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INTERFERENCE OF ZINC SULPHATE WITH TRAMADOL AND DIAZEPAM IMMUNOASSAY TESTING

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Abstract

Drug abuse is a significant public health issue in many countries worldwide. Tramadol used to help relieving moderate to moderately severe pain. Tramadol works similar to opioid (narcotic) analgesics in their mechanism of action. Diazepam belongs to a class of drugs known as benzodiazepines. Diazepam used to treat medical conditions such as alcohol withdrawal, anxiety and seizures. It could be used to relieve muscle spasm and to provide sedation before medical procedures. Immunoassay tests are commonly used health care field. Depending on those circumstances; this study investigated the potential interference of zinc ion used as a direct adulterant for enzyme multiplied immunoassay technique (EMIT)-based drugs of abuse testing in urine samples. For confirmatory testing, Ultra Performance Liquid Chromatography with photodiode array detector method was utilized. The effect of zinc sulfate existence on two drugs of abuse, tramadol and diazepam, was explored and a minimum concentration of 10 mg/ml was found to be effective for adulteration purpose

Keywords: Adulteration; Zinc Sulphate; Tramadol; Diazepam.

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1. Introduction

Drug testing is commonly used in health care field, workplace, and criminal issues, it has become widespread during the past decades. Screening of urine for drugs has been the most common method for analysis because of ease of sampling. Due to the simplicity of handling and access to rapid results have increased the need for immunoassays testing. If results are not confirmed by a secondary method of analysis, such as gas or liquid chromatography, False positive results of immunoassays may lead to serious medical, social or legal consequences. There are common areas for drug testing like the workplace (eg, pre-employment and random testing), athletics, the military fields, criminal and legal situations (eg, post-accident testing, rehabilitation testing), and health care (eg, treatment, compliance monitoring, cause of death). drug tests misinterpretation can have

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dangerous consequences, like risk of prison sentence, unjust termination from a job, inappropriate exemption from a sporting event, and inappropriate medical treatment in emergency cases [1].

Immunoassay tests are based on the ability of an antibody to interact with a drug (2). There are physicians prescribing opioids for sever pain patients follow guidelines established by the American Pain Society [3]. The American Pain guidelines put specifications on the regular or periodic use of urinary drug testing as a component of treatment, this includes steps like upon assessing potential risk for substance abuse, misuse or addiction. The final review of the results is an essential component of any drug testing program. A positive laboratory drug test result does not automatically identify an applicant as an illegal drug user, nor does a laboratory result of invalid, adulterated, or substituted automatically identify specimen manipulation. In the context of information obtained from the applicant or patient interview, an individual with a detailed knowledge of possible alternative medical explanations must interpret drug test results. For each specimen, a specimen validity testing must be performed. For example, creatinine and pH must be determined for each one, specific gravity must be determined for each specimen with creatinine less than 20 mg/ld. Laboratories are required to perform a confirmatory drug test method different from the initial screening test method (i.e., immunoassay), to specifically identify and quantify the parent drug or the drug metabolite. [4-6].

Chromatographic techniques such as gas chromatography (GC) and liquid chromatography (LC) are commonly used to separate and analyze mixtures of chemical substances or drugs. After the chromatographic instrument has separated the analytes in a sample, the constituents enter the mass spectrometer (MS), which identifies and quantifies the separated substances. The MS converts the molecules into charged masses (ions) and separates them according to their mass-to-charge (m/z) ratios. For each substance, the ions produce unique mass spectra, which are used to identify different chemicals or drugs. Urine specimens must undergo a preparation process (extraction) prior to GC/MS analysis and may require an extraction process prior to LC/MS analysis [7].
This study focuses on the manipulating effects of zinc sulfate as a potential adulterant in the urine drug testing. The effect of zinc sulfate on two drugs of abuse, tramadol and diazepam, was explored.

2. Experimental

2.1. Materials, Reagents and Chemicals

- Tramadol hydrochloride and Diazepam pure drugs were obtained as gift sample from Minapharm, Cairo, Egypt.
- Zinc sulphate, acetonitrile (HPLC) (Sigma-Aldrich, USA), Methanol (HPLC grade), (Merck, Germany)
- All other reagents and materials were of analytical grade and supplied from commercial sources.
- Immuonoassay reagents (Syva, Emit plus)

2.2. Sampling

- Drug-free urine specimens were pooled from healthy volunteers
- Urine samples were collected from **30 healthy volunteers**, ranging in age from 23 to 40 years. and used as a matrix to create fortified samples.
- All urine samples were collected and immediately frozen at –20°C until analysis.

2.3. Standard Solutions and Calibration Curves

The primary stock solutions of tramadol, diazepam and IS were prepared to concentration of 1 mg/ml in acetonitrile (ACN) using volumetric flasks. These were then stored at -20°C. Appropriate dilutions of stock standard solutions prepared by diluting appropriate volumes in 10 ml glass tubes.

2.4. Extraction of Tramadol from Urine Samples

The urine samples were left on the bench to thaw naturally at room temperature and they were vortexed prior to use. urine extraction was accomplished with liquid-liquid extraction. To 2 ml of urine, 10 µl of internal standard I.S. (phenacetine working solution 100 mg/ml), 0.5 ml of 0.1 M sodium hydroxide solution were added consecutively. followed by vortexing. Then 4 ml of ethyl acetate-n-hexane solution were added in a ratio 1:5 (v/v), vortexed for 30 s, centrifuged at 3000 g for 20 min. The organic phase containing TRM and internal standard was transferred into another glass tube and evaporated to dryness at 50°C under a stream of nitrogen. The dried residue was reconstituted in 500 µl of the mobile phase and a 25 µl sample was injected into the UPLC system.

2.5. Extraction of Diazepam from Urine Samples

Urine samples were left on the bench to thaw naturally and were vortexed prior to use. Urine extraction was accomplished with liquid-liquid extraction. To 2 ml of urine, 5 µl of internal standard (phenacetine working solution 100 mg/ml), 1 mL saturated sodium carbonate and 6 mL 1-chlorobutane to each tube, were added consecutively, followed by vortexing. Then Capped and rotated tubes for 30 minutes, centrifuged at 3000 g for 20 min. The organic phase containing diazepam and internal standard was transferred into another glass tube and evaporated to dryness at 50°C under a stream of nitrogen. The dried residue was reconstituted in 500 µl of the mobile phase and a 25 µl sample was injected into the UPLC system.

2.6. UPLC Method Development

Apparatus: Waters UPLC with Photodiode array detector and integrator, Acquity H class apparatus with an autosampler. C18 UPLC column, 150 x 2.1 mm I.D, particle size 1.7 µm, (Waters). The mobile phase composed of 0.05 M of NaH_2PO_4 buffer at flow rate 0.4 ml/min, was filtered through 0.2 mm cellulose acetate membrane filters (Sartorius Stedim Biotech S.A.; Aubagne Cedex, France) with a solvent filtration apparatus.

This method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision, recovery and accuracy according to the international guidelines on Bioanalytical Method Validation [8]. calibration curves were obtained by spiking the blank urine with known

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concentration of each analyte and IS to provide concentrations of 10, 20, 50, 100, 200, 500 and 1000 ng/ml, for tramadol, and 20, 40, 80, 100, 200, 400, 800 and 1000 ng/ml for diazepam. peak area versus concentration calibration curves of tramadol and diazepam were plotted. Least squares regression parameters for each calibration curve were calculated, and the concentrations of the test samples were interpolated from regression parameters. Samples concentrations were determined using the formula Y= mX+ b, where Y= peak area, X= concentrations of standard, where m= the slope of the curve and b= the intercept with Y axis. Correlation coefficients for each of the calibration curves were > 0.99.

2.7. Preparation of Zinc Sulphate in Urine Samples

Stock solutions diluted in glass tubes to reach final concentrations of 100,200, 300 ng/ml for tramadol. Working solution of diazepam was prepared by diluting the Stock solution to a final volume of 10 ml in urine, these solutions diluted in glass tubes to reach final concentrations of 50, 70, 120 and200 ng/ml. Zinc sulphate stock solution was added to the previously prepared drug urine samples to obtain concentrations of 10-100 mg/ml for each drug level.

2.8. Effect of pH

Drug-free urine was split into two separate tubes. To one of them, zinc sulfate solution (10-100 mg/mL) was added. The pH of the urine samples was then measured using a pH meter.

3. Results and Discussion

3.1. UPLC Detection Method Development

An Isocratic UPLC-PDA method was developed for the determination of tramadol and diazepam in human urine samples. It includes the extraction of these compounds by liquid–liquid extraction, this method agreed with the previous validated HPLC–UV method by D Szkutnik-Fiedler) [9]. The procedures gave sharp, symmetrical and well separated peaks for tramadol, diazepam and I.S with consistent and convenient retention times of 1.8, 3.00 and 2.45 min, respectively. No interferences were observed among analytes, IS and peak impurities deriving from matrices. In the analysis of the standard solutions using photodiode array detector, 230 nm was chosen as the maximum detection wavelength.

3.2. Validation of the Method

Linearity and sensitivity The method was validated for linearity, precision, accuracy and sensitivity. The limit of quantitation (LOQ) of the method was defined as the lowest concentration of the analyte in the sample that could be quantitatively detected, with a signal-to-noise ratio (peak height) in excess of 10. The limit of detection (LOD) was defined as the lowest concentration of the analyte could be qualitatively detected and maintain retention time. Five replicates of each concentration level were analyzed to determine the LOD and LOQ of the assay. The data of the linear regression equations, LOD and LOQ are reported in table 1.

شيليها خالصPrecision could be expressed as the percent relative standard deviation for a statistically significant number of samples. Precision, in terms of coefficient of variation, was determined at three levels for each drug. The specimens, were fortified with all drugs at concentrations of 10,100 and 1000 ng/mL for tramadol and for 20, 200 and 1000 ng/mL diazepam were prepared. five replicates of each concentration were analyzed within day (intra-day precision) and for five consecutive days (inter-day precision) as reported in Table (1).

Stability of the extracts was investigated. Previously analyzed sample vials containing extracts were left on the bench, exposed to light and room temperature, for a period of two days and then reanalyzed. Any change in the concentration between the days was not noted.

3.3. Immunoassay Studies

This study focuses on the effects of zinc sulfate as a potential adulterant in tramadol and diazepam urine drug testing.

Based on the results observed in table 2 and 3, semi-quantitative for immunoassay data, it is evident that urine samples containing zinc sulfate showed gradual decrease in concentration of either tramadol or diazepam relevant to zinc sulphate increasing concentration. For tramadol samples; 40 - 50 mg/ml of Zn sulphate showed successful masking of positive samples containing different concentrations of tramadol to about 50% of its original concentration. Concerning diazepam samples; 20 - 30 mg/ml of Zn sulphate showed successful masking of positive samples containing different concentrations of tramadol to about 50% of its original concentration. A slightly detectable white precipitate forms, which appears as a turbid urine sample and may sediment in 10–15 min at room temperature. The amount of precipitate formed in urine sample varies in proportion to the amount of zinc sulfate added. According to the revised SAMSHA guidelines, abnormal color, odor, and excessive foaming are indicative of urine adulteration.

The false-negative results were observed for immunoassay tests for tramadol and diazepam are most likely due to the inhibition of the glucose-6- phosphate dehydrogenase enzyme (G-6-PD) by zinc, that came in accordance with studies performed by Abhishek Venkatratnam and Nathan H. Lents. [10]

All semi-quantitated samples were injected into UPLC/PDA to be quantitated and the results showed that there was no masking effect of zinc sulphate on them.

3.4. Effect of Zinc Sulfate on pH of Urine Samples

The extent to which the addition of zinc sulfate alters the pH of collected urine samples was examined. pH values were recorded in triplicate both before and after adding zinc sulfate. As seen in Table 4, the addition of zinc sulfate does cause a measurable change in pH. relevant to successive increase in Zn SO4 concentration; where pH value decreased from 6.5 ± 0.02 at zero concentration of ZnSO4 to a value of 4.02 ± 0.01 at 100 mg/ml concentration of ZnSO4.

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4. Conclusion

We can conclude that our study results support the possibility that addition of zinc directly to urine samples can produce false-negative results with EMIT-based immunoassay testing. therefore, zinc ion (Zn2+) is a potential adulterant for drugs in routine workplace drug screening.

We have no conflicts of interest to disclose. All procedures performed in studies involving human participants were in accordance with the ethical standards. Informed consent was obtained from all individual participants included in the study.

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