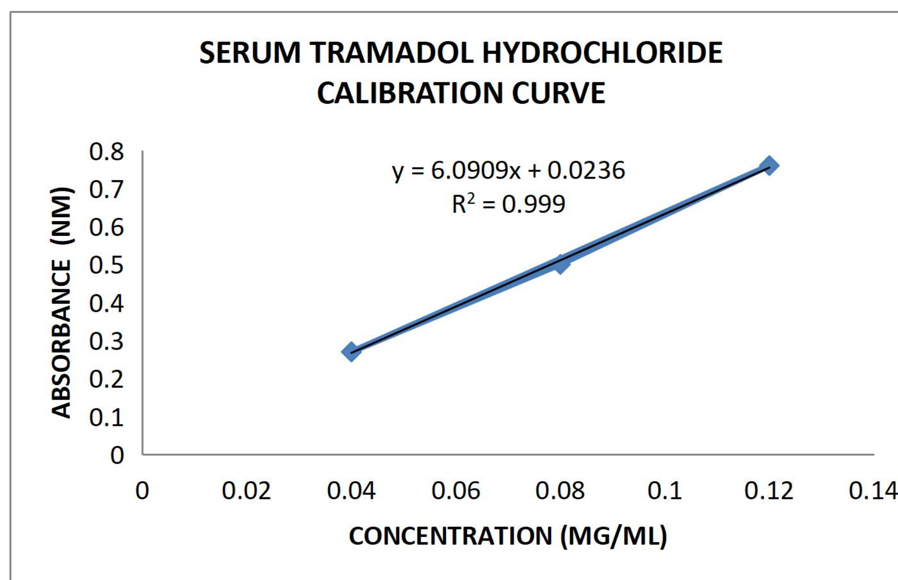

Figure 1: Serum Tramadol Calibration Curve

Table 2 presents the mean values of tramadol concentration in serum at different durations post-exposure for control, low dose, and high dose groups, with all control groups consistently showing no detectable tramadol concentration, while the low dose and high dose groups exhibited varying concentrations over time.

The low dose group, administered with tramadol, displayed a detectable concentration of tramadol in serum with concentrations decreasing over time reaching undetectable concentration after one week. Conversely, the high dose group exhibited higher initial concentrations at 24 hours post-exposure, gradually decreasing over subsequent time intervals, ultimately reaching undetectable levels similar to the low dose group after one week post-exposure.

Table 2: Showing mean value of Tramadol concentration in serum

Duration	Control (mg/ml)	Low dose (mg/ml)	High dose (mg/ml)
1b (24 hours post exposure)	0.00	0.08	0.11
1c (3 days post exposure)	0.00	0.05	0.06
1d (1 week post exposure)	0.00	0.02	0.03
1e (1 month post exposure)	0.00	0.00	0.00

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Figure 2 shows the generation of the calibration curve using spectrophotometric analysis. Absorbance values are plotted on the y-axis in nanometres, while tramadol concentrations in milligrams per millilitre are shown on the x-axis. The linear regression equation derived from this curve is:

$$y=6.090x+0.023$$

In this equation:

y represents the absorbance values obtained from the spectrophotometer,

x signifies the concentration of tramadol in the hair shaft.

The slope of the line is approximately 6.090, indicating that for each unit increase in concentration (x), the absorbance (y) increases by roughly 6.090 units. The y-intercept is approximately 0.023, which denotes the absorbance when the concentration of tramadol is zero.

Additionally, the correlation coefficient (R^2) associated with the calibration curve is reported as 0.999, indicating a highly robust linear relationship between absorbance and concentration. This substantial correlation coefficient highlights the dependability and precision of the calibration curve in accurately representing the connection between absorbance and tramadol concentration in hair shaft samples.

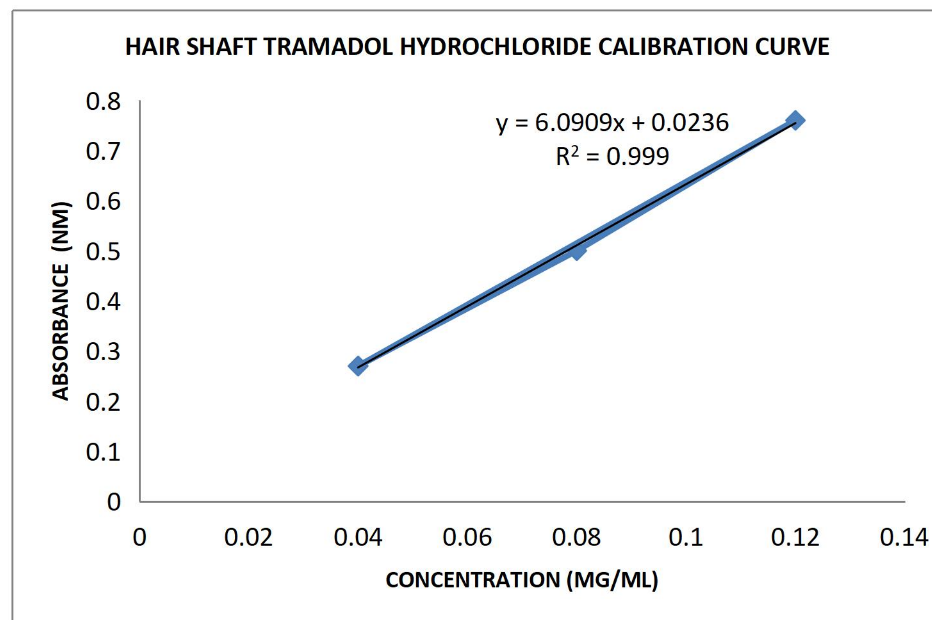

Figure 2: Hair Shaft Tramadol Calibration Curve

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Table 3 shows the mean values of tramadol concentration in hair at various time points post-exposure for control, low dose, and high dose groups. The control group consistently showed no detectable tramadol concentration in hair, while both low dose and high dose groups exhibited detectable concentrations, with higher doses leading to higher concentrations. Concentrations generally increased over time post-exposure, peaking at one week post-exposure for the high dose group, followed by a slight decrease and possible maintenance of concentration levels at one month post-exposure.

Table 3: Showing mean value of Tramadol concentration in hair

Duration	Control (mg/ml)	Low dose (mg/ml)	High dose (mg/ml)
1b (24 hours post exposure)	0.00	0.04	0.09
1c (3 days post exposure)	0.00	0.8	0.12
1d (1 week post exposure)	0.00	0.12	0.16
1e (1 month post exposure)	0.00	0.04	0.06

DISCUSSION

The investigation conducted in this study aimed to evaluate how effectively tramadol can be detected in albino rat hair follicles and serum samples. The results provide valuable insights into the potential forensic uses of tramadol detection. Notably, the findings reveal varying patterns of tramadol distribution and longevity in both serum and hair samples, which has implications for the interpretation and detection of drug usage.

In line with previous research by Dan Wagener,[4] the study found that there is a substantial decline in serum tramadol indicating that serum tramadol levels exhibit a relatively short duration of detectability, diminishing to undetectable levels after one week post-exposure. This observation aligns with the known pharmacokinetics of tramadol, characterized by rapid metabolism and elimination, as documented by Li et al.[14] The transient nature of tramadol in serum highlights the limited window of detection for acute tramadol exposure using this matrix.

Conversely, the study revealed that tramadol concentrations in hair persisted beyond one month post-exposure, with detectable levels observed up to the study endpoint. This prolonged retention of tramadol in hair is consistent with previous literature indicating the capacity of hair to retain and integrate drugs over extended periods, as discussed by Pascal et al.[15] The stability of tramadol in hair underscores its potential utility as a retrospective indicator of drug exposure, offering insights into past usage patterns beyond the acute phase.

These findings have significant implications for forensic investigations and clinical assessments. While serum analysis may provide valuable information regarding recent tramadol ingestion, its limited detection window necessitates prompt sample collection for accurate interpretation. In contrast, hair analysis offers a longer-term perspective, enabling retrospective assessment of tramadol exposure over extended periods.

CONCLUSION

In conclusion, the study provides valuable insights into the forensic implications of this commonly abused opioid analgesic. Through comprehensive analysis, the distinct patterns

of tramadol distribution and persistence was observed, with serum concentrations diminishing rapidly after one week post-exposure, while tramadol levels in hair exhibited prolonged retention over extended periods.

These findings underscore the complementary nature of serum and hair analyses in tramadol toxicology assessments, offering distinct temporal perspectives on drug exposure history. While serum analysis provides insights into recent tramadol ingestion, hair analysis enables retrospective assessment over longer time frames, facilitating comprehensive evaluations of drug use patterns.

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