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Case Report

Toxicological Analysis of Exhumed Specimens: A Challenge for Toxicologists

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Abstract

The objective of this work is to describe the type of biological samples submitted for toxicological analysis after exhumation. Forensic toxicologists receive a variety of biological samples, but exhumed biological specimens with varying degrees of putrefaction pose a greater challenge for analysis. Usually, immunoassay and colorimetric screening are the first line approach for toxicological analysis. Suitable samples can be selected for direct analysis using chromatographic techniques with a mass spectrometer, providing reliable results. The authors report two case studies where exhumed specimens were submitted for the determination of possible intoxication. The deceased were severely injured and remained hospitalized for more than 24 hours before death. Their corpses were exhumed for chemical analysis. For the toxicological analysis, selected samples were analyzed by immunoassay and a gas chromatograph mass spectrometer (GC/MS) with electron impact ionization. Although the samples were negative for drugs of abuse (benzodiazepines and opiates), by immunoassay, midazolam (benzodiazepine) was detected in putrefied material using GC/MS, thus indicating the hospitalization before death.

Keywords: Exhumation, GC/MS, GC/FID, Toxicology challenges, Putrefied specimen

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التحاليل السمية لعينات الجثث المستخرجة من القبور: تحدٍّ أمام أخصائيي علم السموم

المستخلص

إن الهدف من هذه الدراسة هو توصيف نوع العينات البيولوجية الواردة بهدف إجراء التحاليل السمية لعينات الجثث المستخرجة من القبور، حيث يستقبل أخصائيي علم السموم الشرعي مجموعة متنوعة من العينات البيولوجية، وتتميز العينات البيولوجية التي يتم الحصول عليها بعد نبش القبور بوجود درجات متفاوتة من التعفن وهذا ما يشكل تحدياً كبيراً فيما يخص عملية التحليل. تجرى الفحوصات المناعية واللونية عادة كفحوصات كشف نوعي ومسحي حيث تعد المرحلة الأولى في التحاليل السمية. عندما تكون العينات الحيوية مناسبة يمكن اختيار تحليل مباشر تأكيدي وكمي باستخدام تقنيات الكروماتوغرافيا مع مطياف الكتلة ومن ثم الحصول على نتائج يمكن الاعتماد عليها. تعرض هذه الدراسة تقريري حالة تم فيهما تحليل عينات حيوية مأخوذة من جثث مستخرجة من القبور لتحديد إمكانية حصول التسمم قبل الوفاة. كان كل من المتوفين قد أصيبوا بجروح بالغة وبقي كل منهما في المستشفى لأكثر من 24 ساعة قبل الموت، وتم استخراج جثتيهما بهدف إجراء التحاليل السمية. وقد تم تحليل عينات مختارة بواسطة الفحوصات المناعية وكذلك فحوصات الكروماتوغرافيا الغازية المقترنة بمطياف الكتلة (GC/MS). وعلى الرغم من أن العينات كانت سلبية

بالنسبة لتعاطي المخدرات (البنزوديازيبينات والمواد الأفيونية) من خلال الفحوصات المناعية تم الكشف عن الميدازولام (البنزوديازيبين) في العينات المتحللة باستخدام تقنية GC/MS، وتشير هذه النتائج إلى المعالجة التي قدمت في المستشفى قبل الوفاة.

الكلمات المفتاحية: نبش القبور، GC/FID، GC/MS، العينات المتحللة، تحديات علم السموم.

1. Introduction

Forensic toxicologists receive variety of biological samples for toxicological analysis. Among these, exhumed biological specimens with varying degrees of putrefaction are common. Possible causes of putrefaction are enzymolysis, autolysis, and bacteriolysis [1-2]. Usually, enzyme linked immunosorbent assay (ELISA), radio- immunoassay (RIA) [3-4], chromatographic techniques like thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) [5], and colorimetric tests are the first line approach of toxicological screening [6]. These analytical methods are validated for the analysis of fresh biological samples [7-9], but exhumed specimens pose a problem of matrix interference. Owing to the unavailability of blood and urine samples after exhumation, a variety of putrefied biological tissues are submitted for toxicological analysis; the identification of tissues may not be ascertained. Selection of samples is very important as far as the pharmacology of drugs and poisons is concerned [10]. Suitable exhumed samples can be selected for direct analysis using chromatographic techniques with mass spectrometer, giving reliable results. Furthermore, availability of intoxication history (acute or chronic) can be helpful in selecting suitable samples.

This study aims to describe the toxicological analysis of exhumed biological samples by suitable analytical techniques. The authors have also evaluated the application of gas chromatography (GC) coupled with mass spectrometer (MS) in disentangling the problems faced in the toxicological analysis of exhumed biological specimens in the light of presented case studies.

2. Materials and Methods

2.1 Chemicals

Methanol, 1-chlorobutane, dichloromethane, sulfuric acid, zinc phosphide and N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) were purchased from Sigma Aldrich, USA. Potassium cyanide, ammonium chloride, ammonium hydroxide, and sodium hydroxide were purchased from E. Merck, Germany. Desalkyl flurazepam, nordiazepam, diazepam, flunitrazepam, midazolam, bromazepam, oxazepam, chlordiazepoxide, temazepam, lorazepam, clonazepam, alprazolam, triazolam, α-OH-Alprazolam, α-OH-Triazolam, amitriptyline, nortriptyline, promethazine, codeine, verapamil, oxycodone, chlorpheniramine, brompheniramine, doxepin, clomipramine, ketamine, venlafaxine, cyclobenzaprine, sertraline, fentanyl, haloperidol, imipramine, desipramine, chlorpromazine, trazodone, mepridine, phencyclidine, tramadol, mirtazepine, clozapine, metoprolol, paroxetine, and zolpidem were purchased from Cerilliant Corporation, USA. Synthetic negative blood, benzodiazepines and opiate ELISA kits were purchased from Immunalysis Corporation, Pomona, CA. De-ionized water was used in this work.

2.2 Sample Preparation

2.2.1 Confirmation for Benzodiazepines

For the confirmation of benzodiazepines using GC/MS, putrefied material was diluted and homogenized with distilled water. After the addition of 150 μL of desalkyl Flurazepam (methanolic solution of 2 μg/mL as internal standard) to a 2 mL homogenized sample (or spiked sample of drugs listed in Table-1), 2 mL of ammonium chloride/ammonium hydroxide buffer solution was added. Saturated aqueous solution of ammonium chloride adjusted to pH 9.2 with concentrated ammonium hydroxide was used as buffer solution. Samples were extracted with 5 mL of 1-chlorobutane. Back extraction of samples was performed in aqueous layer by using 2 M sulfuric acid (2 mL). Acidic aqueous layer was finally basified with ammonium hydroxide (500 μL) and re-extracted into 1-chlorobutane (5 mL). The organic layer was separated, dried under stream of nitrogen and derivatized with 50 μL of MTBSTFA.

2.2.2 Screening for basic drugs

For the extraction of basic drugs, homogenized putrefied material (2.0 mL) was sampled in a 15 mL polypropylene tube. After the addition of 50 μL of caffeine (methanolic solution of 100 μg/mL as internal standard), the sample (or spiked sample of basic drugs as in section 2.3) was basified with 0.1 M sodium hydroxide solution (1 mL) and extracted with 5 mL of 1-chlorobutane. Samples were then back extracted in aqueous layer by using 2.0 mL of 2 M sulfuric acid. Acidic aqueous layer was finally basified with ammonium hydroxide

Table 1- *Analytical parameters of GC/MS for benzodiazepine confirmation*

	GC (7890A)		
Inlet	Split less		
Inlet Temperature	275 °C		
Carrier gas	Helium (99.999%)		
Inlet Pressure	9.87 psi		
Column	DB-5ms capillary column (30 m x 0.25 mm, 0.25 μm)		
Oven	150 °C (for 1 min)→ 50 °C/min → 250 °C (for 2 min) 6 °C/min → 310 °C (5 min), total Run : 20 min		
	MS (5975 C)		
Transfer line	280 °C		
Mode	SIM		
	SIM Parameters		
Group	**Drug name**	**Start time (min)**	**Ions**
1	Nor-diazepam	0.00	327, 329, 383
	Diazepam		256, 283, 284
	Desalkylflurazepam (IS)		345, 347
	Flunitrazepam		312, 285, 286
	Midazolam		310, 312, 325
2	Bromazepam	10.12	374, 372, 346
	Oxazepam		457, 459, 513
	Clordiazepoxide		356, 282, 358
	Temazepam		357, 283, 255
	Lorazepam		491, 513, 493
	Clonazepam		372, 374, 326
3	Alprazolam	14.02	279, 204, 308
	Triazolam		313, 342, 238
	α-OH-Alprazolam		381, 382, 383
	α-OH-Triazolam		415, 416, 417

Table 2- *Analytical parameters of GC/MS for basic drug screen*

	GC (7890A)
Inlet	Split less
Inlet Temperature	280 °C
Carrier gas	Helium (99.999%)
Inlet Pressure	14.69 psi
Column	DB-5ms capillary column (30 m x 0.25 mm, 0.25 μm)
Oven	100 °C (for 1 min)→10 °C/min → 325 °C (6.5min), total Run : 30 min
	MS (5975 C)
Transfer line	280 °C
Mode	Scan
Scan Range	50-550 m/z

(500 μL) and re-extracted into 5 mL of dichloromethane. The organic layer was separated, dried under a stream of nitrogen and reconstituted with 50 μL of isopropanol.

2.2.3 Confirmation for poisonous gases

In order to confirm the presence of phosphine and cyanide in putrefied materials, about 1.0 mL of homogenized sample (or spiked sample of potassium cyanide and zinc phosphide) was added into a 20 mL clear glass headspace vial (Agilent Technologies, USA). The vial was quickly sealed after acidification with 5 mL of 10 M Sulfuric acid. The sample was analyzed by HS-GC/FID (7890A GC System, Agilent technologies, Palo Alto, CA) in a sequential analysis mode. Agilent GC Chemstation was used for the data analysis in external standard mode.

2.3 Instrument Conditions

Exhumed specimens were analyzed by using a Gas Chromatograph (7890A GC System, Agilent technologies, Palo Alto, CA) interfaced with a mass spectrometer (5973 quadruple Agilent technologies, Palo Alto, CA) and 7963 auto sampler. In addition, static head space (SHS) GC coupled with a flame ionization detector (FID) (Agilent technologies, Palo Alto, CA) was used for the confirmation of poisonous gases. Tables-1-3, describes the instrument conditions and analytical parameters used for the analysis. The selected samples were screened for the presence of drugs of abuse (benzodiazepines and opiates) using ELISA and confirmed by GCMS in SIM mode (Table-1).

Basic drugs (amitriptyline, nortriptyline, promethazine, codeine, verapamil, oxycodone, chlorpheniramine, brompheniramine, doxepin, clomipramine, ketamine, venlafaxine, cyclobenzaprine, sertraline, fentanyl, haloperidol, imipramine, desipramine, chlorpromazine, trazodone, mepridine, phencyclidine, tramadol, mirtazepine, clozapine, metoprolol, diazepam, paroxetine, midazolam, zolpidem and alprazolam) were screened by GCMS (Table-2) after liquid-liquid extraction with organic solvents. Screening of poisonous gases (phosphine and cyanide) [11] was performed by colorimetric technique and later confirmed by GC/FID (Table-3) [12] on the same day in order to avoid gaseous loss from the sample, if any.

3. Case Reports

3.1 Case Report-1

A 30-year-old man was involved in a road traffic accident and was brought to the hospital for treatment. He

Table 3- *Analytical parameters of GC/FID for phosphine and cyanide confirmation*

	GC (7890A)
Loop size	1 mL
Vial Pressure	8.5 psi
Oven	60 °C
Loop temperature	70 °C
Transfer line temperature	80 °C
Vial equilibration time	7.0 min
Pressurization	0.2 min
Loop fill time	0.2 min
Injection time	0.5 min
	MS (5975 C)
Inlet	Split
Split ratio	1:1
Inlet Temperature	200 °C
Carrier gas	Nitrogen (99.999%)
Inlet Pressure	160 KPa
Column	HP-Innovax (Polyethylene glycol bonded) column (30 m x 0.25 mm, 0.25 μm)
Oven	40 °C →16 °C/min → 120 °C, total Run : 5.0 min
	FID
Flame temperature	300 °C
Hydrogen flow	30 mL/min
Air flow	400 mL/min

died after one day of hospitalization and was buried on the same day without postmortem analysis. Due to the suspicion of homicide, exhumation of the corpse was performed after 46-days of the burial. The corpse was at an advanced stage of putrefaction. After the postmortem examination, putrefied material, skin, tissue, hair and control samples of soil were collected and sent for chemical analysis to the authors' laboratory. Putrefied material from the corpse was selected for toxicological examination.

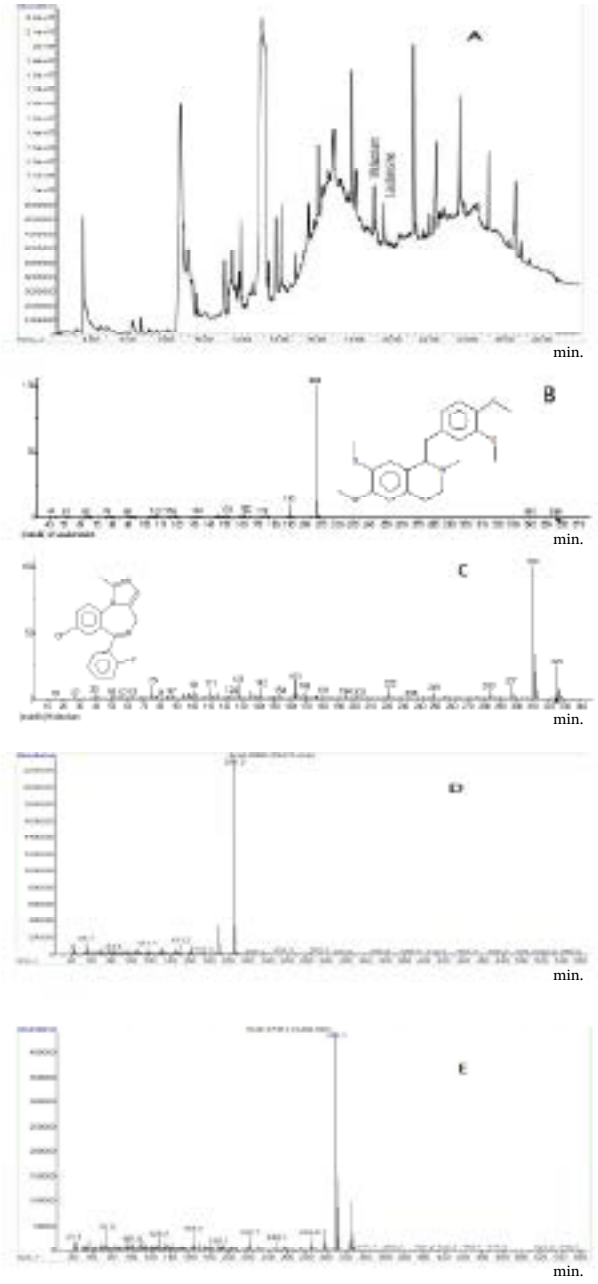

Figure 1- *A-TIC Chromatogram of putrefied material (Case Study 1), Midazolam (19.261) and Laudanosine (19.675 min), B- Library (NIST) matched spectra of Laudanosine, C- Library (NIST) match spectra of Midazolam, D- Spectra of Laudanosine from putrefied material of case study-1, E- Spectra of Midazolam from putrefied material of case study-1*

3.2 Case Report-2

A 29-year-old male was shot and he died after 10-days of hospitalization. His body was buried without postmortem examination. The corpse was exhumed after eight months of burial for postmortem analysis, and the medicolegal officer requested a chemical examination of the specimen. The Authors' laboratory received a piece of coffin, some soil from the grave, putrefied material from the abdominal area, hair, nail, putrefied muscles, skin, ulna, radius and a control sample. Putrefied material from the abdominal area was subjected to toxicological examination.

4. Results and Discussion

Hair, nail, teeth and putrefied materials are the most commonly submitted exhumed samples in addition to soil samples [6]. Selection of soil samples is vital in the absence of hair and nail samples where suspicion of heavy metal poisoning cannot be overruled. Control soil samples are then used to rule out the natural presence of heavy metals in soil samples. Hair is the first choice for the detection of heavy metals (mercury, lead and arsenic etc.) and certain drugs along with their metabolites as they may accumulate in hair after chronic use [13-14]. Quantitation of drugs is not possible in exhumed specimens because of the sample degradation, except in appropriately collected hair samples where section wise analysis provides reliable results [14].

Sometimes, only the bony skeleton is available after exhumation. Gautam et al. have detected morphine, diazepam, ketamine, fentanyl, amitriptyline and olanzapine in bones [15]. Certain in-vitro studies have shown that most drugs can be detected both in blood and bone tissues, but there is a need to develop the correlation of drug concentrations in the tissue and the blood. In some cases however, drugs have been found only in bones but not in blood [15], and the correlation of drug concentration in both tissues cannot be developed. There is still a need to do more work as very limited data is available regarding the concentration of drugs in bone [16]. As for drug analysis in buried human bones, authors were only able to find one study regarding the detection of morphine from the bone sample of a heroin addict [17]. Owing to the availability of very limited research on bones, detection of any drug in bone can only be corresponded as the evidence of exposure to that drug. Results of quantitation of drugs from bony tis-

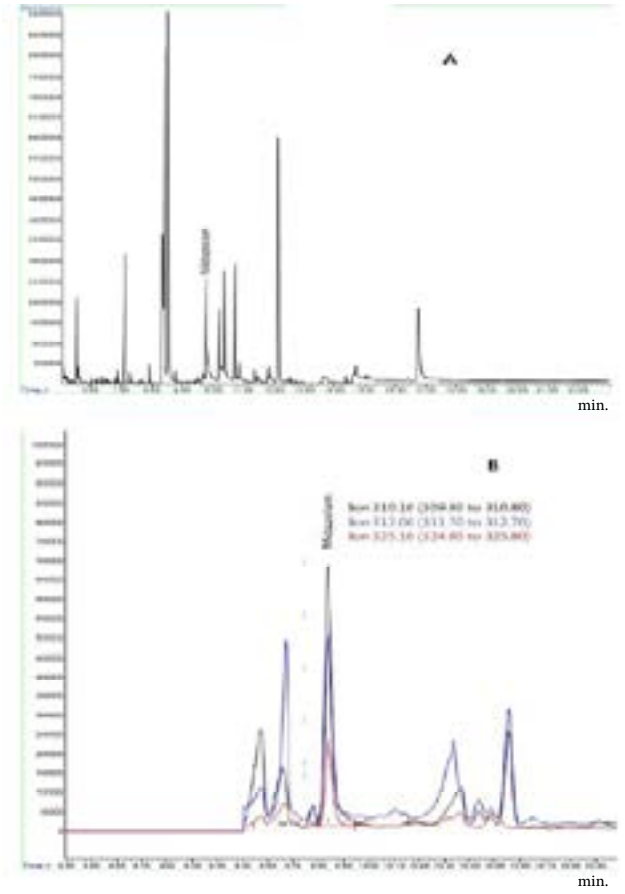

Figure 2- *A- SIM mode TIC Chromatogram of putrefied material of abdomen (Midazolam at 9.839 min) of the case study 2, B- SIM ions abundance of Midazolam in putrefied material of abdomen, the case study 2.*

sues can be compromised as there are multiple factors (collection method, extraction procedure, analytical techniques and storage conditions) that can produce variable results from the same sample. Further work is required to relate the blood levels of the drugs with the concentration in bone [16].

In both of the case studies, the deceased were hospitalized after the injuries, providing the clue for the detection of certain drugs used during their treatment. No drug of abuse was detected when samples were screened with ELISA. In case study 1, laudanosine (metabolite of atracurium) and midazolam (benzodiazepine) were detected in putrefied material when screened with GCMS (Figure-1). The results were contrary to the findings of the ELISA screening where it was negative for the class of benzodiazepines.

In case study 2, putrefied material from the abdominal region was subjected to confirmation of benzodiazepines using GC/MS in SIM mode (Figure-2). The specimen was confirmed to contain midazolam (Figure-2) while the screening test was negative during ELISA procedure. The selected sample also tested false positive for the presence of phosphine and later found negative when confirmed by using GC/FID (Figure-3). In this case study, soil, hair and bone samples were not used due to the nature of injuries and death. The case corresponds to death after hospitalization and rules out the possibility of chronic heavy metal exposure as the cause of death.

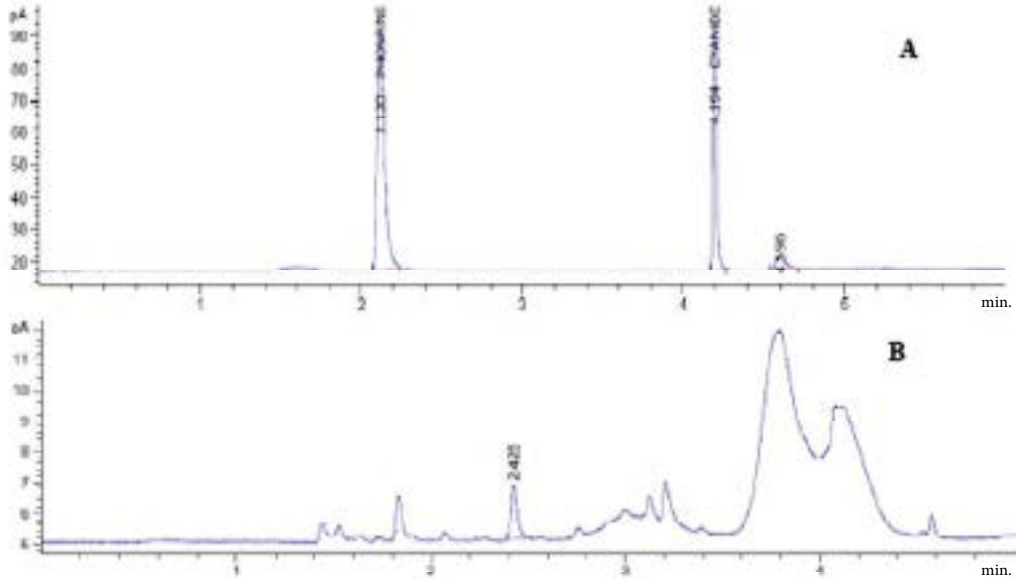

Figure 3- *A- FID chromatogram of standard of phosphine and cyanide, B- FID chromatogram of putrefied abdominal material from case study 2.*

In both of the described cases, midazolam was detected by GCMS while it was not detected at the screening stage. Midazolam and atracurium were used during the surgical procedure of the hospitalized patients. Atracurium besylate injections are used for skeletal muscle relaxation during surgery [18], and its metabolite (laudanosine) was detected in case study-1 as shown in Figure-1.

5. Conclusion

Usually, after the negative immunoassay and colorimetric screening, confirmation procedure is not required. It is concluded from the study that common immunoassay and colorimetric techniques may not reveal the correct results of exhumed specimens owing to the matrix interferences. In such cases, extraction (liquid/liquid or solid phase) of the putrefied samples and screening by GC coupled with MS or FID detectors is a suitable choice for the detection of any analyte.

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