Detection and Quantitation Analysis of Cocaine and Metabolites in Fixed Liver Tissue and Formalin Solutions

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Abstract

This study reports the results of the detection and quantitation of cocaine and its metabolites in liver tissues fixed in formalin and in the formalin solutions in which the same tissues were fixed. Toxicological analyses were performed on formalin-fixed liver samples from four cases of death of cocaine abusers and on formalin solutions (10% buffered, pH 7) in which the samples were preserved. Analyses carried out at the time of autopsy on body fluids and tissues allowed identification of cocaine and the metabolite benzoylecgonine. Liver tissue samples were preserved in formalin solutions for four weeks before analysis. Results only showed the presence of benzoylecgonine in the studied materials. The mean levels of recovery of benzoylecgonine in fixed tissues were 12.31% in liver and 84.47% in formalin from liver. Results indicated that benzoylecgonine has good stability, even in biological specimens subjected to chemical fixation.

Introduction

The use of alternative matrices to plasma and urine in forensic toxicology is a field of increasing interest, as demonstrated by the inclusion of this subject in the main topics of the meeting of the International Society of Forensic Toxicologists since 1998 (1). Interest focuses mainly on drug analysis of hair, saliva, and sweat. In deceased persons, the range of specimens for drug analysis may be extended to solid tissues and gastric contents. Other matrices are not often used, particularly as regards histological specimens (2–5), whereas forensic toxicology is required with increasing frequency to perform analysis of histopathological specimens obtained from medico-legal autopsies of toxin-related deaths. These specimens are usually stored in formalin solutions. Some studies have analyzed drugs in formalin-fixed tissues (2–5) but, to our knowledge, until now there have been no studies concerning the detection and quantitation of cocaine in formalin-fixed tissues.

Analysis of cocaine in biological specimens is complicated by its instability in various biological matrices. It is particularly vulnerable to hydrolysis in cholinesterase-containing specimens, including blood and plasma. Conventional postmortem specimens like urine or blood are sometimes not available for drug analysis. In these cases, alternative specimens may be analyzed. Liver is traditionally used in forensic toxicology to measure drug concentrations. Cocaine is metabolized in liver and is present in postmortem specimens (6,7), so it is an appropriate biological matrix to detect the presence of this substance. The aim of the present study was to examine the presence and concentration of cocaine and its primary metabolite (i.e., benzoylecgonine) in formalin-fixed human liver tissue and in the formalin solutions in which the same tissue was fixed.

Cocaine and its metabolites in biological material were detected by means of gas chromatography (GC) with flame ionization detection (8) and nitrogen-phosphorus detection (9,10). GC–mass spectrometric (MS) (6,7,11,12) and high-performance liquid chromatographic (HPLC) methods (13,14) were also applied.

Materials

Toxicological analyses were performed on samples of fresh, formalin-fixed liver and formalin solutions (10% buffered, pH 7) in which the samples were preserved. These biological materials came from four cases of death of cocaine abusers. For each case, at time of autopsy, a sample of liver was drawn that was separated in two portions; the first one was analyzed immediately, the second was put in formalin. Tissue samples were preserved in formalin solutions for four weeks before analysis. Tissue weights, before and after formalin fixation, were checked and did not turn out to be appreciably modified by preservation (5).

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Analytical-grade chemicals and reagents were Isolute Certify Extraction columns (International Sorbent Technology, Hengoed, Mid Glamorgan), sodium hydroxide (Merck, Darmstadt, Germany), methanol (Carlo Erba, Milan, Italy), hydrochloric acid (Carlo Erba), ammonium hydroxide (Sigma, St. Louis, MO), dichloromethane (Baker, Phillipsburg, NJ), isopropyl alcohol (Sigma), *N*-methyl-*N*-trimethyl-silyl-trifluoroacetamide (MSTFA) (Sigma), cocaine (Alltech, Los Alamos, CA), benzoylecgonine (Alltech), and scopolamine (Sigma).

Equipment included a homogenizer (Ultra Turrax, Ica Labor Technik, Milan, Italy), sonicator (model 1210, Bramson, Danbury, CT), centrifuge (model 4218, ALC, Milan, Italy), vortex mixer (Falc, Bergamo, Italy), Analytichem Vac Elut (SPS 24, Varian, Bergon op Zoom, The Netherlands), temperature bath (Carlo Erba thermostatic bath), and GC–MS (Fisons Instruments, Milan, Italy).

Methods

Preparation of samples

Tissues. Five grams of both fresh and formalin-fixed tissues, both from autopsy sources, were separately crushed and homogenized in distilled water (1:1) and sonicated for 7–8 h. One milliliter of HCl 0.1M was added to homogenized solutions, followed by sulfosalicylic acid for deproteinization. The solutions were centrifuged, and the pH of the supernatant was adjusted to 6 with ammonium sulfate and NaOH (8% in water). One milliliter of scopolamine solution (1 mg/L in water) as internal standard (IS) (15) and 2 mL of 0.1M phosphate buffer (pH 6) were added, and the solutions were centrifuged before the following phase of extraction.

Formalin solutions. Five milliliters of sample were drawn off. Solutions were evaporated to dryness at room temperature. Five milliliters of distilled water were added to the residue. One millilier of scopolamine solution (1 mg/L in water) as IS (15) and 2 mL of 0.1M phosphate buffer (pH 6) were added, and the solutions were centrifuged before the following phase of extraction.

Solid-phase extraction

Isolute Certify Extraction columns were used.

Preparation of columns. Columns were conditioned sequentially with 2 mL of methanol and 2 mL of 0.1M phosphate buffer, pH 7.

Specimen application. Samples were slowly drawn through the columns under low vacuum (at least 2 min).

Column rinsing. Columns were rinsed sequentially with 2 mL of water, 3 mL of 0.1N HCl, and 2 mL of methanol and then dried for 5 min under full vacuum.

Cocaine elution. Analytes were eluted twice with 1 mL of a dichloromethane/isopropyl alcohol solution (8:2) with 2% ammonium hydroxide, made fresh daily; eluates were transferred to 3-mL silanized gradual tubes and evaporated to dryness at 50ºC in a temperature bath under a slow stream of nitrogen.

Derivatization

Extracts were reconstituted with 50 µL of MSTFA and incubated for 15 min at 75ºC in sealed silanized gradual tubes.

GC–MS analysis

The following conditions were applied: a well-coated open-tubular capillary column (fused silica 30 m × 0.32 mm) was used; the carrier gas was helium at a low flow rate of 1.5 mL/min. The temperature program was started at 100ºC and increased first to 180ºC at 40ºC/min and then to 310ºC at 10ºC/min. The injection volume was 1 µL (splitless mode). Electron ionization (70 eV) was used. Mass spectra were recorded in the range of 70–500. Figure 1 shows the mass spectrum of benzoylecgonine trimethylsilyl-derivated from a formalin-fixed liver sample.

Results

The analysis mainly detected and quantitated benzoylecgonine in all the studied materials. Cocaine was detected only in one case in fresh tissue, but never in fixed tissues or formalin solutions. Cocaine and benzoylecgonine were identified by comparing the retention time to that of the internal standard

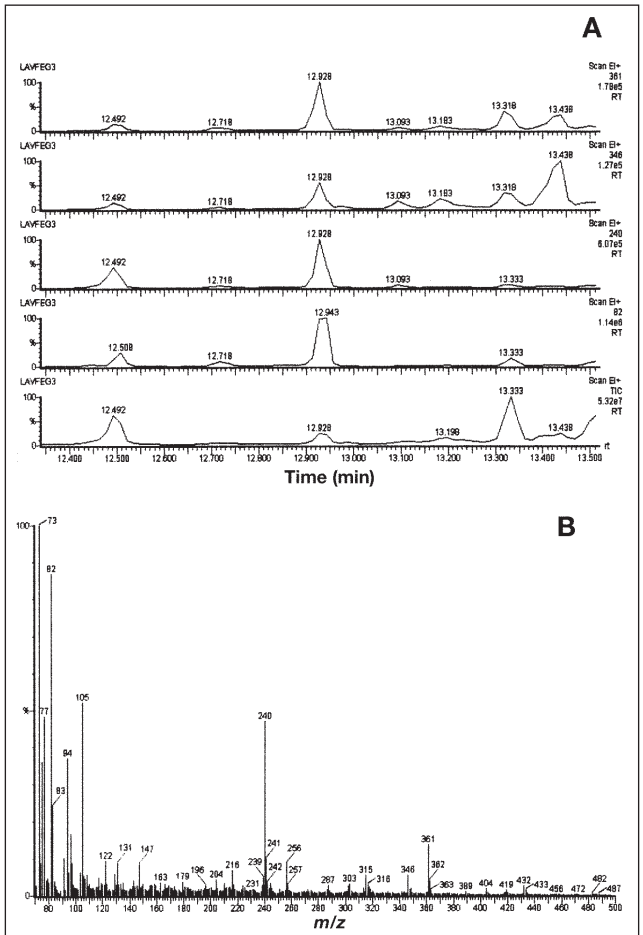

Figure 1. Chromatographic results in fixed liver sample. TIC chromatogram and SIM analysis at *m/z* 82, 240, 346, and 361 (A), and mass spectrum of peak at retention time of 12.928 min of A (B).

scopolamine and the mass spectrum of cocaine and benzoylecgonine standard (cocaine, *m/z* 182, 82, 303, and 272; benzoylecgonine-TMS *m/z* 82, 240, 361, and 346). Quantitation was carried out by monitoring the abundances of *m/z* 182 ions (for cocaine), *m/z* 82 ions (for benzoylecgonine-TMS), and *m/z* 138 ions (for scopolamine-TMS, IS) (15). To verify the linearity of the detector and to calibrate the method, five calibrators were tested: 0.02, 0.05, 0.1, 0.5, and 1.0 mg/L or mg/kg (cocaine and benzoylecgonine in formalin solution and liver tissues previously tested to be negative). Linear regression analysis of the calibration data showed a correlation coefficient near 1. The limit of benzoylecgonine quantitation was set at 0.002 mg/L, with a signal-to-noise ratio of 10. The variation coefficients between runs and days, retesting five calibrators (0.5 mg/L or mg/kg) 10 times each day for 10 days, were 4.5 (for formalin solution) and 5.7 (for liver), respectively. The extraction efficiency of the method was assayed for four liver samples, previously tested to be negative, spiked with an adequate amount of internal standard, cocaine, and benzoylecgonine to give a final concentration of 1 mg/kg. Recovery was 97.8%. The quantitative results for formalin-fixed liver samples and for the formalin solutions in which these tissues were preserved are listed in Table I.

The quantitative values of cocaine and benzoylecgonine obtained in fixed tissues and from the same tissues at the time of autopsy were quite different because detectable quantities are redistributed from tissues into the formalin solution. In many cases, the concentrations of benzoylecgonine in formalin solutions in which liver was preserved were found to be higher than those recovered in the same fixed tissues. Table II lists mean levels of benzoylecgonine recovered from liver (both fixed tissues and formalin solutions in which the tissues were preserved) compared with the mean levels from the same samples at the time of autopsy. The best recovery rates were found in formalin solutions (84.47%). The total sum of recovery rates in formalin solutions and fixed tissues (84.47% + 12.31% = 96.78%) was comparable to those of the extraction efficiency of the method (97.8%) and indicates that cocaine and benzoylecgonine have good stability. This value may be useful in qualitative evaluation of cases.

Table I. Quantitation of Cocaine and Benzoylecgonine in Liver Tissue at Time of Autopsy in Fixed Tissues and Formalin Solutions

Case	Liver at Time of Autopsy (mg/kg)	Fixed Liver (mg/kg)	Formalin Liver (mg/L)
1	COC: 0.376 BZE: 0.423	BZE: 0.056	BZE: 0.885
2	BZE: 0.816	BZE: 0.104	BZE: 0.509
3	BZE: 0.162	BZE: 0.024	BZE: 0.124
4	BZE: 0.815	BZE: 0.135	BZE: 0.672

* Abbreviations: COC = cocaine and BZE = benzoylecgonine.

Table II. Means of Quantitative Results for Benzoylecgonine (Amounts) and Rate of Recovery after Four Weeks

Amount of BZE at Time of Autopsy (means, µg)	Amount of BZE in Fixed Tissues (means, µg)	Amount of BZE in Formalin Solutions (means, µg)	% of Recovery Fixed in Tissues	% of Recovery in Formalin Solutions
3.24[†]	0.399	2.737	12.31	84.47

* Abbreviations: COC = cocaine and BZE = benzoylecgonine.
[†] COC and BZE are considered as BZE.

Discussion

Results indicate that benzoylecgonine could be detected in fixed liver tissue and in the formalin solutions in which the specimens were stored. Samples of liver are easily collected and, as shown also by the procedures used by us, they can be readily homogenized in water or dilute buffer, and the use of ultrasonicating blender is more effective.

Only benzoylecgonine, the main metabolite of cocaine, was found in the analyzed samples. In the living body, cocaine is rapidly metabolized ($t_{1/2}$ = 0.7–1.5 h) to ecgonine methylester and other fragments by serum cholinesterase and liver esterase and to benzoylecgonine by chemical hydrolysis. Cocaine is also hydrolyzed by a liver esterase to benzoylecgonine. Those processes probably continue postmortem in blood and liver (7). Hydrolysis can be slowed by refrigeration and freezing and by decreasing the pH below normal. In addition, it has been shown that, in basic media, the transformation of cocaine into benzoylecgonine is increased (16). The solution of formalin used by us was at neutral pH, and this value (pH 7.0) may not have prevented the transformation of cocaine into benzoylecgonine. So, because many processes may cause cocaine breakdown, cocaine itself may not be found in liver at the time of autopsy, even with timely screening and confirmation (7). Other authors have frequently had cases in which a toxicology screen detects only moderate concentrations of benzoylecgonine and no cocaine (17).

The only case in which we detected cocaine in fresh tissue was a cocaine-related death. In the other three cases, cocaine was incidental to the cause of death. Other authors have also found cocaine in postmortem liver, especially in cases of overdose, but not frequently when cocaine was incidental to the cause of death (7).

Our results highlight the fact that the quantitative values of cocaine and benzoylecgonine obtained in fixed tissues and from the same tissues at the time of autopsy are quite different. The level of benzoylecgonine, increased in the formalin water solutions, demonstrated leaching of the drug from the liver into the formalin solutions. In all cases, the concentrations in formalin solutions in which liver was preserved were found to be higher than those recovered in the same fixed tissues and in fresh tissues. This remarkable fact is probably because of the extraction capability of formalin solution.

Formaldehyde is a highly reactive chemical substance and has recently been shown to react with some drugs (18,19). As regards cocaine and benzoylecgonine, this type of interaction can be excluded. In fact, as shown in Table II, the total sums of drugs in formalin solution and fixed tissues are comparable with those in fresh tissues. The absence of any differences indicates that benzoylecgonine has good stability and does not react chemically with formaldehyde.

Conclusions

The good stability of benzoylecgonine in biological specimens submitted to chemical fixation allows fixed tissues to be used for forensic toxicology diagnosis of cocaine poisoning in cases in which toxicology analysis for the substance was not carried out at the time of autopsy. However, interpretation of quantitative data from fixed tissues requires considerable care. The formalin solutions in which the tissues were preserved can also be used for the same purpose. The double evaluation of benzoylecgonine in fixed tissues and formalin solutions provides a good indication of the original quantity of cocaine in the same tissues before fixation.

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