Analysis of EDTA in Dried Bloodstains

1 Introduction

The collection of blood at crime scenes and for legal proceedings is a common practice that may be used to inculpate or exculpate individuals suspected of being involved in the crime. Occasionally, there are allegations that blood evidence collected from crime scenes was "planted". This issue may be resolved by the determination of exogenous components in the bloodstains (e.g. preservatives) that should not be present in authentic crime scene evidence.

Ethylenediaminetetraacetic acid (EDTA) is an anti-coagulant and enzyme inhibitor that is commonly used in blood specimen collection tubes. Blood specimen collection tubes containing EDTA have lavender-colored tops and are the most common collection tube used to collect reference specimens for DNA testing. Therefore, most allegations of blood evidence "planting" focus on EDTA-preserved blood samples.

EDTA-preserved blood tubes use either the disodium, dipotassium, or tripotassium salt forms of EDTA. The concentration of EDTA in its free acid form in a drawn blood tube is typically 1000-2000 mg/L, depending on the volume of blood and the capacity of the tube. At this concentration, the free acid and salt forms of EDTA are soluble in the blood. EDTA readily forms water-soluble chelates with nearly all heavy metals, so aqueous extractions of dried bloodstains should isolate EDTA.

2 Scope

This procedure allows for the screening and confirmation of EDTA in suspected blood stains.

3 Principle

This method takes advantage of the water solubility of EDTA and EDTA-complexes. A dried bloodstain is first extracted with deionized water and then subjected to ultrafiltration. Following ultrafiltration, the filtrate is analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) in both the positive and negative electrospray ionization (ESI) modes.

4 Specimens

This procedure can be used for assaying bloodstains from a cotton swab or other cotton-based matrices.

5 Equipment/Materials/Reagents

Guidance for the preparation of reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

a. Liquid chromatograph/mass spectrometer equipped with a Hamilton PRP-1 polymeric column (2.1 mm x 150 mm x 5µm) or equivalent

b. Laboratory scissors

c. Millipore Amicon Ultra-4 10,000 Molecular Weight Cutoff Centrifugal Filter Device

d. Wheaton (or similar) pipette – 200 µL

e. Centrifuge

f. EDTA-preserved whole blood

g. EDTA-free whole blood

h. Deionized water

i. Acetonitrile (HPLC grade)

j. Ammonium Hydroxide (HPLC grade)

k. Deionized Water (95%) / Acetonitrile (5%) / Ammonium Hydroxide (0.06%) – Mobile Phase for Positive Electrospray Ionization

l. Acetonitrile (80%) / Deionized Water (20%) / Ammonium Hydroxide (0.03%) – Mobile Phase for Negative Electrospray Ionization

m. Common laboratory supplies such as glassware, Pasteur pipettes, etc.

6 Standards and Controls

a. EDTA LC/MS/MS(ESI) Performance Mix – 100 µg/mL:
Accurately weigh 12.7 mg of the disodium salt of EDTA (reagent grade, Aldrich) and dilute with deionized water to a final volume of 100 mL. Store at room temperature in a clear glass container. Stable for at least 6 months.

b. d_{12}-EDTA Working Internal Standard Solution – 500 µg/mL:
Accurately weigh 5 mg of the free acid of d_{12}-EDTA (reagent grade, Cambridge Isotope Laboratories) and dilute with deionized water to a final volume of 10 mL. Store frozen in a brown glass container. Stable for at least 6 months.

c. Negative Bloodstain Control:
Add 50 µL of EDTA-free whole blood to a cotton-tip applicator. Dry for at least 30 minutes at room temperature before use. Store at room temperature in a glass test tube or paper envelope. Stable for at least 2 years.

d. Positive Bloodstain Control:
Add 50 µL of EDTA-preserved whole blood to a cotton-tip applicator. Dry for at least 30 minutes at room temperature before use. Store at room temperature in a glass test tube or paper envelope. Stable for at least 2 years.

7 Calibration

This procedure has not been validated for quantitative analysis.

8 Sampling

Not applicable.

9 Procedure

a. Carefully cut the tip from a cotton swab (negative control, positive control, or questioned swab) using clean laboratory scissors.

b. Place the cotton swab tip into a separately labeled molecular weight cutoff filter device.

c. Add 200 µL of the d_{12}-EDTAWorking Internal Standard Solution directly to each cotton swab tip in the molecular weight cutoff filter device. Allow to sit at room temperature for 45 minutes.

d. Centrifuge the molecular weight cutoff filter device for 10 minutes at 2500 RPM.

e. Transfer the filtrate to an autosampler vial and inject 5 µL into the LC/MS/MS system that is in negative ion mode and monitor for both the free acid of EDTA and the EDTA-iron complex.[1] Samples that screen positive are confirmed by injection of 5 µL of the filtrate into the LC/MS/MS system in the positive ion mode, in which the free acid of EDTA can be confirmed.

10 Instrumental Conditions

10.1 Liquid Chromatograph Parameters

10.1.1 Positive Electrospray Ionization Mode

Mobile Phase Composition: Deionized Water (95%) / Acetonitrile (5%) / Ammonium Hydroxide (0.06%)

Column Parameters: Hamilton PRP-1 (2.1 mm x 150 mm x 5µm) at ambient temperature

Isocratic Flow Rate: 0.3 mL/minute

10.1.2 Negative Electrospray Ionization Mode

Mobile Phase Composition: Acetonitrile (80%) / Deionized Water (20%) / Ammonium Hydroxide (0.03%)

Column Parameters: Hamilton PRP-1 (2.1 mm x 150 mm x 5µm) at ambient temperature

Isocratic Flow Rate: 0.3 mL/minute

10.2 Mass Spectrometer Parameters

10.2.1 Positive Electrospray Ionization Mode

Spray Voltage: 4.5 kV

Capillary Temperature: 230°C

Capillary Voltage: +30V

Collision Induced Dissociation: 100%

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[1] See note on carryover in the Limitations section of this procedure (Section 14).

MS/MS Mode: Products of *m/z* 293.0 (EDTA Free Acid)→ (*m/z* 125.0 – 315.0); Collision Energy = 15.0%[2]

Acquisition Time: 10 minutes

10.2.2 Negative Electrospray Ionization Mode

Spray Voltage: 4.5 kV

Capillary Temperature: 230°C

Capillary Voltage: -10V

Collision Induced Dissociation: 0% (Off)

SRM Mode: All Collision Energies Set to 15%

Segment 1 (EDTA Free Acid): *m/z* 291.2 → (*m/z* 246.5-247.5; *m/z* 272.5-273.5)

Segment 2 (d_{12}-EDTA Free Acid): *m/z* 303.2 → (*m/z* 282.8-284.3)

Segment 3 (EDTA Iron Complex): *m/z* 344.2 → (*m/z* 299.5-300.5; *m/z* 325.5-236.5)

Segment 4 (d_{12}-EDTA Iron Complex): *m/z* 356.0 → (*m/z* 311.5-312.5)

Acquisition Time: 10 minutes

11 Decision Criteria

11.1 Performance Mix Suitability

Proper calibration and sensitivity of the LC/MS/MS (ESI) are demonstrated each day samples are analyzed. The EDTA LC/MS/MS (ESI) Performance Mix effectively evaluates system suitability. Proper mass assignments, elution times, and signal-to-noise responses can be assessed by analyzing 5 µL of the Performance Mix. In all instances, the elution time should be ±2% of the retention time (relative or absolute) obtained from the previous run's injection of the Performance Mix. A stacked Gaussian-shaped peak must be present for the EDTA Free Acid analyte with a signal-to-noise ratio exceeding 50:1 for all extracted ions.

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[2] *m/z* 160 and 247 are monitored for EDTA confirmation.

11.2 Analyte Suitability

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control. In most cases, all of the following should be met in order to identify EDTA within a bloodstain:

11.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. Additionally, the following two criteria should be met:

11.2.1.1 Retention Time

The retention time of the peak should be within ±2% of the retention time (relative or absolute) obtained from injection of the EDTA LC/MS (ESI) Performance Mix, extracted Positive Control, or the d_{12}-EDTA internal standard.

11.2.1.2 Signal-to-Noise

To justify the existence of a peak, its signal-to-noise ratio must exceed 3. Further, the baseline signal for the peak from the sample of interest must be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank sample injected just prior to that sample.

11.2.2 Mass Spectrometry

The mass spectral results (whether run in SRM mode or full scan products mode) for the analyte of interest should match that of the appropriate reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (Tox 104) for further guidance.

12 Calculations

Not applicable.

13 Uncertainty of Measurement

Not applicable.

14 Limitations

a. Limit of Detection (LOD): The LOD for EDTA was determined to be 13 µg/mL in both the positive and negative electrospray ionization modes. These detection limits were determined by triplicate analysis of serial dilutions of an EDTA solution. The lowest concentration that reproducibly met the decision criteria listed in Section 11 above was determined to be the LOD.

A separate LOD study was conducted to determine the minimum volume of EDTA-preserved blood that was detectable using this analytical method. Three milliliters of whole blood were placed into a 4-mL lavender-topped blood collection tube containing 7.5 mg of EDTA and thoroughly mixed. The EDTA-preserved blood was placed on a clean, non-porous surface in triplicate at the following volumes: 1 µL, 5 µL, and 10 µL. Following a 1-hour drying period, the blood stains were swabbed using deionized water and cotton-tipped applicators. Each swab was extracted and analyzed to determine the minimum sized EDTA-preserved blood stain required in order to detect EDTA on the swab using the decision criteria requirements of Section 11. The 1 µL drop was readily detectable using this technique.

b. Selectivity: Selectivity was determined by extraction and analysis of 10 matrix blanks (swabs dipped into different blood samples with a variety of non-EDTA preservatives added to them). None of the 10 matrix blanks exhibited peaks of EDTA that met the decision criteria requirements of Section 11. Additionally, d_{12}-EDTA was evaluated for interferences in the analysis and none was observed.

c. Matrix Effects: Five extracted matrix blanks were spiked with an equal amount of an EDTA standard at both low and high concentrations. Following analysis of these samples, the results were compared with equal concentrations of neat EDTA to determine the amount of ion suppression caused by the blood matrix. While ion suppression was not noted in the positive electrospray ionization mode, suppression of 3% and 34% were noted in the negative electrospray ionization mode at EDTA concentrations of 50 µg/mL and 500 µg/mL, respectively.

d. Carryover: It has been reported in the literature that trace amounts of EDTA may be absorbed in the chromatographic system and released in a subsequent analysis of a sample. This carryover was assessed during validation, but was determined to be minor in nature appearing mainly after the injection of a high concentration of EDTA. To demonstrate the

lack of carryover within an analytical run, a minimum of two matrix-matched negative samples (i.e. whole blood extracts) must be analyzed between case samples.

15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

16 References

Miller, M.L., McCord, B.R., Martz, R., and Budowle, B. "The Analysis of EDTA in Dried Bloodstains by Electrospray LC-MS-MS and Ion Chromatography", *Journal of Analytical Toxicology*, Vol. 21, 1997, 521-528.

Sheppard, R.L. and Henion, J. "Determining EDTA in Blood", *Analytical Chemistry*, Vol. 69, 1997, 477A-480A.

Preparation of Chemical Reagents (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

Guidelines for Comparison of Mass Spectra (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Chemistry Unit - Instrument Operation and Support Subunit SOP Manual.

FBI Laboratory Safety Manual.

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