Detection and Quantitation of Morphine in Fixed Tissues and Formalin Solutions

Mariano Cingolani1,*, Rino Froldi2, Roberto Mencarelli1, Dora Mirtella1, and Daniele Rodriguez1
1Institute of Legal Medicine, University of Ancona, Ospedale Regionale, I-60020, Italy and 2Institute of Legal Medicine, University of Macerata, Via Don Minzoni 9, I-62100, Italy

Abstract

The study reports the results of the detection and quantitation of morphine in tissues fixed in formalin and in the formalin solutions in which the same tissues were preserved. Toxicological analyses were performed on formalin-fixed liver and kidney samples from five cases of opiate poisoning and on the 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 opiate as the cause of death and its quantitation. Tissue samples were preserved in formalin solutions for 12 weeks before analysis. The mean levels of recovery of morphine in fixed tissues were 36.29% in liver, 29.41% in kidney, 74.93% in formalin from liver, and 42.17% in formalin from kidney. Results indicated that this particular toxic substance shows good stability even in biological specimens subjected to chemical fixation.

Introduction

The use of formalin-fixed tissue samples for toxicological analyses is an interesting field in the laboratory application of forensic toxicology. Many studies have been conducted on formalin-fixed human tissues obtained from medicolegal autopsies of toxin-related deaths (1–9). The aim of the present study was to detect and quantitate morphine in formalin-fixed tissues and in the formalin solutions in which the same tissues were fixed.

Morphine is the primary alkaloid substance of crude opium; it has narcotic and analgesic properties and remains a useful drug against severe pain (10). It is rarely used by addicts, but it is also the most important metabolite of heroin, one of the most frequently used drugs of abuse (11). Both morphine and heroin are currently listed in Table I of the Italian drugs law. Levels of morphine in cases of heroin overdose found in postmortem body fluids and tissues depend considerably on the overall history of the subject. The best expressions of recent

use are whole blood levels; liver, kidney, bile, and urine levels indicate past exposure to the drug (10). In heroin fatalities, blood levels (expressed as total morphine) vary from (mean) 0.30 to 0.43 mg/L (range 0.01–3.0 mg/L) (10). However, there are considerable difficulties in connecting blood levels with clinical effects and in interpreting toxicological data in order to establish the cause of death (12).

Heroin metabolites in biological fluids and tissues were detected by means of gas chromatography (GC) with flame ionization (FID) (13), nitrogen-phosphorus (NPD) (14), and electrochemical (ECD) detection (15). Gas chromatographic–mass spectrometric (GC–MS) (16–18) and high-performance liquid chromatographic (HPLC) methods (19,20) were also applied; very sensitive GC–MS (21) and liquid chromatographic–mass spectrometric (LC–MS) (22) methods have recently been reported.

Materials

Toxicological analyses were performed on samples of formalin-fixed liver and kidney and formalin solutions (10% buffered pH 7) in which these samples were preserved. These biological materials came from five cases of acute opiate poisoning in which analyses carried out at the time of autopsy on body fluids (blood, urine, bile) and tissues (liver and kidney) allowed the detection and quantitation of morphine and the identification of this substance as the cause of death. Toxicological data on these cases are listed in Table I. Tissue samples were preserved in formalin solutions for 12 weeks before analysis. Tissue weights, before and after formalin fixation, were checked and did not prove to be appreciably modified by preservation.

Analytical-grade chemicals and reagents used were Bond Elut Certify Extraction columns (Varian), sodium hydroxide (Merck), methanol (Carlo Erba), hydrochloric acid (Carlo Erba), methylene chloride (Sigma), ammonium hydroxide (Sigma), isopropyl alcohol (Sigma), N-methyl-N-trimethyl-silyl-trifluoroacetamide (MSTFA) (Sigma), morphine (Sigma), and nalorphine (Sigma).

Equipment used included a homogenizer (Kinematica
Microtron MB1), a sonicator (Bramson 1210), a centrifuge (ALC 4218 Centrifuge), a vortex mixer (Velp Rx3), an Analytichem Vac Elut (Varian SPS 24), a temperature bath (Carlo Erba Thermostatic bath), and a GC–MS (Saturn 4D GC–MS, Varian).

Methods

Preparation of samples (8)

Tissues. 5 g of both fresh and formalin-fixed tissues, both from autopsy sources, were separately crushed and homogenized in distilled water (1:1) and sonicated for 30 min.

Formalin solutions. Five milliliters of sample was drawn off. Solutions were evaporated to dryness in a temperature bath.

Acid hydrolysis (23)

One milliliter of nalorphine solution (1 mg/L in water), as internal standard, and 1 mL of hydrochloric acid (37% in water) were added to the samples (5 g of tissue homogenate and 5 mL of water solution from the formalin sample). The mixture was incubated at 125°C for 20 min in a 10-mL sealed glass tube and then kept overnight at room temperature. After filtration and centrifugation, the solution was alkaliniized to pH 8.9–9 with sodium hydroxide (25% in water).

Solid-phase extraction (24)

Bond Elute Certify Extraction columns were used.

Preparation of columns.

Columns were conditioned sequentially with 2 mL of methanol and 2 mL of 0.1M phosphate

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<tr>
<th>Table I. Quantitation of Morphine in Body Fluids and Tissues at Time of Autopsy in Fixed Tissues and in Formalin Solutions</th>
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Figure 1. Chromatographic results in fixed liver sample. A, TIC chromatogram and SIM analysis at m/z 429, 414, and 401. B, mass spectrum of peak at retention time 8.91 min of A.

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<th>Table II. Means of Quantitative Results for Morphine (Amounts) and Rate of Recovery after 12 Weeks</th>
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<tr>
<td>Kidney</td>
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<td>Total</td>
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buffer at 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.

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

Derivatization (25)

Extracts were reconstituted with 50 μL of N-methyl-N-trimethyl-silyl-trifluoroacetamide (MSTFA) and incubated for 15 min at 75°C in sealed silanized gradual tubes.

GC–MS analysis (25)

The following conditions were applied: a DB-5MS capillary column (fused silica, 30 m x 0.25 mm) was used; carrier gas was helium at a flow rate of 1.8 mL/min. The temperature program was started at 120°C and increased to 320°C at 15°C/min. The injection volume was 1 μL (splitless mode). EI (70eV) was used for ionization. Mass spectra were recorded in the range m/z 40–650. Figure 1 shows the mass spectrum of morphine-bis-TMS from a formalin-fixed liver sample.

Results and Discussion

The above analyses allowed the detection and quantitation of morphine in all the studied. Morphine was identified by comparing the retention time of the internal standard naltrexone and with the mass spectrum of morphine standard (morphine-bis-TMS m/z 429, 414, 401). Quantitation was carried out by monitoring the abundance of m/z 429 ions (for morphine-bis-TMS) and m/z 455 ions (for naltrexone-bis-TMS, I.S.) (25). To verify the linearity of the detector and to calibrate the method, five calibrators were tested: 0.5, 1.0, 2.0, 4.0, and 8.0 mg/L (morphine in formalin solution and in liver tissues previously tested to be negative). Linear regression analysis of the calibration data showed a correlation coefficient near 1.0. The variation coefficients between runs and days, retesting 5 calibrators (1.0 mg/L) 10 times each day for 10 days, were 4.3 (for formalin solution) and 5.8 (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 and morphine to give a final concentration of 1 mg/kg. Recovery was 90.72%. The quantitative results for formalin-fixed liver and kidney samples and for formalin solutions in which these tissues were preserved are reported in Table I.

The quantitative values of morphine obtained in fixed tissues and from the same tissues at the time of autopsy were quite different, since detectable quantities are redistributed from the tissue into the formalin solution. In many cases, the concentrations of morphine in formalin solutions in which liver and kidney were preserved were found to be higher than those recovered in the same fixed tissues. This remarkable fact is probably due to the extraction capability of formalin solutions. Table II lists mean levels of morphine recovered from five different specimens (both fixed tissues and formalin solutions in which the tissues were preserved) compared with the mean levels from the same sample at the time of autopsy. The best recovery rates were found in formalin solutions (74.93% for liver formalin; 42.17% for kidney formalin); evaluation of mean values (Table II) confirmed a high redistribution of morphine in formalin solutions. The total sums of recovery rates in formalin solutions and fixed tissues (32.95% + 59.00% = 91.95%) are comparable to those of the extraction efficiency of the method (recovery 90.72%) and indicate that morphine has good stability. This value may be useful in quantitative evaluation of cases.

Conclusions

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

References


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