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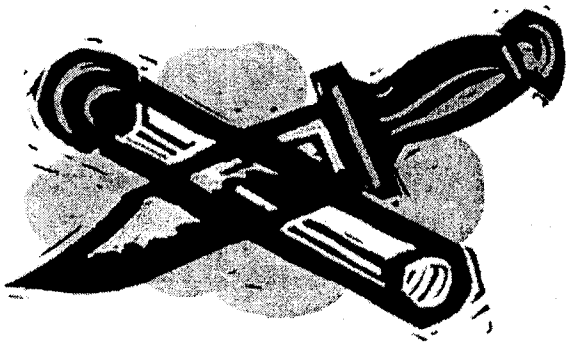

Determining EDTA in Blood

A murder trial sheds light on the need for a better analytical method

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It is not often that a story on the national evening news uses words such as "liquid chromatography" and "mass spectrometry". Analytical chemists who heard such reports during the murder trial *The State of California v. Orenthal James Simpson* immediately perked up their ears, amazed that the analytical details of FBI laboratory testing were actually making headlines.

The subject of the testing was EDTA (ethylenediaminetetraacetic acid) issue was whether police had "planted" or tampered with blood evidence in an attempt to shore up the case against Simpson. The possible outcomes of the testing were simple: Either EDTA was present or it wasn't. Qualitative testing began (*1*), but as is usually the case, nothing is that simple. There was evidence of some EDTA, at levels much lower than in EDTA-preserved blood. The questions "How much EDTA is there?" and "Are the detected levels consistent with 'normal' levels or those that would result from tainted blood collected in EDTA anticoagulant blood tubes?" arose immediately.

The lead prosecutor, Marcia Clark, tried her best to present this scientific evidence. But how do you convince a jury of citizens that knows little about analytical chemistry that the EDTA came not from a lavender-stoppered tube but from a bleeding O.J. Simpson? Although it may not have been the only weak point in the prosecution's case, it certainly was a factor in the trial's outcome. Because of this criminal case, determining EDTA in human blood has become a topic of renewed interest.

What was wrong with the laboratory testing? First, it was not clear whether the method had ever been

used before. Most likely the method was developed quickly under a great deal of time pressure. In retrospect, FBI chemists now believe that the EDTA detected may have been injection carryover in the LC/MS/MS (*2*) instrumentation because a water blank instead of a matrix blank had been run before the sample. Second, the EDTA concentration was not rigorously quantitated. Certainly, the volume of the blood stain could have been estimated. EDTA is present at about 4.5 mM (~1300 ppm) in EDTA-preserved blood, which would be a very concentrated sample and easily detected by electrospray LC/MS/MS. It appeared that the amount of EDTA detected in the forensic blood samples was orders of magnitude below 4.5 mM. Regardless of what happened in the Simpson trial, it became apparent that a definitive and valid method for determining EDTA in human blood was needed.

EDTA BASICS

EDTA is a metal-complexing agent that has been popular since its commercialization in the early 1950s (*3*). The free-acid structure with a molecular weight of 292.1 is shown in Figure 1a, and a three-dimensional representation of EDTA complexed with nickel(II) with a molecular weight of 347.0 is shown in Figure 1b. EDTA has four acidic protons that are sequentially ionized at solution pH values of 2.0, 2.67, 6.16, and 10.26, respectively (*4*). The disodium salt is commonly used as an anticoagulant (*5*), and the familiar lavender-stoppered blood collection tubes contain enough EDTA to give a final concentration of ~ 4.5 mM. Upon mixing with blood, the EDTA immediately chelates the available calcium. Because calcium is necessary for the formation of fibrin, coagulation cannot take place (*5*).

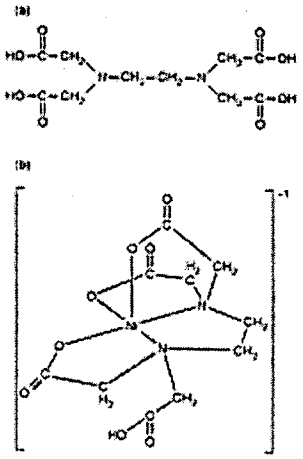

Figure 1. (a) EDTA's free-acid structure and (b) three-dimensional structure of Ni-EDTA.

EDTA is also used extensively as a food preservative, a water-softening agent, and to deliver trace minerals in animal feeds (*6*). Despite its ubiquitous presence, metabolism studies have shown that little, if any, EDTA should be present in human blood. In 1954, a metabolism study using $^{14}C_4$-labeled calcium-EDTA given intravenously showed that EDTA was detectable in the plasma but not in the blood cells (*7*). On average, 95% of an oral dose was recovered in the urine and feces within three days of administration with no EDTA detected in the plasma, and the remaining 5% was detected in the urine within 18 h. More recent metabolism studies using the NaFe(III)-EDTA complex report that it dissociates during digestion and confirm that only about 5% of the EDTA is absorbed and excreted in urine (*8*).

DETERMINING EDTA IN BLOOD AND PLASMA

Although there are numerous published methods for determining EDTA in various matrices such as

mayonnaise (*9*), wastewater (*10*), and ophthalmic solutions (*11*), our lab and one other group have recently attempted to develop improved methods that could be used for the forensic measurement of EDTA in biological matrices (*12*, *13*).

There are numerous ways to prepare samples containing free or chelated EDTA. The first and most obvious is no sample preparation at all. If the pH of the sample is not adjusted and if metal contamination can be eliminated, the natural distribution of EDTA and its metal chelates can be determined. This is not the most sensitive approach because the EDTA signal would be distributed among several different metal species or peaks. In addition, quantitation would be difficult because any change in the sample matrix could change the complex equilibria. Therefore, all the methods used to measure EDTA itself focus on preparing a singular EDTA-containing peak or compound.

For GC, the analyte must be reasonably volatile and thermally stable. Because EDTA is not volatile, it is usually derivatized by esterifying the four carboxyl groups with methanol, butanol, or isopropyl alcohol (*13-15*). This liquid-liquid extraction is a labor-intensive, multistep procedure that is difficult to automate. For LC or capillary electrophoresis (CE), the most logical way to convert all available EDTA to a single compound is to add an excess of one metal that strongly complexes with EDTA. Although iron(III) and copper(II) are commonly used (*11*, *16*), we found that nickel(II) complexes with EDTA just as effectively, and it has several advantages: Heating is not required, the complex is stable down to pH\t3, and the complex gives a higher signal by ion spray MS than either the iron or copper complexes.

DEVELOPING A CE/MS/MS METHOD

Several reports in which CE was used to determine metals using EDTA as a complexing reagent have been published in the literature (*17*, *18*). Using these reports as a starting point, we predicted that an "MS-friendly" CE separation could be developed that would minimize the chemical additives introduced into the mass spectrometer.

The intricacy of interfacing the capillary to a mass spectrometer is the reason the technique is considered nonroutine. Although many qualitative CE/MS applications have been reported, very few quantitative methods have been described in the literature (*19*, *20*). Therefore, we used this opportunity to achieve two goals: to offer an improved technique for measuring EDTA in biological matrices and to demonstrate that CE/MS can be used in a routine manner for quantitative bioanalytical applications.

Although MS may not be the most sensitive CE detector available, it offers a high level of selectivity as well as wider applicability than, for example, laser-induced fluorescence (*21*). For example, the full-scan CE/MS separation shown in Figure 2a displays the EDTA distributed as five different metal complexes. Figure 2b shows the same EDTA standard in which the predominant ion *m/z* 347, caused by Ni-EDTA, has been extracted from the total ion current data. This type of selectivity can be exploited by using the selected ion monitoring mode in which only the ion(s) of interest would be detected.

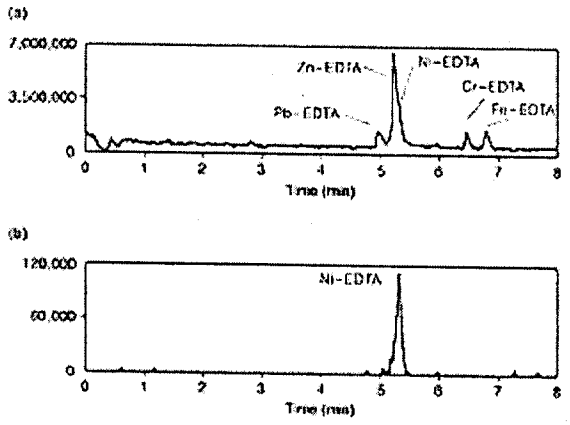

Figure 2. (a) Full-scan CE/MS electropherogram of five EDTA-metal chelates and (b) extracted ion electropherogram of Ni-EDTA.

Additional selectivity can be achieved using MS/MS by selecting a precursor ion that is characteristic of the analyte, fragmenting it into one or more ions, and selectively detecting one specific fragment from the selected precursor ion. For example, a full-scan single mass spectrum of the Ni-EDTA complex in Figure 3a shows the deprotonated molecular anion at *m/z* 347, which corresponds to the $[Ni^{2+}.H^{+}.EDTA^{4-}]^{-}$ ion. This precursor ion may be fragmented using collision-induced dissociation within the second-quadrupole region of a triple-quadrupole mass spectrometer. The full-scan product ion mass spectrum from the fragmentation of *m/z* 347 in Figure 3b shows product ions *m/z* 329 and 257, which are characteristic of the Ni-EDTA complex.

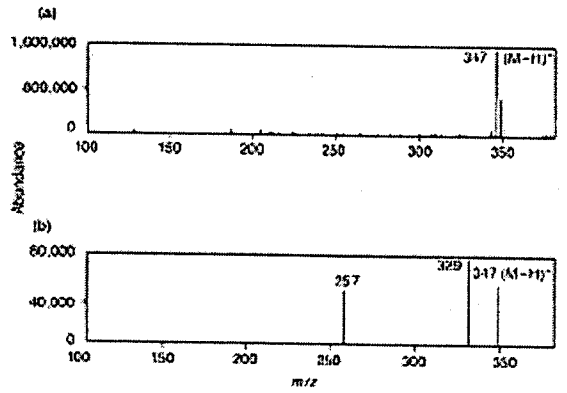

Figure 3. (a) Full-scan mass spectrum and (b) full-scan product ion scan of Ni-EDTA.

This additional selectivity can be put to use in the selected reaction monitoring (SRM) mode by monitoring only the specified transition *m/z* 347 fragmenting to 329. Further selectivity is achieved via the initial CE separation, which gives a characteristic migration time for Ni-EDTA. It is highly unlikely that a chemical compound other than Ni-EDTA would result in this characteristic transition at the specified migration time under the described experimental conditions.

To illustrate this point, blank and Ni-EDTA-spiked plasma samples were simply diluted with water, filtered, and analyzed by CE with UV detection and CE with SRM detection. The blank plasma in Figure 4a and the 1-μM Ni-EDTA-spiked plasma in Figure 4b are indistinguishable by CE/UV because of the excessive chemical background detected at 200 nm. In contrast, when analyzed by SRM-CE/MS, the same blank plasma sample is free of all matrix peaks (Figure 4c); whereas the Ni-EDTA-spiked

plasma displays only the targeted Ni-EDTA peak (Figure 4d). This example clearly shows the advantage of using tandem MS for this type of targeted analysis.

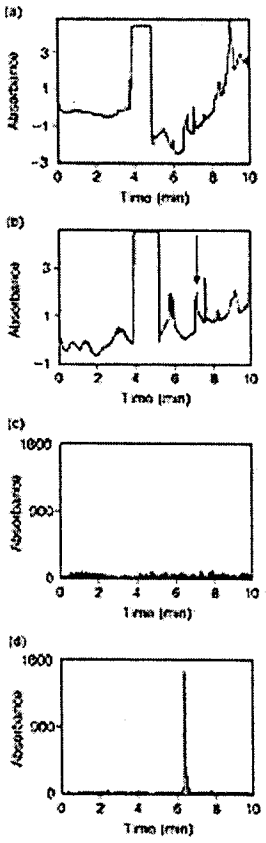

Figure 4. UV detection versus SRM detection.

CE/UV analysis of (a) blank plasma and (b) Ni-EDTA-spiked plasma. SRM-CE/MS analysis from *m/z* 347 to 329 of (c) blank plasma and (d) Ni-EDTA-spiked plasma.

To further minimize the possibility of interference, we developed an automated anion-exchange solid-phase extraction procedure. The complete SRM-CE/MS procedure uses 100 μL of plasma, to which is added 50 ng of ($^{13}C_4$)EDTA internal standard, brought to pH 9-10 with ammonium hydroxide, and complexed using nickel nitrate. The sample is diluted 1:45 with 0.05% formic acid (pH 3) and then extracted using strong anion-exchange solid-phase extraction media. The sample is eluted, evaporated, and reconstituted in 30 μL of water. The extract is injected for 0.1 min at 950 mbar inlet pressure onto a 50 μm X 60 cm amine-coated capillary. The separation is performed using a CE running buffer of 30 mM ammonium formate at pH 3 (adjusted with formic acid) and -30 kV with 50-mbar inlet pressure throughout the run. A homemade self-aligning liquid junction CE/MS interface is used with a makeup liquid of 5 mM ammonium formate in 95% methanol at 10μL/min (*22*, *23*). A triple-quadrupole mass spectrometer is used in the negative-ion mode with SRM of the transitions *m/z* 347-329 for Ni-EDTA and *m/z* 351-333 for the internal standard Ni-($^{13}C_4$)EDTA. The complete method and validation are described in this issue in reference 24.

Using this sample preparation procedure and the SRM-CE/MS method, we achieved a detection limit of 7.3 ng/mL EDTA in human plasma and a lower level of quantitation (LLQ) of 15 ng/mL (~6 fmol injected). If this method was used to determine whether a forensic blood stain had been "planted", this

LLQ corresponds to "planting" 1-3 nL of EDTA-preserved blood. Because it would be physically difficult to manipulate such a small volume, any such forensic sample would probably contain at least 1 μL. This hypothetical scenario illustrates the excellent sensitivity and potential forensic usefulness of the method. Similarly, the GC/MS/MS method developed by Ballard and colleagues demonstrated a comparable detection limit of 10 ng/sample, which corresponds to ~7 or 8 nL of EDTA-preserved blood (*13*).

CONCLUSIONS

Many techniques have been used over the years to determine EDTA in various matrices, and most can be adapted to biological samples. However, SRM-CE/MS provides the highest specificity and the best detection level of any method currently published.

We have been able to demonstrate that typical human plasma samples do contain detectable EDTA, but at levels that are lower than the LLQ reported in this work. The LLQ of our method, at 15 ng/mL, is a factor of 10^5 lower than the typical concentration found in EDTA-preserved blood (4.5 mM or 1.3 X 10^6 ng/mL). But more importantly, we have demonstrated that CE/MS methods can be used for routine bioanalytical analysis with acceptable precision, accuracy, and adequate detection levels for quantitation of trace-level concentrations (*24*).

CE/MS techniques will undoubtedly become an important forensic technique because of the low volumes of sample required for analysis, as well as the ability to use the mass spectrometer to achieve selectivity higher than with any other on-line detector.

The question of blood-evidence tampering in a criminal trial has led not only to improved analytical techniques for the determination of EDTA, but also to the demonstration that a relatively new technique is ready to be used as credible scientific evidence in the courtroom.

REFERENCES

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