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ORIGINAL ARTICLE

Influence of dose-death interval on colchicine and metabolite distribution in decomposed skeletal tissues

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Abstract The semi-quantitative analysis of decomposed bone of rats exposed to colchicine and euthanized following different time intervals postexposure (i.e., dose-death interval, DDI) is described. Rats received colchicine (50 mg/kg, i.p.) and were euthanized 30 min (DDI1; n=4), 60 min (DDI2; n=4), or 180 min (DDI3; n=4) postdose. Drug-free animals (n=3) served as negative controls. Perimortem heart plasma was collected. Remains were decomposed to skeleton outdoors and then collected and sorted (skull, vertebrae, rib, pelvis, femur, tibia). Bones were dried, pulverized, and prepared by microwave-assisted extraction and microplate solid-phase extraction (MAE-MPSPE), followed by analysis for colchicine, 3-demethylcolchicine (3DMC), and 2-demethylcolchicine (2DMC) by ultra-high-performance liquid chromatography with photodiode array detection (UHPLC-PDA) at 350 nm. Bone type was a main effect (Kruskall-Wallis, $p<0.05$) with respect to drug level (expressed as mass-normalized response ratio, RR/m) for each analyte, at each DDI. For all samples, DDI was a main effect (Kruskall-Wallis, $p<0.05$) with respect to analyte level, and the ratio of analyte levels (RR_{3DMC}/RR_{COLCH}, RR_{2DMC}/RR_{COLCH}, and RR_{2DMC}/RR_{3DMC}). Bone COLCH levels varied by 19-fold, 12-fold, and 60-fold across all bone types in the DDI1, DDI2, and DDI3 groups, respectively. Bone 3DMC levels varied by 12-fold, 11-fold and 17-fold across all bone types in the DDI1, DDI2, and DDI3 groups, respectively. Bone 2DMC levels varied by 20-fold, 14-fold, and 14-fold across all bone types in the DDI1, DDI2, and DDI3 groups, respectively. Values of RR_{3DMC}/RR_{COLCH} varied by 16-fold, 5-fold, and 5-fold across all bone types in the DDI1, DDI2, and DDI3 groups, respectively. Values of RR_{2DMC}/RR_{COLCH} varied by 10-fold, 6-fold, and 12-fold across all bone types in the DDI1, DDI2, and DDI3 groups, respectively. Values of RR_{2DMC}/RR_{3DMC} varied by 3-fold, 5-fold, and 2-fold across all bone types in the DDI1, DDI2, and DDI3 groups, respectively. Measured analyte levels in bone correlated poorly with corresponding levels in blood (r=−0.65–+0.31). Measured values of RR_{2DMC}/RR_{COLCH} and RR_{2DMC}/RR_{3DMC} in bone also correlated poorly with corresponding values in blood. Measured values of RR_{3DMC}/RR_{COLCH} were well correlated with corresponding blood levels for all bone types except skull (r=0.91–0.97).

Keywords Forensic toxicology · Bone · Colchicine · Microwave assisted extraction · UHPLC

Introduction

The utility of bone tissue as a matrix for toxicological analysis has been the subject of various investigations in recent years [1–7]. From those investigations, it may be noted that the utility of quantitative measurements of bone drug concentrations remains unclear, as various studies have shown that there is often a poor correlation between drug levels in bone and those in corresponding blood samples [1, 3, 4]. This phenomenon is compounded by the large inter-bone variation in drug and metabolite levels that has been observed in a given body and may be further compounded by influences of postmortem environment and the extent of decomposition on drug and metabolite concentrations and relative distribution. As a consequence, the interpretative value of any given drug or metabolite concentration in bone is limited at this time, and it may be

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that any interpretive value will only be gleaned by analysis of bone under various conditions of drug exposure, delay between exposure and death, and postmortem environment.

Recent work in our laboratory has suggested that different patterns of drug exposure may be discriminated by evaluation of the relationship between levels of drug and those of one or more of its metabolites [4, 8, 9]. In particular, we have observed that the ratio of levels of parent drug and metabolite (or of different metabolites) may be more discriminatory between certain circumstances of acute vs. repeated exposures. This observation may be due in part to similar patterns of distribution of drug and metabolites to the different bones, such that measurement of the ratio of levels of drug and metabolite offsets the variation in concentration between different bones. It is also important to characterize the kinetics of drug and metabolite distribution in bone after a given exposure. Drugs that undergo rapid, moderate drug distribution into bone may provide kinetic profiles that mirror that in blood, while those drugs with more complex kinetics may not.

This study was done to assess the impact of the time interval between exposure and death on the relative distribution of colchicine and its metabolites, 3-demethylcolchicine and 2-demethylcolchicine, in skeletal remains. To achieve this, a recently reported method for semi-quantitative analysis of skeletal tissue based on microwave-assisted extraction (MAE), microplate solid-phase extraction (MPSPE), and ultra-high-performance liquid chromatography (UHPLC) [10] was applied to the remains of rats exposed to a given dose of colchicine, with variable time intervals between exposure and death (i.e., dose-death interval).

Methods

Chemicals

Colchicine (COLCH) and demecolcine (DEM), used as an internal standard, were purchased from Sigma Aldrich (Oakville, ON). 3-Demethylcolchicine (3DMC) and 2-demethylcolchicine (2DMC) were obtained from Toronto Research Chemicals (Toronto, ON). Methanol and acetonitrile used in extraction were high-performance liquid chromatography grade and purchased from EMD Chemicals (Gibbstown, NJ). Water, acetonitrile, and formic acid used for UHPLC mobile phase was LC/MS grade and obtained from Fisher Scientific. All other chemicals were reagent grade and were obtained from EMD Chemicals.

Colchicine administration and bone sample preparation

Wistar rats (n=15) were divided into three groups (n_i=5; DDI1, DDI2, DDI3). All animals received 50 mg/kg colchicine (i.p). Animals in groups DDI1, DDI2, and DDI3 were euthanized by CO_2 asphyxiation approximately 30, 60, or 180 min postdose. Three animals remained drug-free control to serve as negative controls. All animals were allowed to decompose to skeleton in secure caging in a wooded area at Laurentian University in Sudbury, Ontario. All bones were collected, sorted, and separated according to animal (e.g., DDI1-1) and type (e.g., femur). Each bone type was then rinsed sequentially with 3 mL phosphate buffer (PB6: 0.1 M, pH=6), 3 mL methanol, and 3 mL acetone and dried under ambient conditions overnight. Bones were ground using a Micro-Mill® grinder (Bel-Art Products, Pequannock, NJ) and then further pulverized to powder using a SPEX 5100 Mixer/Mill (SPEX SamplePrep, Methuchen, NJ).

Microwave-assisted extraction, microplate solid-phase extraction, and ultra-high-performance liquid chromatography methods

The validation of the MAE, MPSPE, and UHPLC methods used in analysis of pulverized bone has been described in a previously published report [10]. MAE was done using a MARS 6 microwave oven (CEM Corporation, NC), equipped with a 40-position rotor and 25 mL PTFE extraction vessels. Stirring was used in all vessels. Bone (0.5 g) from a given type collected from each animal was combined with 10 mL methanol in a PTFE vessel with magnetic stirring bar. Following brief vortex mixing, samples underwent microwave irradiation (1200 W, 80 °C) for 30 min. Methanol fractions were recovered and bone was washed with a second fraction (10 mL) methanol, which was then recovered and combined with the original extract. Methanolic extracts were then evaporated to dryness under a gentle stream of air at 70 °C, reconstituted in 1 mL phosphate buffer (PB6; 0.1 M, pH 6.0), and internal standard (250 ng DEM) was added. Each sample underwent precipitation of lipids and proteins by addition of 3 mL acetonitrile/methanol (1:1) and incubation at −20 °C for 2 h. Samples were centrifuged at 1100×g and supernatants were recovered and evaporated to approximately 1 mL under a gentle stream of air at 70 °C.

All samples underwent SPE in a 48-well plate format using Styre Screen DVB sorbent (60 mg/well, United Chemical Technologies, Bristol, PA). Wells were conditioned with 3 mL methanol, 3 mL distilled water, and 3 mL PB6 prior to sample loading. After loading samples by gravity, wells were washed with 3 mL 0.1 M HCl and 3 mL 0.1 M HCl in 20:80 methanol/water (i.e., 0.1 M HCl prepared using 20:80 methanol/water as the diluent). Wells were dried under vacuum at approximately 350 mmHg for 10 min and then eluted with 3 mL 3 % ammonium hydroxide in ethyl acetate/isopropanol (80:20 v/v). Extracts were evaporated under a gentle stream of air at 70 °C and reconstituted in 250 μL of mobile phase A (0.1 % formic acid (v/v) in 90:10 acetonitrile/water). Samples were centrifuged for 10 min at 13,000×g and

then transferred to autosampler vials, and 15 μL of sample was injected into the UHPLC.

Ultra-high-performance liquid chromatography

A Waters Acquity™ Ultra Performance LC (Waters, Milford, MA) with photodiode array detection (PDA) was used for the analysis of COLCH, 3DMC, and 2DMC in extracts. The column used was a Kinetex C18 column (150 mm×2.1 mm, 1.7-μm particle size; Phenomenex, Torrance, CA). Samples were run using a binary gradient elution (A—0.1 % (*v*/*v*) formic acid, 10 % (*v*/*v*) acetonitrile, and 90 % (*v*/*v*) water; B—acetonitrile). The mobile phase gradient began with 90:10 A/B, held for 3 min, followed by a linear increase to 65:35 A/B over 3 min, followed by a linear increase to 10:90 A/B over 2 min, and reverted back to 90:10 A/B over 2 min. The mobile phase was then held at 90:10 A/B for 1 min for a total run time of 11 min at a constant flowrate of 0.400 mL/min. The autosampler was maintained at 25 °C, with column temperature set to 50 °C. The photodiode array detector range was set from 220 to 400 nm, and quantitative comparisons were made using the response ratio (i.e., RR—the ratio of peak areas for drug and internal standard) measured at 350 nm.

Validation of the MPSPE and UHPLC methods involved preparation of standard analyte samples in drug-free bone tissue extract (BTE) as the analytical matrix, as has been described elsewhere [4, 8]. Method validation was based on extraction and measurement of drug from skeletal tissue extracts. Standard curves were prepared in triplicate on each of six different days to assess precision, limits of detection and quantitation, and linearity of the analytical method. The response ratio was linear (1/x weighting; $R^2 \geq 0.99$) from 10 to 2000 ng/mL and was verified ($n=3$) separately to be linear up to at least 10,000 ng/mL for all drugs. The precision, measured as the coefficient of variation (CV) of triplicate analyses of extracted standards over the linear range, was <20 % (0.3–18 %) on each of six different days. The limit of detection for the method is approximately 10 ng/mL for each analyte, defined as the lowest concentration assayed where the analyte could still be detected, but was not subject to precision criteria using CV ≤20 %. The limit of quantification for the method is approximately 25 ng/mL for each analyte, defined as the lowest concentration assayed where the analyte was detected, and precision was within CV ≤20 %. Accuracy was determined through blinded duplicate analysis of standard samples of each analyte in BTE on three different days, at levels 250 and 1300 ng/mL. In those assays, deviation of measured concentrations differed from the actual targets by 13–24 % for COLCH, 14–19 % for 3DMC, and 16–23 % for 2DMC.

Statistical analysis

All statistical analysis was performed using StatPlus 2009 software (AnalystSoft Inc.,www.analystsoft.com/). The Kruskall-Wallis test, a non-parametric analysis of variance, was used to compare measured values to determine whether bone type or DDI significantly influenced measured analyte levels and ratios of analyte levels. This non-parametric test was chosen as it does not presume that measured values within a group are normally distributed.

Results

Expression of data

All analyte levels reported in this work are expressed as normalized response ratios (RR/m), as has been done in various other published studies [3–9]. The response ratio (RR) is defined as 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) minimizes the influence of variation in the mass of bone sampled. Values of RR/m are proportional to analyte concentration, allowing them to be used in semi-quantitative comparison of analyte levels in similarly prepared bone samples that are derived from different anatomic sites or from different animals that have experienced different drug-exposure conditions (e.g., dose-death interval or chronic vs. single exposure). As has been described elsewhere [3–5, 8, 9], analyte levels are reported as RR/m values because the nature of the solid bone matrix precludes accurate measurement and calibration of analyte recovery using techniques conventional to the forensic toxicology laboratory.

Survey of distribution of COLCH, 3DMC, and 2DMC in bone

The levels (RR/m) of each analyte across the various bone types (skull, vertebrae, rib, pelvis, femora, and tibiae) and DDI groups are summarized in Fig. 1. The ratio of analyte levels (i.e., RR_{3DMC}/RR_{COLCH}, RR_{2DMC}/RR_{COLCH}, RR_{2DMC}/RR_{3DMC}) across the various bone types and DDI groups are summarized in Fig. 2. The measured levels (RR/m) of each analyte in all samples measured (i.e., pooled data) are shown in the box-and-whisker plots in Fig. 3, while the measured values for the ratio of analyte levels are shown in the box-and-whisker plots in Fig. 4.

The levels (RR/m) of COLCH in the various bone types examined and for all DDI values examined are illustrated in Fig. 1a. Overall, the largest COLCH levels were observed in the vertebral and femoral bone and the lowest COLCH levels were observed in the rib. COLCH levels varied by 2–33-fold within an animal and by 19-fold, 12-fold, and 60-fold across

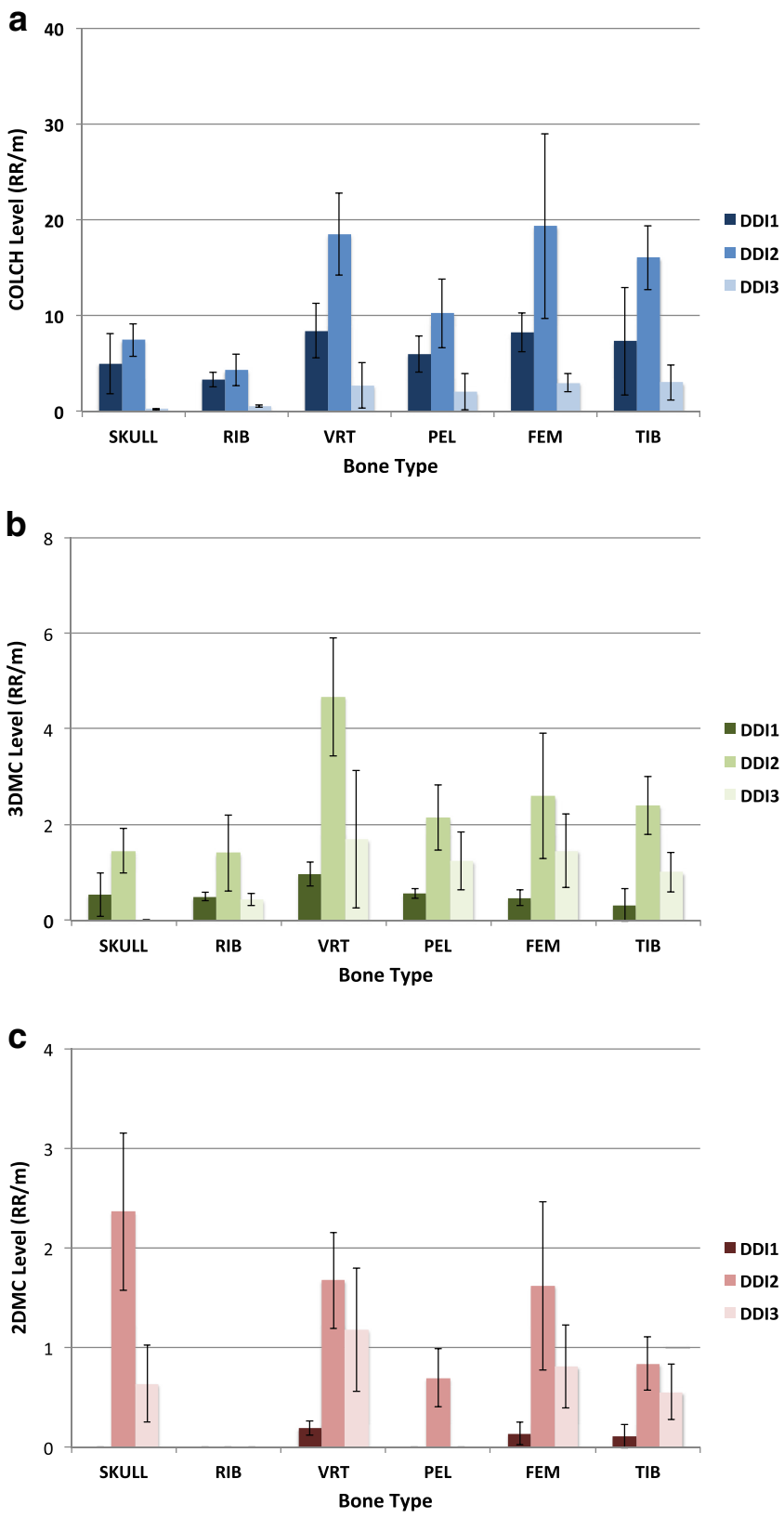

Fig. 1 Mean (±S.D.) analyte levels, expressed as mass-normalized response ratios (*RR/m*), for each bone type sampled, at various dose-death intervals (*DDI*). **a** COLCH, **b** 3DMC, **c** 2DMC

all of the samples from the DDI1, DDI2, and DDI3 groups, respectively. The Kruskall-Wallis non-parametric analysis of variance showed that bone type was a main effect for COLCH level in the DDI2 and DDI3 groups ($p<0.05$), but not for the

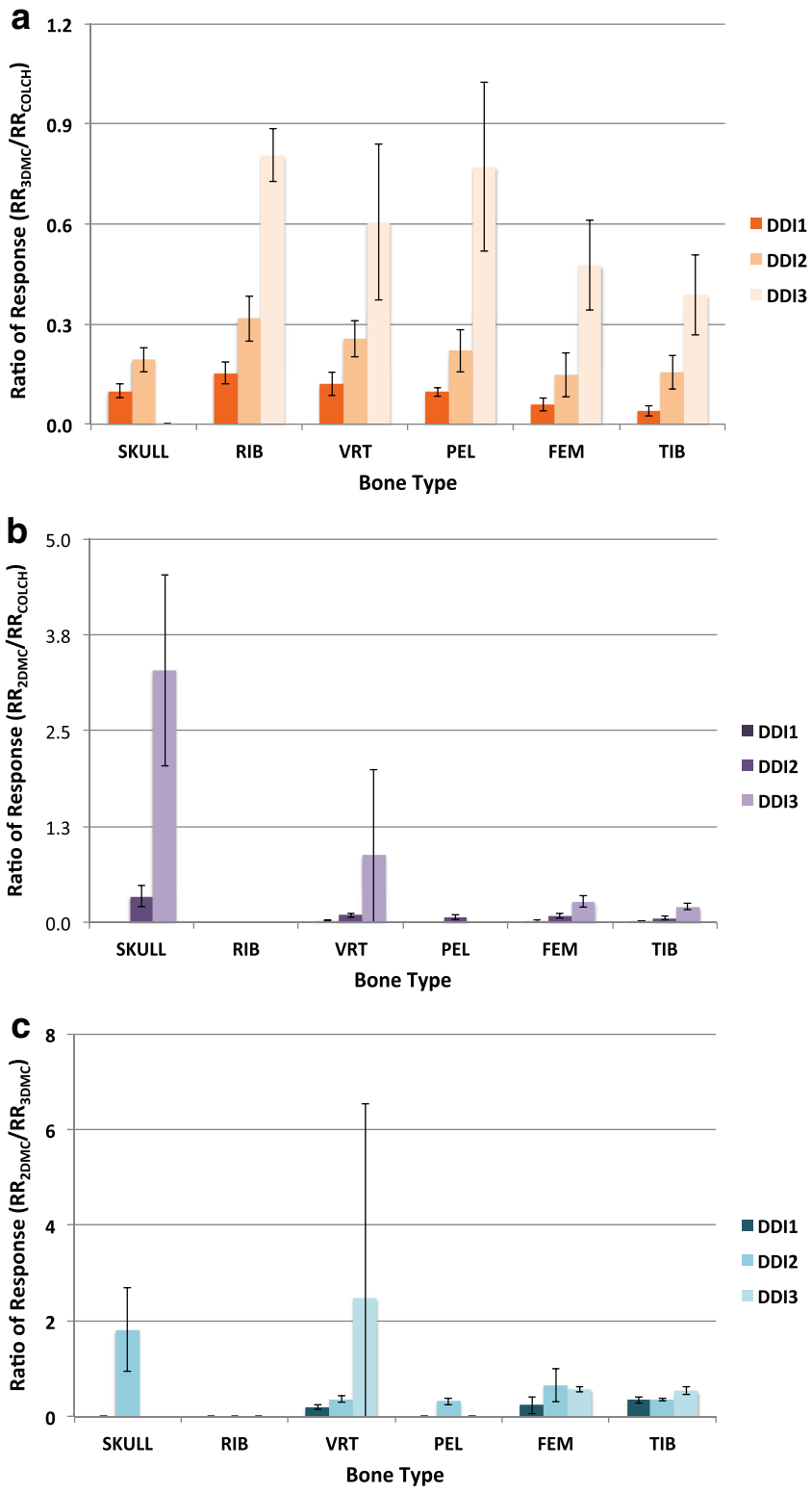

Fig. 2 Mean (±S.D.) values of the ratio of analyte levels, (RR_i/RR_j), for each bone type sampled, at various dose-death intervals (*DDI*). **a** RR_{3DMC}/RR_{COLCH}, **b** RR_{2DMC}/RR_{COLCH}, **c** RR_{2DMC}/RR_{3DMC}

DDI1 group ($p>0.1$), and that DDI was a main effect for COLCH level (when data from all bone types was grouped for a given DDI). Based on the response ratios of calibrators, prepared in BTE, collected over the concentration range 10–2000 ng/mL in the same manner as described in the method validation study [10], bone COLCH levels corresponded to concentrations of approximately 0.20–2.9, 0.39–4.2, and 0.07–0.95 μg/g in the DDI1, DDI2, and DDI3 groups, respectively. However, based on the limitations in the ability to accurately quantify drugs and metabolites in the solid bone

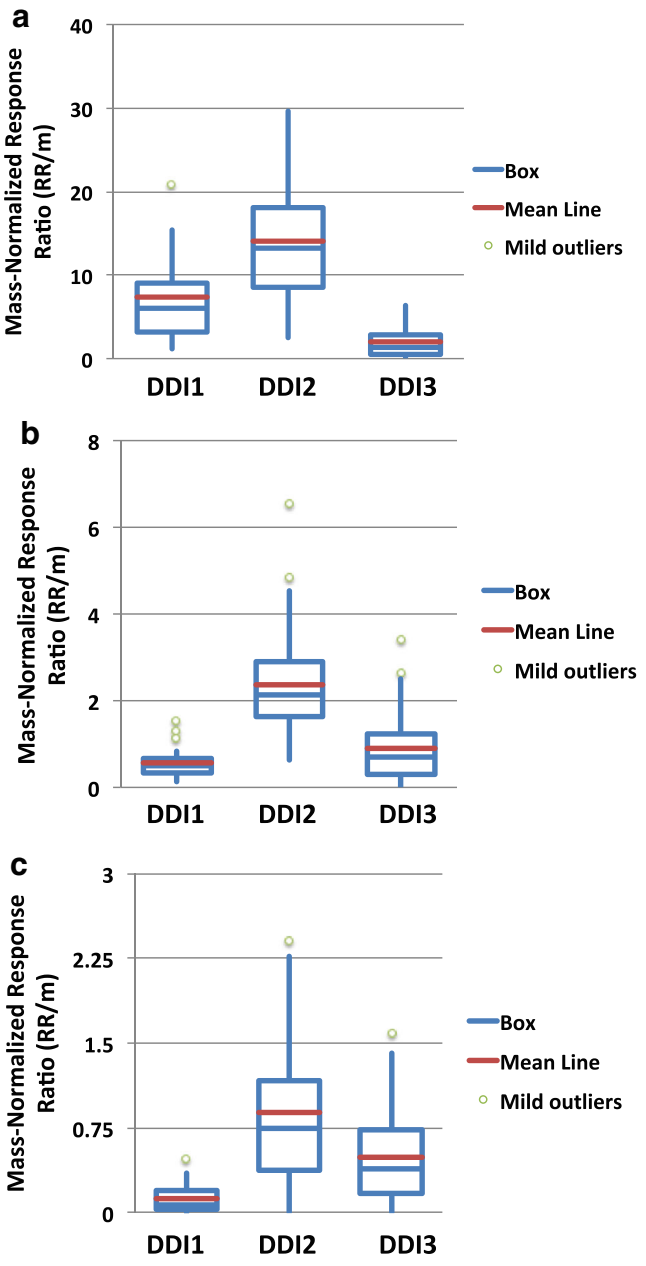

Fig. 3 *Box-and-whisker plots* illustrating analyte levels, expressed as mass-normalized response ratios (RR/m), for all samples assayed, at each dose-death interval (*DDI*). **a** COLCH, **b** 3DMC, **c** 2DMC

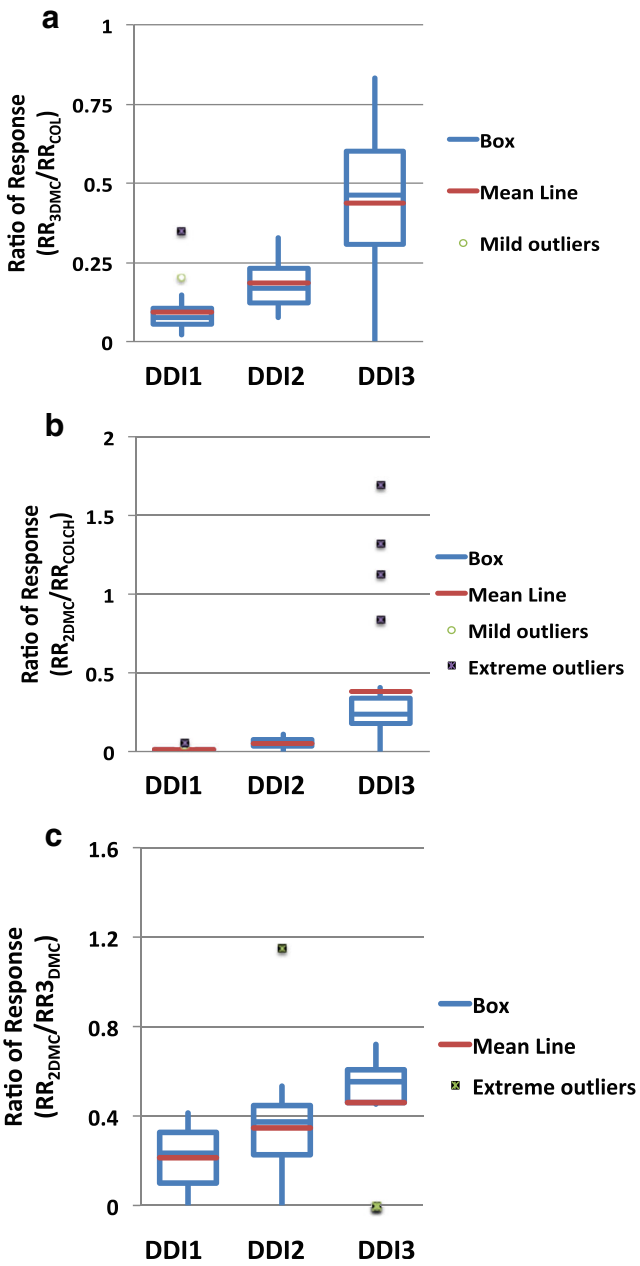

Fig. 4 *Box-and-whisker plots* illustrating the ratio of analyte levels, (RR_i/RR_j), for each bone type sampled, at each dose-death interval (*DDI*). **a** RR_{3DMC}/RR_{COLCH}, **b** RR_{2DMC}/RR_{COLCH}, **c** RR_{2DMC}/RR_{3DMC}

matrix as outlined above, these values must be taken as estimates only.

The levels (RR/m) of 3DMC in the various bone types examined, and for all DDI values examined, are illustrated in Fig. 1b. The largest 3DMC levels were generally observed in the vertebral bone in the DDI2 group. Where detectable, the lowest levels were observed in the tibial bone in the DDI1 group. 3DMC was not detected in the skull bone samples from the DDI3 group. Where detectable, levels varied by 4–8-fold within an animal and by 12-fold, 11-fold, and 17-fold in all of the samples from the DDI1, DDI2, and DDI3 groups, respectively. The Kruskall-Wallis non-parametric analysis of variance showed that bone type was a main effect for 3DMC level in the DDI2 and DDI3 groups ($p<0.05$), but not for the DDI1 group ($p>0.1$), and that DDI was a main effect for 3DMC (when data from all bone types was grouped for a given DDI). Estimates of bone 3DMC levels corresponded to concentrations of approximately 0.06–0.41, 0.16–1.79, and 0.05–0.93 μg/g in the DDI1, DDI2, and DDI3 groups, respectively.

The levels (RR/m) of 2DMC in the various bone types examined and for all DDI values examined are illustrated in

Fig. 1c. The largest 2DMC levels were generally observed in the skull in the DDI2 group. 2DMC was not detected in any of the samples from the rib (DDI1, DDI2, DDI3), from the pelvis (DDI1 and DDI3), and from the skull (DDI1). Where detected, the lowest 2DMC levels were observed in the tibial bone samples from the DDI1 group. In samples with detectable 2DMC, levels varied by 2–7-fold within an animal and by 20-fold, 14-fold, and 14-fold in all of the samples from the DDI1, DDI2, and DDI3 groups, respectively. The Kruskall-Wallis non-parametric analysis of variance showed that bone type was a main effect for 2DMC level in all DDI groups ($p<0.05$) and that DDI was a main effect for 2DMC level (when data from all bone types were grouped for a given DDI). Estimates of 2DMC levels corresponded to concentrations of approximately 0.06–0.18, 0.06–0.92, and 0.05–0.60 μg/g in the DDI1, DDI2, and DDI3 groups, respectively.

The Kruskall-Wallis analysis showed that dose-death interval was a main effect ($p<0.05$) for all the measured ratio of levels of analytes measured (i.e., RR_{3DMC}/RR_{COLCH}, RR_{2DMC}/RR_{COLCH}, RR_{2DMC}/RR_{3DMC}). Measured levels of RR_{3DMC}/RR_{COLCH} varied by 16-fold, 5-fold, and 5-fold in the DDI1, DDI2, and DDI3 groups, respectively. Measured levels of RR_{2DMC}/RR_{COLCH} varied by 10-fold, 6-fold, and 12-fold in the DDI1, DDI2, and DDI3 groups, respectively. Measured levels of RR_{2DMC}/RR_{3DMC} varied by 3-fold, 5-fold, and 2-fold in the DDI1, DDI2, and DDI3 groups, respectively.

Survey of distribution of COLCH, 3DMC, and 2DMC in perimortem plasma

The levels (RR/m) of each analyte in perimortem plasma are illustrated in Fig. 5, for the DDI1, DDI2, and DDI3 groups, respectively. Overall, the largest mean analyte levels were observed in DDI1, with progressively lower levels, on average, observed in the DDI2 and DDI3 groups, respectively. In the DDI1 group, plasma COLCH, 3DMC, and 2DMC levels ranged from 11 to 39, 0.96 to 2.7, and 0.06 to 0.35 μg/mL, respectively. In the DDI2 group, plasma COLCH, 3DMC, and 2DMC levels ranged from 2.7 to 11, 0.45 to 1.7, and 0.03 to 0.06 μg/mL, respectively. In the DDI3 group, plasma COLCH and 3DMC levels ranged from 0.58 to 0.95 and 0.14 to 0.32 μg/mL, respectively. 2DMC was not detected in any plasma samples from the DDI3 group (Fig. 6).

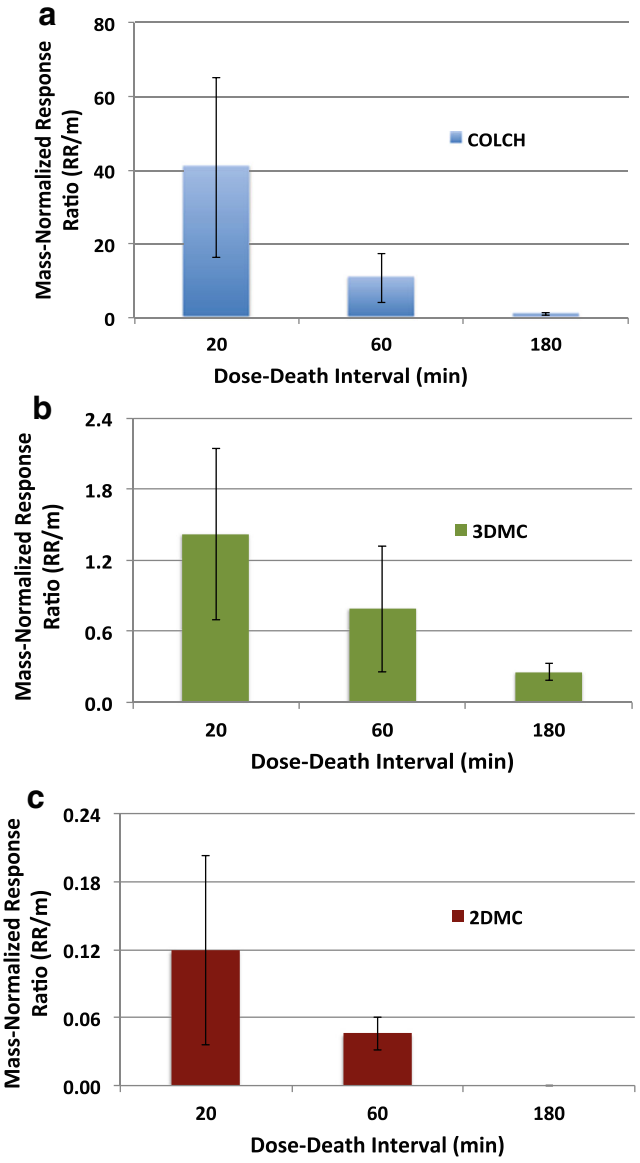

Fig. 5 Mean (±S.D.) analyte levels, expressed as mass-normalized response ratios (*RR/m*), in perimortem plasma, at various dose-death intervals (*DDI*). **a** COLCH, **b** 3DMC, **c** 2DMC

Discussion

The primary purpose of this work was to examine the influence of the time interval between COLCH exposure and death on the relative levels of COLCH, 2DMC, and 3DMC across different bone types. The application of the previously published method for determination of those analytes [10] was valuable to this work, by allowing for the parallel processing of 40 samples, which provided a dramatic improvement in analytical throughput relative to what was possible in the earlier work [3, 4, 8, 9]. This study contributes to a larger body of work that aims to determine if, and under what circumstances, different patterns of drug exposure may be discriminated through analysis of decomposed skeletal tissues. Work in our laboratory to date [4, 8, 9] suggests that different exposure patterns may be discriminated through examination of the relationship between drug and metabolite levels (e.g., ratio of levels between parent drug and metabolite or between different metabolites). As was observed in those studies, the levels of drug and metabolites in bone observed in this work were widely variable within and between animals. Ratios of analyte levels (i.e., RR_{3DMC}/RR_{COLCH}, RR_{2DMC}/RR_{COLCH},

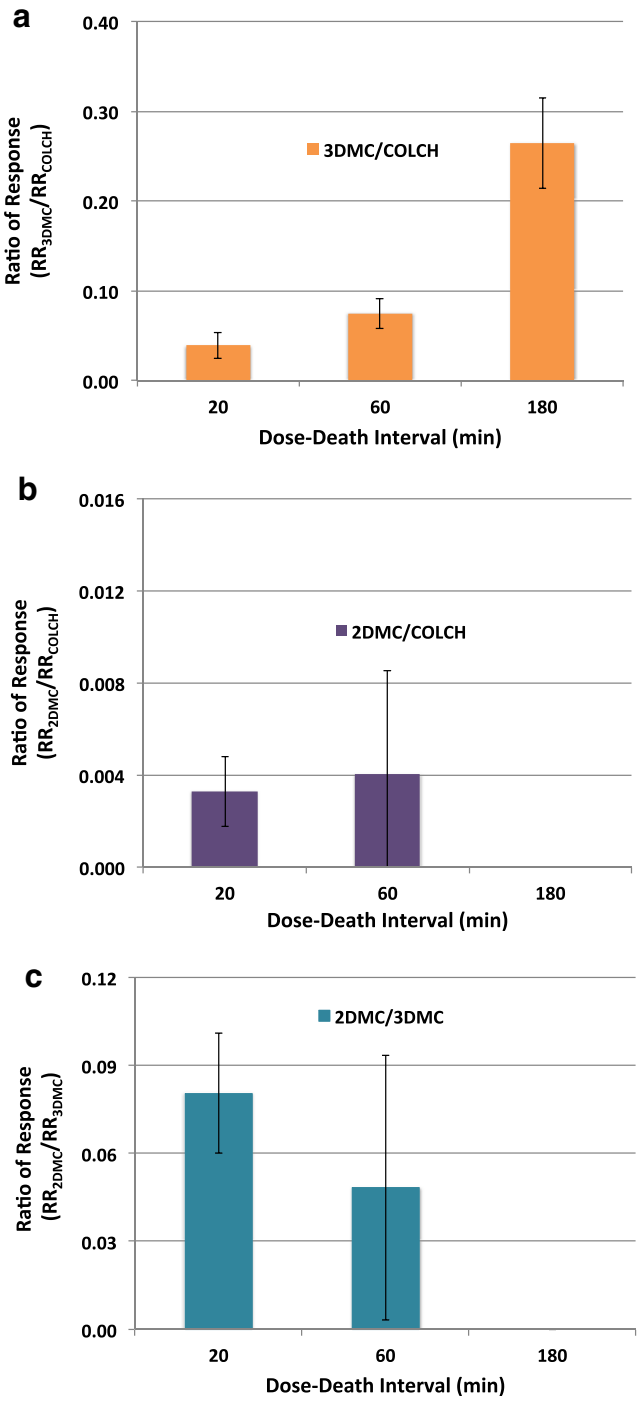

Fig. 6 Mean (±S.D.) values of the ratio of analyte levels, (RR_i/RR_j), perimortem plasma, at various dose-death intervals (*DDI*). **a** RR_{3DMC}/RR_{COLCH}, **b** RR_{2DMC}/RR_{COLCH}, **c** RR_{2DMC}/RR_{3DMC}

RR_{2DMC}/RR_{3DMC}) were generally less variable than absolute analyte levels. This may be the result of some correction for the variation in bone analyte distribution between different bones since the extent of analyte partition into the various bones may be similar for each analyte.

It is clear from the data that the drug levels in bone were poorly correlated to those in perimortem plasma (r=−0.65–+0.31). Similarly, measured values of RR_{2DMC}/RR_{COLCH} and RR_{2DMC}/RR_{3DMC} in bone also correlated poorly with corresponding values in blood (r=−0.51–+0.24 and r=−0.63–+0.04, for measured values of RR_{2DMC}/RR_{COLCH} and RR_{2DMC}/RR_{3DMC}, respectively). Measured values of RR_{3DMC}/RR_{COLCH} were well correlated with corresponding blood levels for all bone types except the skull (r=+0.91–+0.97). The correlation in RR_{3DMC}/RR_{COLCH} values between the plasma and skull bone was complicated by the fact that 3DMC was not detected in skull bone samples in the DDI3 group.

Interestingly, the data in Figs. 1 and 5 suggest differences in the time course of the analyte levels in plasma and those in bone. While mean analyte levels in plasma decreased with progressively longer DDI, those in bone were generally highest in the DDI2 group. Further, COLCH levels in the DDI1 group were generally higher than those in the DDI3 group, while metabolite levels (2DMC and 3DMC) in the DDI3 group were generally higher than those in the DDI1 group. These data may be suggestive of a pattern wherein the analytes are distributing very extensively into bone tissue, perhaps via lipid-rich marrow, such that bone levels continue to rise after blood levels have reached their maximum value and have begun to fall. A study by Rochdi et al. [11] reported one case of a human fatality wherein colchicine appeared to accumulate significantly in the bone marrow. A weakness of this study is that the measurements were made using a validated immunoassay, although cross-reactivity with the 2DMC, 3DMC, and a third metabolite (*N*-deactylcolchicine) was shown to be very low in the concentration. While there is little data in the literature describing the volume of distribution of colchicine and metabolites in rats, Leighton et al. [12] reported various parameters of colchicine pharmacokinetics in a group of Sprague Dawley rats. That study reported a mean observed volume of distribution of 1.839±0.145 L/kg for colchicine. Volumes of distribution for 2DMC and 3DMC were not reported. It will be important to examine whether this distribution pattern is common in experiments with other drugs wherein extensive analyte partitioning into marrow is possible. Overall, these findings also support the notion that the quantitative relationship between parent drug and metabolite levels may be of value in interpretation of toxicological measurements in bone.

Conclusion

The previously published MAE-MPSPE-UHPLC method for analysis of COLCH, 3DMC, and 2DMC was successfully applied to this study of the influence of the delay between COLCH exposure and death on the levels of COLCH, 3DMC, and 2DMC. Analyte levels were widely variable across different bone types, whereas values of the ratio of

analyte levels were less variable across bone types. Results suggested that COLCH, 3DMC, and 2DMC may partition extensively into skeletal tissues, such that bone drug levels may continue to rise after corresponding blood levels have begun to decline. This may have implications for the interpretation of measurements of colchicine and its metabolites in bone tissue samples.

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