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Analysis of dextromethorphan and dextrorphan in decomposed skeletal tissues by microwave assisted extraction, microplate solid-phase extraction and gas chromatography- mass spectrometry (MAE-MPSPE-GCMS)

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Analysis of decomposed skeletal tissues for dextromethorphan (DXM) and dextrorphan (DXT) using microwave assisted extraction (MAE), microplate solid-phase extraction (MPSPE) and gas chromatography-mass spectrometry (GC-MS) is described. Rats (n = 3) received 100 mg/kg DXM (i.p.) and were euthanized by CO_2 asphyxiation roughly 20 min post-dose. Remains decomposed to skeleton outdoors and vertebral bones were recovered, cleaned, and pulverized. Pulverized bone underwent MAE using methanol as an extraction solvent in a closed microwave system, followed by MPSPE and GC-MS. Analyte stability under MAE conditions was assessed and found to be stable for at least 60 min irradiation time. The majority (>90%) of each analyte was recovered after 15 min. The MPSPE-GCMS method was fit to a quadratic response ($R^2 > 0.99$), over the concentration range 10 10 000 ng·mL^{-1}, with coefficients of variation <20% in triplicate analysis. The MPSPE-GCMS method displayed a limit of detection of 10 ng·mL^{-1} for both analytes. Following MAE for 60 min (80 °C, 1200 W), MPSPE-GCMS analysis of vertebral bone of DXM-exposed rats detected both analytes in all samples (DXM: 0.9-1.5 μg·g^{-1}; DXT: 0.5-1.8 μg·g^{-1}). Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: forensic toxicology; bone; microwave assisted extraction; dextromethorphan; dextrorphan

Introduction

Toxicological analysis of bone has been the subject of renewed interest in recent years.[1–10] The results of studies since 2000 done with both human tissues derived from forensic casework[1–3] and animal tissues derived from studies involving controlled drug exposure and post-mortem conditions[4–12] have shown that detection of a wide variety of drugs and metabolites is possible, but there remains uncertainty with respect to the interpretive value of quantitative measurements in bone, due to poor correlation of drug levels in bone and those in corresponding blood samples. Furthermore, the use of bone as a substrate for toxicological analysis may be expected to occur primarily in cases involving extensive decomposition, where few or no other conventional sample types are available. This adds a further measure of complexity to the interpretation of results if the influence of decomposition on the levels of a given drug or metabolite in bone are unclear.

Recent work in our laboratory, involving controlled drug exposure to experimental animals followed by natural decomposition to skeleton, suggests that it may be possible to discriminate between different patterns of drug exposure in some cases. Such discrimination is based on the quantitative relationship between drug and metabolite levels in decomposed bone. In order to expand on that work, it is necessary to analyze a substantially larger number of samples to improve statistical power, and to investigate a wider range of toxin exposure conditions, where analytical throughput is a limiting factor. The work presented here describes the characterization of a method for the analysis of dextromethorphan, an antitussive compound with abuse potential at high doses, and its primary metabolite, dextrorphan, in bone. Drug extraction was done using a novel microwave assisted extraction (MAE) technique, followed by clean up by microplate solid-phase extraction (MPSPE) and analysis by gas chromatography-mass spectrometry (GC-MS) in the selected ion monitoring (SIM) mode.

Materials and methods

Chemicals

Dextromethorphan (DXM), dextrorphan (DXT), and deuterated dextrorphan (D3-DXT) were obtained from Cerilliant (Round Rock, TX, USA) as 1.0 mg mL^{-1} methanolic solutions and diluted as needed. N-methyl-N-(trimethylsilyl) trifluroacetamide with 1% trimethylchlorosilane (MSTFA + 1%TMCS), obtained from United Chemical Technologies (Bristol, PA, USA), was used as the

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derivatizing agent. All other reagents used were obtained from EMD Chemicals (Gibbstown, NJ, USA).

Dextromethorphan administration and animal care

Animals used in this work were housed in the Laurentian University Animal Care Facility, and all procedures used were approved by the Laurentian University Animal Care Committee. Male Wistar rats were obtained Charles Rivers Laboratories (St Constant, QC, Canada) and were housed at a temperature of 20 °C and placed on 12 h light/dark cycle. The rats were stored 3 per cage and permitted free choice of water and Harlan Teklad Laboratory Diet 864.

The rats (n = 3), used as part of a separate study, received 100 mg/kg dextromethorphan (i.p.), and were then euthanized by carbon dioxide asphyxiation 30 min post-dose. The remains of these rats as well as one drug-free rat used as a control were allowed to decompose under secure mesh wire in a forested area on Laurentian University Campus in Sudbury, Ontario (August 2013). Vertebral bone was harvested and rinsed sequentially with 3 mL PBS (pH = 6), 3 mL methanol, and 3 mL acetone. The bones were allowed to dry overnight then ground using a Micro-Mill® grinder (Bel-Art Products, Pequannock, NJ, USA). The bones were then pulverized using a SPEX 5100 Mixer/Mill (SPEX SamplePrep, Methuchen, NJ, USA). The resulting powder was then prepared by MAE, followed by a GC-MS method validation.

MPSPE and GC-MS method

Drug-free bone tissue extract (BTE) was used as the analytical matrix for the preparation of the standard analyte samples to characterize and validate the MPSPE and GC-MS method. BTE was prepared by sonicating 100 g of pulverized drug-free bone in 500 mL phosphate buffer. All calibrant samples (ranging from 0 to 10 000 ng ml^{-1}) along with 200 ng of internal standard (D3-DXT) were fixed to 1 mL in BTE. 3 mL of acetonitrile:methanol (1:1) was then added before a 12 h incubation at -20 °C to precipitate lipids and proteins. Samples were centrifuged (1100 x g) for 10 min and the supernatant was isolated and concentrated to 1 mL by vacuum centrifugation using a CentriVap Acid-Resistant vacuum concentrator (Labconco, Kansas City, MO, USA). Samples were then primed for SPE by adding 3 mL of PBS and acidifying with 100 μL of glacial acidic acid.

All samples underwent SPE in a 48-well plate with CleanScreen XCEL I sorbent (130 mg, United Chemical Technologies, Bristol, PA, USA). Wells were first conditioned using sequential additions of 3 mL methanol, 3 mL distilled water, and 3 mL of PBS (0.1 M, pH 6.0). Samples were then loaded into the wells by gravity, and then washed with 3 mL PBS and 3 mL of 0.1 M acetic acid. The wells were then dried under vacuum (~350 mmHg) for 5 min. A final 3 mL of methanol was added to complete the wash and then wells were dried again under vacuum (~350 mmHg) for 10 min. Analytes were eluted using 3 mL of 3% NH_4OH in 80:20 ethyl acetate:isopropanol solution. Extracts were evaporated to dryness by vacuum centrifugation (70 °C, 1725 RPM).Samples were reconstituted in 50 μL of MSTFA + 1%TCMS and 50 μL of ethyl acetate then vortexed for 30 s. Samples were then incubated for 1 h at 70 °C.

A Clarus 600C GC-MS instrument (PerkinElmer LAS, Shelton, CT, USA) equipped with a 30 m Zebron ZB-Drug-1 column from Phenomenex (Torrance, CA, USA) was used in selected ion monitoring (SIM) mode for all analyses. Extracts of 2 μL were injected into an injection port held at 250 °C. The oven was held at 100 °C for 3 min, then ramped at 10 °C min^{-1} to 300 °C, and finally held at 300 °C for 5 min, (total run time: 28 min). The source and transfer line temperatures were both 250 °C. DXM was identified using retention time relative to pure standard (t_R =18.4 min) and *m/z* values of **271**, 214 and 150. DXT was identified using retention time relative to pure standard (t_R = 18.6 min) and *m/z* of **329**, 272, and 150. D3-DXT was identified using retention time relative to pure standard (t_R =18.6 min) and *m/z* of **332**, 275, and 153. Ions used for quantitative comparison are shown in bold font.

Standard curves were prepared in triplicate on each of three separate days to assess precision, concentration dependence, and limits of detection and quantitation. The accuracy of the MPSPE-GC-MS method was tested by blinded assay of standard samples prepared in BTE, at concentrations of 250 and 1500 ng mL^{-1} on each of three different days. The stability of derivatized analytes while resident on the instrument autosampler was assessed over a 36-h time period.

MAE – method development and characterization

Analyte stability in methanol as extraction solvent

MAE was performed using a MARS 6 microwave oven (CEM, Matthews, NC, USA), equipped with a 40-position rotor and 25 mL PTFE extraction vessels. To develop an MAE method for extraction analytes from solid bone substrate, the stability of analytes within the system and optimal irradiation time must be determined. Analyte stability was determined by preparing solutions (of 200 ng mL^{-1} or 2000 ng mL^{-1} in methanol (10 mL) in the presence of drug-free bone in the PTFE vessels. The solutions were prepared in triplicate with one solvent control. Stirring was used in all vessels using a magnetic stir bar to ensure sample agitation throughout irradiation. Samples were assayed before irradiation (t = 0 min), during irradiation (t = 30 min), and after irradiation (t = 60 min). The samples were allowed 20 min to cool before aliquots of 1 mL were removed at these intervals. The assays were evaporated to dryness under a gentle stream of air at 70 °C. They were reconstituted in 1 mL PBS (0.1 M, pH 6.0), extracted by the MPSPE method, derivatized and analyzed by GC-MS described by the method above.

Measurement of optimal irradiation time

Establishment of an optimal extraction time required the preparation of solutions (n = 3) containing 0.5 g drug-positive bone in 5 mL methanol in the PTFE vessels. One drug-free bone solution was also prepared as a control. Stirring was actuated in all samples using a magnetic stir bar. At defined intervals (15 min, 30 min, and 60 min), the samples were removed from irradiation, allowed to cool for 20 min and all solvent was removed and replaced with clean methanol. After removal of the methanol, the sample was rinsed once with methanol and the rinse was pooled with the original solvent to maximize analyte recovery. The analyte response at each interval was observed to determine the irradiation time required to achieve maximum extraction yield. After solvent recovery, samples were evaporated under a gentle stream of air at 70 °C. They were reconstituted in 1 mL PBS (0.1 M, pH 6.0), extracted by the MPSPE method, derivatized and analyzed by GC-MS described by the method above.

Results

Expression of results

All analyte levels reported in this work are expressed as mass-normalized response ratios (RR/m), as has been done in various

other published studies.[4,7–11] The response ratio (RR) is the ratio of analyte peak area to that of the internal standard. Normalization of RR to the mass of bone sampled (i.e., RR/m) accounts variation in the mass of bone sampled. Values of RR/m are proportional to analyte concentration and therefore may be used in semi-quantitative comparison of analyte levels in different bone types or in tissues derived from animals exposed to drug under different exposure conditions (i.e., dose, dose frequency, delay between dose and death). Results are reported in this format because the nature of the solid bone matrix precludes accurate measurement and calibration of analyte recovery using techniques conventional to the forensic toxicology laboratory.

Validation of the MPSPE-GC-MS Analysis

The limits of detection and quantification were 10 ng mL^{-1} for DXM and DXT. Precision (% coefficient of variation, % CV) of the ratio of measured responses of *m/z* 271 (DXM) and 329 (DXT)) was acceptable (<25%), ranging from 2% to 19% over the concentration range examined. The assay response was shown to be concentration dependent to at least 10 000 ng mL^{-1}. Both DXM and DXT were fit with quadratic regression lines (DXM R^2 = 0.995–0.999; DXT: R^2 = 0.995–0.996). Standards prepared in bone-tissue extract (n_i =3; 250 and 1500 ng mL^{-1}) were assayed in a blind format to assess the accuracy of the MPSPE-GC-MS method. The accuracy of the method was found to be acceptable (<25%) with bias ranging between 12 and 19%. Analyte stability while resident in extracts on the instrument autosampler was assessed by injection of extracts (post-derivatization) of standard mixtures of analytes at two different concentration levels (100 ng/mL or 1000 ng/mL in each analyte,; n = 3, respectively) that were incubated on the autosampler at ambient temperature at intervals of 0, 6, 12, 18, 24, 30, and 36 h. The criteria for stability was no loss in analyte response to in excess of 25% of the standard corresponding to t = 0 h. For both DXM and DXT, there was no change in RR that differed by more than 25% relative to that of the sample corresponding to t = 0 h for each concentration level, indicating that both DXM and DXT (derivatized) remained stable while resident on the instrument autosampler over that time period.

Analyte stability during irradiation

The effect of microwave irradiation on the stability of the analytes of interest in methanolic solvent was tested (Figure 1). To conclude that an analyte was stable, the measured response ratio (RR ±25%) post-irradiation was required to concur with the measured response ratio pre-irradiation (RR ±25%). The response ratio for both analytes at both concentrations fell within the accepted range after 30 and 60 min of irradiation (<20%) concluding that the analytes are stable in methanol under these microwave conditions.

Extraction time

The effect of extraction time on analyte recovery was investigated to determine an optimal amount of irradiation time with a maximum yield. These results are illustrated in Figure 2. These figures show that the majority (87 95%) of the maximum yield of both analytes is extracted within the first 15 min of irradiation. However both analytes were still marginally detectable at 30 and 60 min. To optimize throughput and yield, 30 min of irradiation was used from that point onwards. Total ion chromatograms (TICs) corresponding to analysis of vertebral bone from drug-free control

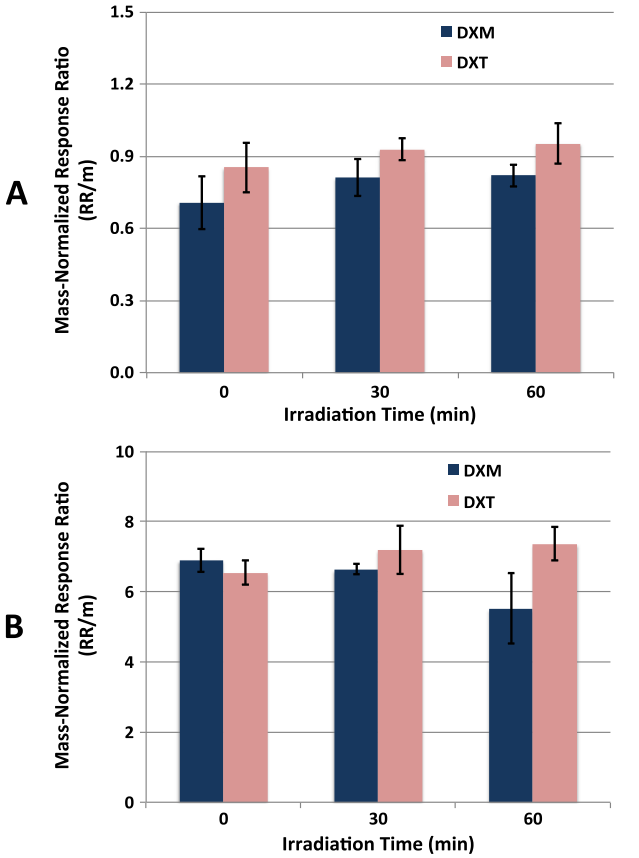

Figure 1. Analyte stability to microwave irradiation (1200 W, 80 oC) in methanol over different time intervals at (A) 100 ng/mL and (B) 1000 ng/mL. Analyte levels are represented as mass-normalized response ratios.

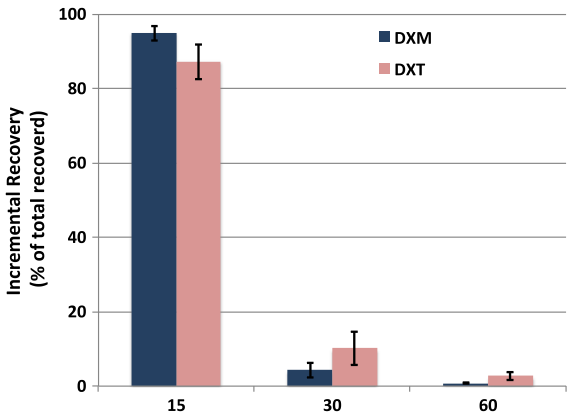

Figure 2. Incremental analyte recovery following successive intervals of MAE in methanol extraction solvent. Incremental recovery is expressed as a percentage of the total amount recovered after 60 min

animals and animals exposed to 100 mg/kg DXM are shown in Figure 3. DXM and DXT were detected in all samples derived from DXM-exposed animals. DXM concentrations estimated to be 0.9–1.5 μg/g; while DXT concentrations were estimated to be 0.5–1.8 μg/g. while DXT concentrations were estimated as: 0.5 1.8 μg·g^{-1}. These values should be regarded as estimates as analyte recovery from the solid bone matrix cannot be accurately measured or calibrated for, using techniques conventional to forensic toxicology, as described above and elsewhere.[4,7–11]

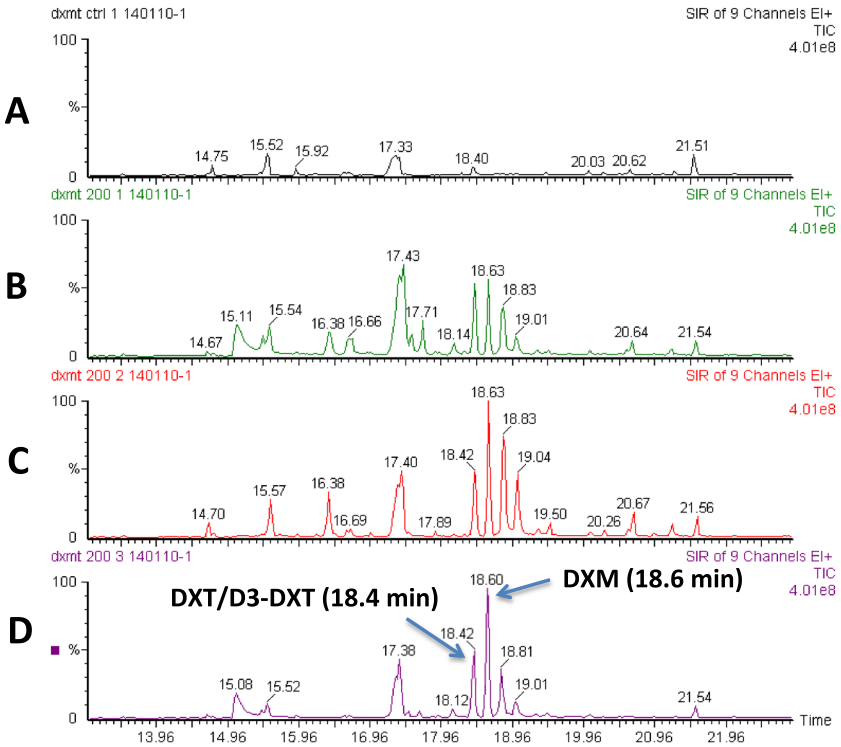

Figure 3. Total Ion Chromatograms (TICs) of extracts of vertebral bone prepared by MAE-MPSPE. A – Drug-free control; B,C,D – DXM-exposed rats (100 mg/kg, i.p.).

Discussion

The purpose of this research was to develop a rapid method for preparation of bone tissue samples for semi-quantitative analysis of DXM and its primary metabolite DXT by GC-MS. The protocol developed is proposed for experiments investigating the effect of different patterns of DXM exposure on measured levels of DXM and its metabolites. This work, in conjunction with other studies, aids in understanding the significance, if any, of drug and metabolite levels in toxicological analysis of bone. Recent work in our laboratory[7,8,11,12] suggests that the relationship between drug and metabolite levels may be useful in the discrimination of different patterns of drug exposure (e.g. acute, high dose vs. repeated low dose exposures). In order to further investigate this prospect, a large number of samples derived from different exposure conditions (dose, frequency, delay between exposure and death, etc.) must be analyzed. Accordingly, sample preparation time is a limiting factor in throughput in such studies. The method described here provides for rapid and parallel processing of 40 bone samples, with extraction times that are substantially shorter than what has been used in previously published work, including that from our own laboratory,[4,5,7,8,11,12] which used solvent extractions with long incubation times (>18 h).

The protocol was developed with intentions of extending the same method to other metabolites of DXM. Well known are two other metabolites of DXM: an *N*-desmethyl-DXM (3-methoxy morphinan) and *N*-desmethyl-DXT (3-hydroxymorphinan). To allow for the introduction of other metabolites to the protocol, a 30 m column was used and optimized due to its higher power to resolve

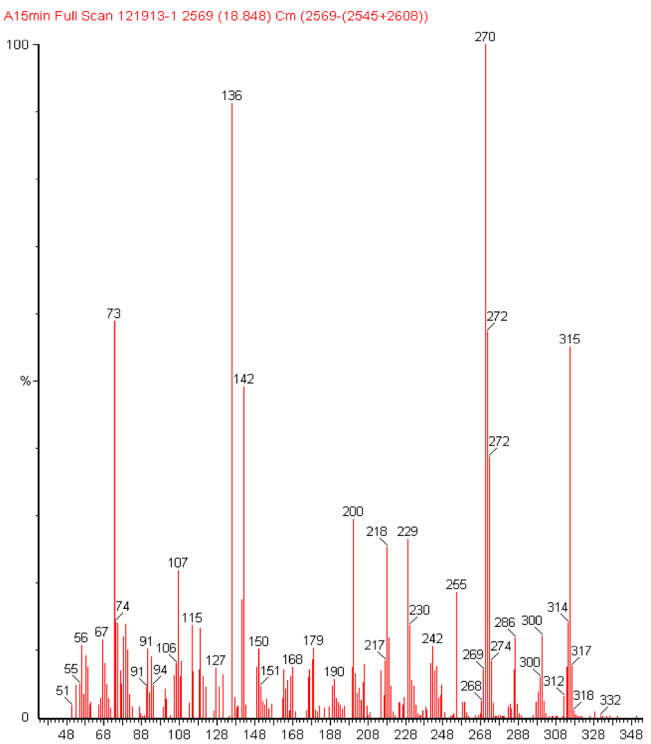

Figure 4. EI mass spectrum (background subtracted) corresponding to peak at t_R =18.8 min (derived from analysis of vertebral bone extract of DXM-exposed rat, obtained from optimization of MAE extraction time, 15-min interval).

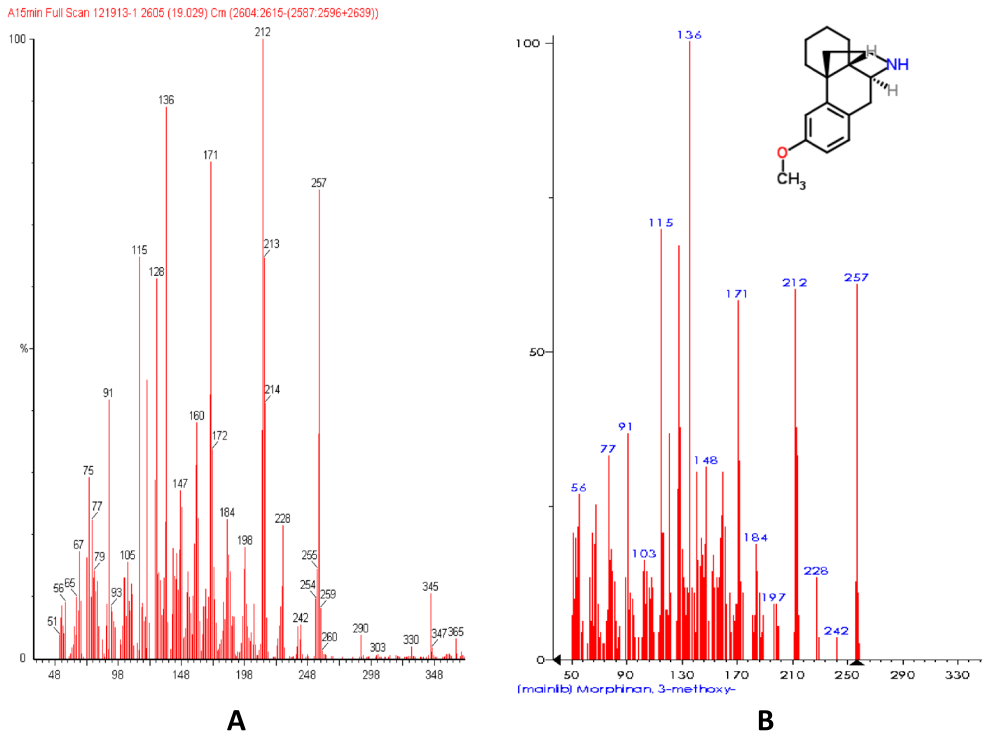

Figure 5. EI mass spectra corresponding to (A) peak at t_R =19.0 min (background subtracted, derived from analysis of vertebral bone extract of DXM-exposed rat, obtained from optimization of MAE extraction time, 15-min interval); (B) reference spectrum of 3-methoxy-morphinan from NIST spectral library (match probability: 0.94)

multiple analytes in one run. These metabolites may hopefully also be extracted using the same SPE parameters set out in this study. Indeed, examination of the chromatograms shown in Figure 3 shows that while resolution of DXM and DXT from each other and from adjacent peaks was strong, there are clear peaks present in TICs corresponding to DXM-exposed animals that are absent in that corresponding to the drug-free control, which may reasonably be assigned to potential DXM metabolites. The peaks with t_R = 18.8 min displayed EI mass spectra (Figure 4) that may correspond to the trimethyl silyl (TMS) derivative of *N*-desmethyl DXT, with putative M^+ ion with *m/z* =315, and prominent fragments at *m/z* =270, and 136. At the time of this work, *N*-desmethyl DXT was not included as part of the method validation, and the EI mass spectrum was not included in our standard spectral library, so the identification of the compound with t_R =18.8 min is inconclusive. The peaks with t_R =19.0 min displayed EI mass spectra which generated a most probable match with 3-methoxy morphinan using the NIST standard spectral library. The standard spectrum and observed spectrum (t_R =19.03 min) are shown for comparison in Figure 5. Again, 3-methoxy morphinan was not included as part of the method validation, so the identification of the compound with t_R =19.0 min also remains inconclusive at this time.

Conclusions

The utility of the MAE-MPSPE-GC-MS approach in the assay of dextromethorphan and dextrorphan in decomposed skeletal tissues was illustrated. The data generated will support future work applying this method to studies of the effects of different dextromethorphan exposure patterns, including acute vs. repeated dosing and varying the delay between exposure and death on the relative levels of dextromethorphan and dextrorphan in bone.

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References

[1] I.M. McIntyre, C.V. King, M. Boratto, O.H. Drummer. Post-mortem drug analyses in bone and bone marrow. *Ther. Drug Monit.* **2000**, *22*, 79.
[2] K.K. McGrath, A.J. Jenkins. Detection of drugs of forensic importance in postmortem bone. *Am. J. Forensic Med. Pathol.* **2009**, *30*, 40.
[3] T. Delabarde, C. Keyser, A. Tracqui, D. Charabidze, B. Ludes. The potential of forensic analysis on human bones found in a riverine environment. *Forensic Sci. Int.* **2013**, *228*, e1.
[4] J.H. Watterson, N.A. Desrosiers, C.C. Betit, D. Dean, J.F. Wyman. Relative distribution of drugs in decomposed skeletal tissue. *J. Anal. Toxicol.* **2010**, *34*, 510.
[5] J.H. Watterson, J.E. Botman. Effects of tissue type and the dose-death interval on the detection of acute diazepam exposure in bone and marrow with solid-phase extraction, ELISA and liquid chromatography tandem mass spectrometry. *J. Forensic Sci.* **2009**, *54*, 708.
[6] P. Guillot, M. de Mazancourt, M. Durigon, J.C. Alvarez. Morphine and 6-acetylmorphine concentrations in blood, brain, spinal cord, bone marrow and bone after lethal acute or chronic diacetylmorphine administration to mice. *Forensic Sci. Int.* **2007**, *166*, 139.
[7] J.H. Watterson, J.P. Donohue, C.C. Betit. Comparison of relative distribution of ketamine and norketamine in decomposed skeletal tissues following single and repeated exposures. *J. Anal. Toxicol.* **2012**, *36*, 429.
[8] J.H. Watterson, H.M. Cornthwaite. Discrimination between patterns of drug exposure by toxicological analysis of decomposed skeletal

tissues, Part II: Amitriptyline and citalopram. *J. Anal. Toxicol.* **2013**, *37*, 565.

[9] H.M. Cornthwaite, J.H. Watterson. Microwave assisted extraction of ketamine and metabolites from skeletal tissues. *Anal. Methods* **2014**, *6*, 1142.

[10] H.M. Cornthwaite, J.H. Watterson. The influence of body position and microclimate on ketamine and metabolite distribution in decomposed skeletal remains. *Forensic Sci. Int.* **2014**, *242*, 261.

[11] T.R. Wiebe, J.H. Watterson. Analysis of tramadol and o-desmethyltramadol in decomposed skeletal tissues following acute and repeated tramadol exposure by gas chromatography mass spectrometry. *Forensic Sci. Int.* **2014,** DOI: 10.1016/j.forsciint.2014.07.010.

[12] J.H. Watterson, D. Man. Discrimination of Single and Repeated Xylazine Exposures in Skeletal Tissues by LC-TOF MS. Society of Forensic Toxicologists Annual Meeting, Orlando, FL, **2013**. (October 28-November 1, 2013)