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Application of a chemiluminescence immunoassay system and GC/MS for toxicological investigations on skeletonized human remains

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ABSTRACT

Hair, larvae and cardiac muscle, the only biological samples present on a skeletonized human body found in a rural area, were used for forensic toxicological analyses in order to determine possible causes of death. Since no information about the victim or the circumstances of death was available (except for the place where the corpse was found, known to be a gathering place for drug addicts), the first approach for the analysis of non-conventional matrices involved the screening of different classes of active principles, using a chemiluminescence-based screening assay designed for whole blood. The immunoassay test results showed positivity to amphetamines, cocaine and opiates on water/methanol extract from cardiac tissue, larvae and hair samples. Gas chromatography–mass spectrometry (GC/MS) analyses confirmed the immunoassay results, except for amphetamines. The minimal sample preparation (hydration and extraction in an ultrasonic bath), the reduced sample volume required for the analyses, together with the correctness of results as confirmed by GC/MS, showed the suitability of the screening test for forensic applications on non-conventional matrices. Quantitative analyses in GC/MS allowed the cause of death to be ascertained on the basis of the ratio between parent drugs and metabolites.

1. Introduction

It can be extremely challenging to ascertain the cause of death when the body is totally or partially skeletonized, especially if the causes of death are to be found in soft tissue, which commonly applies in the case of sudden death. In such cases, traditional biological specimens are commonly not available and alternative matrices, such as larvae, fragments of tissues or organs and hair, assume extreme importance. Determination of the colonization interval of a corpse ("postmortem interval") has been a major issue for forensic entomologists since the 19th century [1,2]. For the above "classical" purpose, larvae may now be seen as the substrate for toxicological analyses: in recent decays, sensitivity and specificity of mass spectrometry-based methods have allowed the analysis of larvae and/or insects to evidence the possible presence of drugs that could be contributory causes of death [3–5]. Larvae are of particular interest, since a drug can still be present and detected in insects that have fed on a corpse, while it may no longer detectable in organs or tissue remains. During the period between 1977–2016 over 60 papers were published on the detection of toxicants, as reviewed by Da Silva et al. [6]. Nevertheless, the possibility of referring findings from alternative matrices to causes of death are, in a certain way, limited to the availability of published data that can provide support with the interpretation of results, with particular attention to pharmacological and physiological mechanisms regulating drug disposition in the species of interest.

Mass spectrometry-based methods allow unique and irrefutable identification of certain chemicals, while immunochemical methods offer the possibility to perform rapid and less expensive screening of several classes of substances [4,7]. Moreover, immunoassay screening tests are less sample-consuming and require reduced sample pretreatment. Consequently, immunoassays focusing on the accuracy of determinations of different drugs in typical forensic matrices have been widely tested [4,6–11]. The possibility of performing forensic toxicological screening on alternative matrices is limited due to the difficulty in testing their efficacy on real samples.

Recently, the legal authorities ordered the forensic autopsy and toxicological analysis of skeletonized human remains. This case gave us the opportunity to verify the applicability of an analytical strategy for the analysis of non-conventional matrices, based on

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chemiluminescence immunoassay for the rapid screening of different classes of drugs, followed by confirmation analyses in gas chromatography/mass spectrometry. GC/MS data also allowed the cause of death to be determined.

2. Case report

Skeletonized remains of an unknown subject were found in a field, known to be a gathering place for drug addicts. The discovery of the body followed an anonymous call to police. During inspection of the area, the police recovered an empty woman's bag worn over the body. In one of the outside pockets of the bag there was an empty jar of hand cream containing a needle. The bag also contained a pink plastic watch. Two bracelets (one of dark rubber and one of steel), together with a clasp knife, two lighters and a syringe (fitted with a needle), were found on the scene after body removal.

External examination revealed the absence of appreciable injury. The corpse was in an advanced state of decomposition due to the action of Diptera larvae still present on the skeletal remains. Results of anthropological analyses (data not shown) evidenced that the corpse was that of a young adult male (between 18 and 25 years old), rather thick-set, of medium-low height (172 cm ± 3.5 cm). The corpse presented few dental elements in both arches, all characterized by peculiar erosion of the mesial and lateral margins of the upper anterior teeth (central incisors), presumably due to dental decay or to an erosive process of another nature. The body presented a deformed left collarbone, probably due to a previous fracturing process, lacking the acromial portion.

The corpse presented only a few cutaneous shreds on the back, where small channels, compatible with the action of larvae, were clearly visible. Furthermore, there were fragments of paravertebral, parasternal, and proximal femoral quadriceps, strongly altered by decomposition processes. The heart within a residual pericardial sac was the only recognizable organ and presented an altered flattened and dried shape. The almost complete skeletonization and the action of micro and macro fauna did not allow accurate estimation of the post-mortem interval, presumably dating back at least 30 days, also in the light of climate conditions (the corpse was found in late summer). Complete toxicological analyses were performed on hair, larvae and on the completely dehydrated cardiac muscle.

3. Material and methods

Certified standard solutions of drugs of abuse used for confirmation analysis in gas chromatography/mass spectrometry (GC/MS) were from Cerilliant (Sigma-Aldrich, Milan, Italy), N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) and heptafluorobutyric acid (HFBA) derivatizing agent from Acros (Morris Plains, NJ, USA), and HPLC grade-solvents from Carlo Erba (Milan, Italy).

GC/MS analyses were performed using a DSQII single quadrupole mass spectrometer directly linked to a TraceGC 2000 series gas chromatograph equipped with a *split-splitless* autosampler AS 3000, all from ThermoFisher (San José, CA, USA). Gas chromatographic separations were performed with a Rxi®-5MS (30 m × 0.25 mm × 0.25 μm) capillary column (Restek, Bellefonte, PA, USA). Data were processed using the Xcalibur software (version 2.0.7) from ThermoFisher.

Immunochemical screening tests were performed on Randox Evidence Investigator (Randox Toxicology. Country Antrim, UK), using DoA I + WB SQ and DoA II WB whole blood tests for AMP/MAMP/MDMA, barbiturates, benzodiazepines, buprenorphine, cannabinoids, cocaine, methadone, opiates, phencyclidine, tricyclic antidepressants, fentanyl, ketamine, LSD, methaqualone, oxycodone and propoxyphene.

3.1. Immunochemical screening test

3.1.1. Sample preparation for chemiluminescence immunoassay

3.1.1.1. Larvae. Larvae were gently washed with bidistilled water to remove dirt and soil debris. Aliquots of 150 mg were added with 2 mL of methanol and put in ultrasonic bath overnight. After filtration, organic extract, dried under nitrogen stream, was reconstituted using 200 μL of the specimen dilution buffer of the immunoassay kit.

3.1.1.2. Cardiac tissue. In an attempt to obtain a blood surrogate, after addition of 250 mL of bidistilled water the entire organ was placed in ultrasonic bath for 15 min in order to dissolve the dried blood present on the organ. The liquid was filtered under vacuum, stored at −20 °C and used for subsequent toxicological analyses. Immunoassay tests were performed on 100 μL aliquot, processed according to immunoassay system specifications.

3.1.1.3. Hair. Hair was washed by adding water until complete coverage; samples were gently vortexed, and the liquid discharged. The procedure was repeated until clear waste water was obtained. The washing step was repeated with acetone. Hair was left at room temperature until dried and finely cut. Aliquots of 50 mg were used for immunoassay tests; the sample was prepared following the same procedure used for larvae (2 mL methanol, overnight in ultrasonic bath, organic extract dried and reconstituted in specimen dilution buffer).

For all the tested matrices, screening tests were performed according to the manufacturer's specifications for whole blood samples, after specimen preparation as specified above. The immunochemical system used an enhanced chemiluminescent substrate with a horseradish peroxidase label for the detection of antibodies or analytes bound to the biochip surface of the device. Although the immunoassay results were in terms of semi-quantitative data, given the nature of specimens analyzed, the results are reported in terms of positive/negative.

3.2. GC/MS analysis

3.2.1. Sample preparation for GC/MS analyses

3.2.1.1. Larvae. Larvae were gently washed with bidistilled water to remove dirt and soil debris. Aliquots of 360 mg were added with 2 mL bidistilled water, and placed in ultrasonic bath overnight. After filtration, the liquid fraction was recovered, spiked with internal standard and pH-adjusted. Finally, samples were purified by solid phase extraction (SPE) specific to amphetamines, cocaine and opiates (see below).

3.2.1.2. Cardiac tissue. GC/MS analyses were performed on "blood surrogate", obtained as specified above (see cardiac tissue sample preparation for chemiluminescence immunoassay). Five-mL aliquots of filtered liquid spiked with deuterated internal standards were purified by SPE for detecting amphetamines, cocaine and opiates (see below).

3.2.1.3. Hair. Hair was washed as described for the chemiluminescence immunoassay and 50 mg aliquots were used for GC/MS analyses, involving the use of deuterated internal standards and specific hydrolysis and SPE purification (see below).

Biological samples, spiked with the appropriate deuterated internal standards and pretreated as specified above, underwent

SPE purification specific to cocaine and benzoylecgonine, opiates, amphetamines and benzodiazepines (see below).

3.2.1.4. Cocaine, benzoylecgonine and free morphine. Aliquots of larvae and cardiac tissue, pretreated as described above, were spiked with internal standards (d_3-cocaine, d_3-benzoylecgonine and d_3-morphine), and pH-adjusted to 8–9 with 10 M KOH saturated with $KHCO_3$ prior to SPE extraction.

Aliquot of washed hair sample, with the addition of 1 mL of 0.1 HCl, underwent acidic hydrolysis at 45 °C overnight for cocaine and benzoylecgonine quantification. The sample was added with to 2 mL of 0.1 M phosphate buffer (pH 6) and 2 N NaOH until pH 8–9. It was then centrifuged (2000 rpm for 5 min) prior to SPE extraction.

SPE extraction, performed with Screen-C cartridges (200 mg/3 mL), involved drop-to-drop elution at 5 mmHg and the following extraction procedure. Conditioning: 2 mL methanol and 2 mL 0.1 M phosphate buffer (pH 6). Sample loading. Washing: 6 mL bidistilled water; 3 mL 0.1 N HCl; 9 mL methanol and cartridges left to dry for 5 min. Elution: 4 mL (dichloromethane/methanol = 8/2, 2% ammonia, v:v). Eluted fraction, dried under nitrogen stream, was derivatized by adding 50 μL BSTFA and the samples left at 75 °C for 20 min.

3.2.1.5. Total morphine. Aliquots of pretreated larvae and cardiac tissue, spiked with internal standard (d_3-morphine), underwent acidic hydrolysis. After addition of 200 μL of 37% HCl the samples were left at 120 °C for 20 min. They were then cooled to room temperature and pH-adjusted to 8–9 by adding 800 μL 2 M Tris buffer (pH 8) and 200 μL 10 M KOH saturated with $KHCO_3$. The samples were centrifuged (4000 rpm for 40 min) and purified by SPE as described above (see "Cocaine, benzoylecgonine and free morphine" subsection).

Aliquot of hair sample was hydrolyzed and purified as described above (see "Cocaine, benzoylecgonine and free morphine" subsection) for total morphine quantification.

3.2.1.6. Amphetamines. Aliquots of larvae and cardiac tissue, pretreated as described above, were spiked with internal standards (d_6-amphetaine, d_5-methamphetamine and d_5-MDMA), and pH-adjusted to 6 with 0.1 M phosphate buffer, and then centrifuged (4000 rpm for 20 min). Purification through SPE extraction involved the use of BondElute Certify (200 mg/3 mL) cartridges, with the drop-to-drop elution at 5 mmHg and the following extraction procedure. Conditioning: 2 mL methanol and 2 mL 0.1 M phosphate buffer (pH 6). Sample loading. Washing: 1 mL 1 M acetic acid and cartridges were dried for 5 min. 6 mL methanol and cartridges were dried for 5 min. Elution: in a test tube with the addition of 50 μL (methanol/HCl = 9/1): 4 mL (isopropanol/dichloromethane/ammonia = 78/22/2, v:v). Eluted fraction, dried under nitrogen stream, was derivatized by adding 100 μL HFBA and the samples were left at 70 °C for 20 min.

3.2.1.7. Benzodiazepines. Aliquots of larvae and cardiac tissue, pretreated as described above, were spiked with internal standard (d_5-prazepam), and pH-adjusted to 6 with 0.1 M phosphate buffer. An aliquot of washed hair sample, spiked with internal standard (d_5-prazepam) was added with 1 mL methanol and underwent hydrolysis at 45 °C overnight. All samples were diluted with 3 mL 0.1 M phosphate buffer, then mixed/vortexed for 5 min and centrifuged (4000 rpm for 40 min).

Purification through SPE extraction involved the use of Screen C (200 mg/3 mL) cartridges, with the drop-to-drop elution at 5 mmHg and the following extraction procedure. Conditioning: 3 mL methanol and 3 mL 0.1 M phosphate buffer (pH 6). Sample loading. Washing. 3 mL (5% acetonitrile in 0.1 M phosphate buffer (pH 6), v:v) and cartridges were dried for 5 min. 3 mL hexane and cartridges were dried for 5 min. Elution. 6 mL (ethyl acetate/ammonia = 98:2, v:v). Eluted fraction, dried under nitrogen stream, was derivatized by adding 50 μL BSTFA and the samples were left at 75 °C for 20 min.

All samples were analyzed in GC/MS *full scan* mode and quantified by MS-Single Ion Monitoring (MS-SIM) mode (three ions characteristic of each analyte were acquired).

4. Results

4.1. Forensic toxicological analyses

Results of screening tests on larvae, cardiac tissue and hair are reported in Table 1. The larvae resulted positive to benzodiazepines, cocaine and opiates; cardiac tissue showed a positivity to cocaine and opiates; hair was positive to benzodiazepine, cocaine and opiates. Moreover, immunochemical assay evidenced a positivity to amphetamines for all tested matrices.

GC/MS-SIM analyses confirmed positivity to cocaine and morphine on all matrices; positivity to temazepam and diazepam was also recorded, while immunoassay positivity to amphetamines was not supported by GC/MS-SIM confirmation. Quantitative analysis, performed in SIM mode by using a specific calibration curve, evidenced results reported schematically in Table 2. In the case of morphine, GC/MS analyses on larvae and cardiac tissue were repeated with and without acidic hydrolysis to quantify total and free morphine, respectively. Results obtained with and without hydrolysis are almost superimposable both for larvae and cardiac tissue (see Table 2), thus evidencing that in both specimens positivity to morphine can be traced back to almost free morphine.

GC/MS *full scan* analyses on all specimens showed no further toxicologically relevant analyte.

5. Discussion

The need to investigate the presence of drugs of abuse or other chemicals in highly decomposing body or skeletal remains, as well as ascertain their role as a contributory factor to the cause of death of the subject, represents the drive to perform toxicological analyses on larvae and other non-conventional matrices, such as tissues or organ remains. After encouraging results obtained from the early 1980s onwards, in 1991 Pounder [5] coined the term "*forensic entomotoxicology*" to define the branch of forensic toxicology focusing on the use of insect specimens as indirect

Table 1
Results of chemiluminesce immunoassay tests on larvae, cardiac tissue and hair.

	Larvae	Cardiac tissue	Hair
AMP	+	+	–
MAMP	+	+	+
MDMA	+	+	+
Barbiturates	–	–	–
Benzodiazepines 1	+	–	+
Benzodiazepines 2	+	–	–
Buprenorphine	–	–	–
Cannabinoids	–	–	–
Cocaine	+	+	+
Methadone	–	–	–
Opiates	+	+	+
Phencyclidine	–	–	–
Tricyclic Antidepressants	–	–	–
Fentanyl	–	–	–
Ketamine	–	–	–
LSD	–	–	–
Methaqualone	–	–	–
Oxycodone	–	–	–
Propoxyphene	–	–	–

Table 2
GC/MS-SIM quantitative results for cocaine, morphine and benzodiazepines on larvae, cardiac tissue and hair.

	Larvae (ng/mg)	Cardiac tissue[a] (ng/ml)	Hair (ng/mg)
Cocaine			
Cocaine	5.35	328	43.24
Benzoylecgonine	17.32	509	0.74
Benzodiazepines			
Temazepam	N.D.	N.D.	0.02
Diazepam	N.D.	N.D.	0.03
Morphine			
Free-morphine	2.98	208	–
Total-morphine	3.15	211	1.73

[a] Analyses were performed on aqueous extract of the entire organ, in order to attempt a "blood surrogate", see "Sample preparation for chemiluminescence immunoassay" section.

matrices to elucidate drug-related deaths in all those cases where fluids were no longer available. As recently reviewed by da Silva et al. [6], one of the main aims of forensic entomotoxicology is to verify the possible role of a certain drug in contributing to the cause of death by performing toxicological analyses of insects/larvae found on the body. Moreover, verifying the presence of drugs is extremely useful also for the "traditional" forensic application of entomotoxicology, i.e. estimation of time-since-death. Drugs can, indeed, influence the development rate of insect species [12,13]. While several authors have reported results of toxicological analyses performed on larvae by chromatographic/mass spectrometric methods and a considerable database is available [6,14], the possibility of applying rapid immunochemical screening to alternative matrices has been less extensively investigated.

In the present paper, an immunochemiluminescent assay was applied to the rapid screening of extracts from larvae, cardiac tissue and hair, the only biological matrices available for the forensic toxicological analyses of a skeletonized human body. For all the specimens considered, sample pretreatment was reduced to the minimum, since the water/methanolic extracts were directly applied to the chip of the screening device. Although the device offered semi-quantitative data, results were considered in terms of positive/negative.

As expected, a positivity towards amphetamines was recorded for all tested matrices and not confirmed by GC/MS analyses. Putrefactive amines (phenylethylamine, putrescine, tryptamine, tyramine) may cross-react with immunoassay tests for amphetamines [15]. Moreover, different medication classes present in clinic formulary, such as antihistamines (brompheniramine, phenulpropanolamine or nonprescription nasal inhalers), antidepressants (bupropion, trazodone), antipsychotics (chlorpromazine, promethazine), or other agents (ranitidine, ephedrine), have been identified as potential sources of cross-reactivity with amphetamine/methamphetamine screening tests [16].

Immunoassay positivity recorded for hair extract to the benzodiazepine test was confirmed by GC/MS, evidencing the presence of diazepam and temazepam. Confirmation analyses for benzodiazepines were also repeated on cardiac tissue and larvae samples, and resulted negative. Positivity to the BENZO1 test recorded for hair sample, with a negative result to BENZO2, is in line with assay specificities declared by the manufacturer. The main target of BENZO1 is oxazepam (100% cross-reactivity, %CR); while BENZO2 is designed for lorazepam (100% CR). Among the tested congeners, diazepam and temazepam show high affinity to BENZO1 alone (256% CR and 382% CR, respectively), while no significant cross-reaction (<0.1% CR) is reported for BENZO2 [17]. Thus our results showed the suitability of the immunoassay for the rapid screening of benzodiazepines, whose presence was correctly evidenced in hair extracts. Moreover, performing both benzo tests gives useful indications about the specific drug(s) present in the sample.

In the case of cardiac tissue extract, GC/MS results confirmed that of the immunoassay, thus supporting the hypothesis that the man in question was not under the effect of benzodiazepines when he died. GC/MS analyses on larvae extracts did not confirm the immunoassay positivities. Such a discrepancy could be attributed to the inadequacy of the immunotest for the specimen or to the presence of metabolites (mainly conjugates) that can be detected by the screening test and not evidenced by GC/MS, since no enzymatic hydrolysis was performed.

The results obtained for the cocaine immunoassay evidenced positivity in all tested matrices. Checking the test cross-reactivity, it may be noted that the cocaine immunoassay is designed to react with benzoylecgonine, BE, (100% CR), with poor affinity to cocaine (2.9% CR). Results of GC/MS analyses evidenced higher BE concentrations in all specimens. Thus, also cocaine immunoassay is suitable for the analysis of alternative matrices. Data obtained on larvae and cardiac tissue strongly suggest that the subject was under the effect of cocaine shortly before he died. GC/MS data can only be discussed as a ratio between the parent drug and the metabolite, due to the well known intra-individual variability to cocaine toxicity, the absence of standardized protocols to prepare the available matrices and, in the case of cardiac tissue extract, the complete absence of any literature data. Ratios between cocaine (COC) and BE were 0.309 and 0.644 for larvae and cardiac tissue, respectively. Rees et al. [18] reported cocaine concentrations measured in cardiac and femoral muscles in nine cases, and compared results to blood (femoral and cardiac) and vitreous humor. Ratios between COC and BE in the range 0–0.32 were obtained for skeletal muscle (median 0.14), while values between 0–0.47 (median 0.09) were obtained for cardiac muscle [18]. Considering these values as indicative of a cocaine-related death (blood ratio was: 0–0.53, median 0.12 for cardiac blood; 0.02–0.1, median 0.07 for femoral blood) [18], it may be assumed that the case discussed herein concerns a death resulting from cocaine.

Results of the immunoassay tests evidenced a positivity of all specimens to opiates, which was confirmed by GC/MS analyses. In the case of larvae and cardiac tissue extracts, confirmation analyses performed with and without acidic hydrolysis evidenced similar free- and total-morphine concentrations in all samples, thus showing that morphine (the target molecule of the opiate immunoassay) was the more abundant species. Such results, similarly to those obtained for cocaine, suggest that the subject survived for a short time after drug assumption. As reported by Darke and Duflou, *lower blood concentrations of morphine 3- and 6-glucuronides, and lower ratios of these conjugates to free morphine, are suggestive of a more rapid death, as there has been less time for the metabolism of morphine to occur* [19].

Finally, the results obtained for hair confirmed that the subject was polydrug-addicted. Considering also circumstantial data (the body was found close to a district known to be a gathering place for drug addicts), the medical examiner concluded that death was probably caused by an overdose of cocaine and opiates in a poly-addicted subject.

6. Conclusions

To the best of our knowledge, the present paper is the first to report results of a chemiluminescence-based immunoassay for the forensic screening of extracts of larvae, cardiac tissue and hair sampled from a skeletonized body. Although confirmation analyses, performed in GC/MS, were mandatory, data from immunotesting were useful to set up the GC/MS-SIM analyses. No further analytes were detected, except for those resulting from

screening, evidencing good specificity of the screening tests. Finally, comments on the parent drug/metabolite ratio, quantified by gas chromatography/mass spectrometry, allowed identification of the possible cause of death, attributed to cocaine and opiate acute toxicity in a poly-addicted subject.

Conflict of interests

None.

CRediT authorship contribution statement

P. Basilicata: Formal analysis, Conceptualization. **M. Pieri:** Conceptualization, Writing - review & editing. **A. Simonelli:** Formal analysis. **D. Faillace:** Investigation. **M. Niola:** Funding acquisition. **V. Graziano:** Investigation, Conceptualization.

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