



Analysis of tramadol and *O*-desmethyltramadol in decomposed skeletal tissues following acute and repeated tramadol exposure by gas chromatography mass spectrometry



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ABSTRACT

Decomposed bone and plasma samples of rats exposed to tramadol (TRAM) under different dosing patterns were analyzed. Wistar rats received TRAM as one acute dose ($n = 4$, 45 mg/kg, i.p.) or three doses ($n = 4$, 15 mg/kg, i.p.), 40 min apart. Perimortem heart blood was collected, rats were euthanized and placed outdoors to decompose to skeleton. Recovered bone was ground and subjected to methanolic extraction. Bone extracts and plasma samples underwent solid phase extraction and were analyzed using gas chromatography–mass spectrometry. Levels of TRAM and the primary metabolite *O*-desmethyltramadol (ODMT) were expressed as mass normalized response ratios (RR/m).

Levels (RR/m) for TRAM and ODMT did not differ significantly between exposure types in any of the bone types examined or for the pooled bone comparisons (Mann–Whitney, $p > 0.05$). However, ratios of analyte levels (RR_{TRAM}/RR_{ODMT}) differed significantly between exposure patterns for tibial and skull bone as well as for pooled bone comparisons (Mann–Whitney, $p < 0.05$). Levels of TRAM and ODMT, as well as ratios of analyte levels (RR_{TRAM}/RR_{ODMT}), differed significantly in plasma between exposure patterns. Bone TRAM and ODMT levels were poorly correlated to corresponding plasma levels (TRAM: $r = 0.33$ – 0.57 ; ODMT: $r = -0.35$ – 0.23).

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1. Introduction

Since 2000, there has been a significant increase in the number of reports describing detection of drugs and their metabolites in skeletal tissues [1–7]. A variety of factors have been studied, including the type of tissue examined (e.g., bone vs. marrow) [8,9], both intra-bone and inter-bone drug distribution, and the time course of drug disposition in bone after various dosing patterns and post-mortem intervals [6–9].

A common finding amongst various authors is the poor correlation between drug levels in bone and blood [1,2,7]. This implies that attempts to correlate a drug measurement in bone to an associated blood concentration range for purposes of estimating toxicity will be problematic. Coupled with challenges in accurate measurement of drug concentration in bone, this may suggest that drug measurements in bone tissue may have strictly qualitative value (i.e., present or absent). However, recent work has suggested

that the quantitative relationship between levels of drug and metabolite(s) in bone may improve discriminating power between different patterns of drug exposure [6,7].

The work presented here is one of a series of investigations of the utility of toxicological measurements in bone to discriminate between different drug exposure patterns [6,7]. In this work, we extended our semi-quantitative approach to examine relative drug distribution in bone [5–7] to assess the relative distribution of tramadol (TRAM), a synthetic opioid analgesic with a μ -receptor affinity of approximately one-tenth of that of codeine [10], and its active primary desmethyl metabolite, *O*-desmethyltramadol (ODMT), in decomposed bone tissue following one of two different exposure patterns. One group (acute dosing: ACU) received a single large dose (45 mg/kg, i.p.) while the other (repeated dosing: REP) received three smaller doses (15 mg/kg, i.p.) separated by approximately 40 min. The utility of measured bone drug levels, as well as the ratio of levels of metabolite to parent drug, in discriminating between these exposure patterns was examined. Measured bone drug levels were also compared to corresponding blood levels to determine if any correlation existed.

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2. Methods

2.1. Chemicals

Tramadol, *O*-desmethyltramadol, and D4-meperidine (internal standard) were each obtained as a 1 mg/mL solution in a volume of one millilitre from Cerilliant (Round Rock, TX) and were diluted as needed. The derivatizing agent, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1%TMCS), was obtained from United Chemical Technologies (Bristol, PA). Methanol, ethyl acetate, and all other chemicals were obtained from EMD Chemicals (Gibbstown, NJ) and were reagent grade.

2.2. Animals & drug administration

All procedures performed during the course of this study were approved by the Laurentian University Animal Care Committee. Adult male Wistar rats were obtained from Charles Rivers Laboratories (Saint-Constant, QC) and were housed in two groups with Harlan Teklad 1/4' bedding (Indianapolis, IN). Rats were placed on a 12-h light/dark cycle at a room temperature of 20 °C and supplied with free-choice water and Harlan Teklad Laboratory Diet 8640.

Male Wistar rats ($n = 10$) were separated into two groups. The first group (ACU: $n = 4$) were each given a single, acute dose of 45 mg/kg (i.p.) TRAM and were euthanized by carbon dioxide asphyxiation within 20 min of dose administration. The second group (REP: $n = 4$) were each given three repeated injections of 15 mg/kg (i.p.) TRAM 40 min apart. REP was euthanized by carbon dioxide asphyxiation 40 min following the last dose. The 40-min interval between doses and the delay in asphyxiation among REP was determined by the half-life of TRAM in rats, which is approximately 40 min [11]. The remaining two animals served as drug-free controls

Heart blood samples were taken from each rat perimortem and stored in BD Vacutainer Plus Blood Collection Tubes (Franklin Lakes, NJ) containing 10 mg sodium fluoride (preservative) and 8 mg potassium oxalate to inhibit coagulation. The animal remains were then placed outdoors on a grassy surface under metal wire and allowed to decompose to skeleton for 3 weeks in August on the Laurentian University campus in Sudbury, Ontario. After the decomposition period, the remains of the rats were dissected and the different bone types separated according to bone type and animal. Bones collected for analysis included skull, vertebrae, pelvis, femora, ribs, and tibiae.

2.3. Bone preparation

Bones underwent passive solvent extraction based on previously published protocols [5–7]. Each bone type was lightly washed in 3 mL deionized water, followed by 3 mL of methanol, and finally 3 mL of acetone. Bones were dried overnight and the next day were ground using a domestic grinder. Crushed bone was then weighed (approximately 0.500 g) and placed in a screw-cap test tube. Drug was extracted from the bone by adding 5 mL methanol, vortexing, and placing the sample on a hot plate at 70 °C for 72 h. Throughout the incubation, samples were vortexed periodically. Following incubation, the methanol was pipetted into a new glass test tube and an additional 5 mL was added to the screw-cap test tube, vortexed and then pipetted into the glass test tube used previously. This wash was repeated. The samples in the test tube with methanol were then evaporated at 70 °C under a gentle stream of air and upon complete evaporation were reconstituted in 1 mL PBS (phosphate buffer solution, 0.1 M, pH 6). Blood samples were centrifuged ($1100 \times g$) to separate plasma,

from which 200 μ L plasma was diluted with 800 μ L phosphate buffer (0.1 M, PBS) so that the final volume was 1 mL. D4-meperidine (600 ng) was added to each sample as an internal standard.

Both plasma and bone samples underwent a lipid-protein precipitation. A volume of 3 mL 1:1 acetonitrile:methanol was added to each 1 mL sample. Samples were then placed overnight in a freezer (–20 °C) to ensure that lipids and proteins precipitated. The next day samples were centrifuged ($1100 \times g$) for 10 min, and the supernatant was poured into a new test tube. Supernatants were evaporated at 70 °C under a gentle stream of air until they reached a volume of approximately 1 mL.

2.4. Solid phase extraction

Following precipitation, samples were diluted with 3 mL PBS and acidified with 100 μ L glacial acetic acid. Samples then underwent solid phase extraction using Clean Screen CSDAU203 columns (3 cm³, 200 mg, United Chemical Technologies Bristol, PA). Columns were sequentially conditioned with 3 mL methanol, followed by 3 mL distilled water, and 3 mL PBS. Samples were loaded by gravity, and columns were washed sequentially with 3 mL PBS and 3 mL acetic acid (0.1 M). Columns were dried under vacuum (~ 10 in Hg) for 5 min, washed with 3 mL methanol, and dried again under vacuum (~ 10 in Hg) for 10 min. Elution was achieved by using 6 mL 3% ammonium hydroxide in 20:80 isopropanol: ethyl acetate solution. Eluents were evaporated completely at 70 °C and under a gentle air stream. Samples were reconstituted in 50 μ L ethyl acetate, vortexed, and pipetted into GC injector vials. Each vial had 50 μ L MSTFA + 1%TMCS added and then the vials were vortexed and derivatized for 1 h at 70 °C.

2.5. Gas chromatography–mass spectrometry (GC/MS) analysis

Analysis was done using a Clarus 600 C GC/MS instrument (PerkinElmer LAS, Shelton, CT) in Selected Ion Monitoring (SIM) mode with a Zebron ZB-Drug-1 column (15 m, 0.25 mm i.d., 25 μ m film) from Phenomenex (Torrance, CA). Extracts of 2 μ L were injected into an injection port held at 250 °C in splitless mode. The initial oven temperature was 100 °C and was held for 3 min. The temperature was then increased at a rate of 10 °C per minute until reaching 190 °C, where it was held for 5 min. The temperature was then set directly to 300 °C, where it was held for 3 min, resulting in a total run length of 21 min for each sample. TRAM was measured using the ions with m/z **335**, 58, 135 and ODMT levels were measured using the ions with m/z **303**, 58, 231. D4-meperidine was measured using m/z **251**, 222, and 172. Ions used for quantitative comparison are shown in bold font.

2.6. Precision and linearity

As part of characterization of the analytical method in terms of precision and linearity, duplicate standard curves were produced on each of three separate days. Aqueous rat bone tissue extract (BTE), prepared by sonication of drug free rat bone in PBS, as described elsewhere [7] was used as the analytical matrix in which standard solutions were prepared. Standard solutions with concentrations of 0, 25, 50, 100, 200, 500, 1000 and 2000 ng/mL TRAM and ODMT in 1 mL BTE were prepared in duplicate. Standard samples underwent the same preparation and analysis that the actual specimen samples would undergo, including a protein precipitation, solid phase extraction, derivatization, and analysis by GC/MS. The mean, standard deviations, and coefficients of variance were calculated for each set of samples and using the mean a curve was produced. Curves yielded correlation coefficients (R^2) ranging from 0.997 to 0.9998 in TRAM and 0.983 to

0.996 in ODMT, both of which show sufficient linearity within the concentration range selected. The coefficients of variance were below 25% for TRAM and ODMT samples that were 50 ng/mL or greater. At 25 ng/mL, the coefficients of variance were less than 25% for ODMT and 30% for TRAM. The cutoff for semi-quantitative comparison of bone drug levels for TRAM and ODMT was thus set to 25 ng/mL or 50 ng/g.

2.7. Data treatment

As has been described in published work elsewhere [5–7], measured drug levels in bone are reported as the mass-normalized response ratio (RR/m), where RR represents the ratio of analyte peak area (using the ion chosen for quantitation) to that of the internal standard. Measured RR values were normalized to the mass of bone sampled in each case to account for variability in the mass of tissue sampled. Blood drug levels were expressed in terms of RR/m for purposes of comparison to corresponding bone levels.

Drug levels of ACU and REP exposures were compared using the non-parametric Mann–Whitney *U*-test, where significance was acknowledged when $p < 0.05$. Blood samples were compared to pooled bone samples using Mann–Whitney *U*-test with $p < 0.05$ to determine if bone and blood levels differed significantly among the two exposure groups. The significance of bone type on observed mean drug levels was determined using the non-parametric Kruskal–Wallis test (STATPLUS v. 5.7.6.2 AnalystSoft) where significance was acknowledged at $p < 0.05$.

3. Results

3.1. Drug level comparison in individual bone types

Levels of TRAM and ODMT, as well as the ratio of levels of TRAM and ODMT (RR_{TRAM}/RR_{ODMT}) were compared between acute (ACU) and repeated (REP) exposures for each bone type. ACU and REP mean drug levels in each bone type were compared using the Mann–Whitney *U* test to assess the significance of any observed differences. Mean TRAM and ODMT levels, expressed as the mass-normalized response ratio (RR/m) for all bone types assayed are presented in Fig. 1. The mean ratio of response (i.e., RR_{TRAM}/RR_{ODMT}) for each bone type is also shown in Fig. 1.

For all bone types analyzed, drug levels (i.e., TRAM or ODMT) were not significantly different between exposure types. Values of RR_{TRAM}/RR_{ODMT} differed significantly between ACU and REP exposures in the skull and tibia ($p < 0.05$, Mann–Whitney).

The non-parametric Kruskal–Wallis test was used to determine if bone type was a main effect in the variance of ACU or REP drug levels. Bone type was not shown to be a main effect for TRAM or RR_{TRAM}/RR_{ODMT} levels in either exposure patterns, while bone type was observed to be a main effect for ODMT levels in both ACU and REP groups.

3.2. Drug level comparison among pooled bone levels

Levels of TRAM and ODMT, and the ratio of levels (RR_{TRAM}/RR_{ODMT}) from all bone types were grouped by exposure pattern for data comparison, as summarized in the box-and-whisker plots in Fig. 2. In this pooled data, only RR_{TRAM}/RR_{ODMT} ($p = 0.002$) levels differed significantly between exposure types while TRAM and ODMT levels did not ($p > 0.05$). Fig. 2 shows that the overlap in measured values is reduced in measurements of RR_{TRAM}/RR_{ODMT} relative to those of RR/m for TRAM or ODMT.

Estimates of bone TRAM concentrations ranged from 0.09 to 1.4 $\mu\text{g/g}$ and 0.08 to 0.85 $\mu\text{g/g}$ in the ACU and REP groups, respectively. Estimates of bone ODMT concentrations ranged from 0.05 to 0.42 $\mu\text{g/g}$ and 0.04 to 0.89 $\mu\text{g/g}$ in the ACU and REP groups,

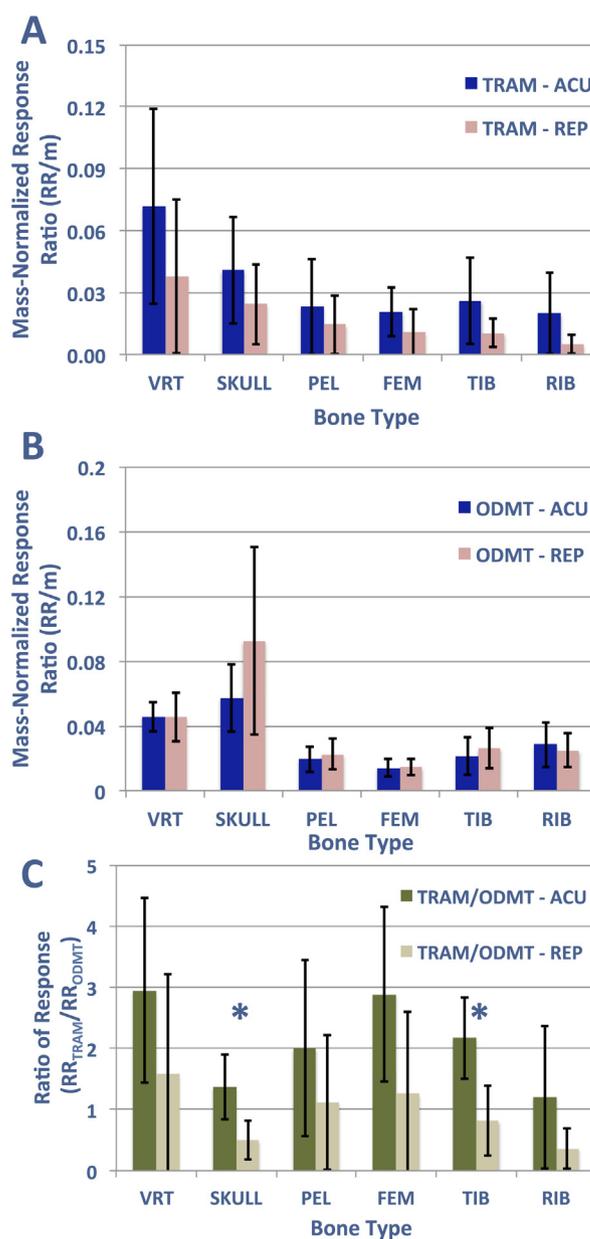


Fig. 1. Mean (\pm SD) (A) TRAM and (B) ODMT levels, expressed as mass-normalized response ratios (RR/m) for each bone type assayed, and (C) mean (\pm SD) ratio of TRAM and ODMT levels (RR_{TRAM}/RR_{ODMT}). * Denotes significant difference in measures between ACU and REP exposure patterns (Mann–Whitney, $p < 0.05$).

respectively. As described in section 2.7, these values should be considered to be approximate due to the inability to accurately determine and calibrate for analyte recovery from the solid bone matrix.

3.3. Drug level comparison in plasma

TRAM, ODMT, and RR_{TRAM}/RR_{ODMT} levels (RR/m) in plasma were measured and compared between the ACU and REP exposures to determine if exposure type could be discriminated using plasma samples. TRAM, ODMT, and RR_{TRAM}/RR_{ODMT} levels were all significantly different between the two exposure types.

Plasma TRAM concentrations ranged from approximately 2.2 to 3.4 $\mu\text{g/g}$ and 0.26 to 0.56 $\mu\text{g/g}$ in the ACU and REP groups, respectively. Plasma ODMT concentrations ranged from 0.49 to

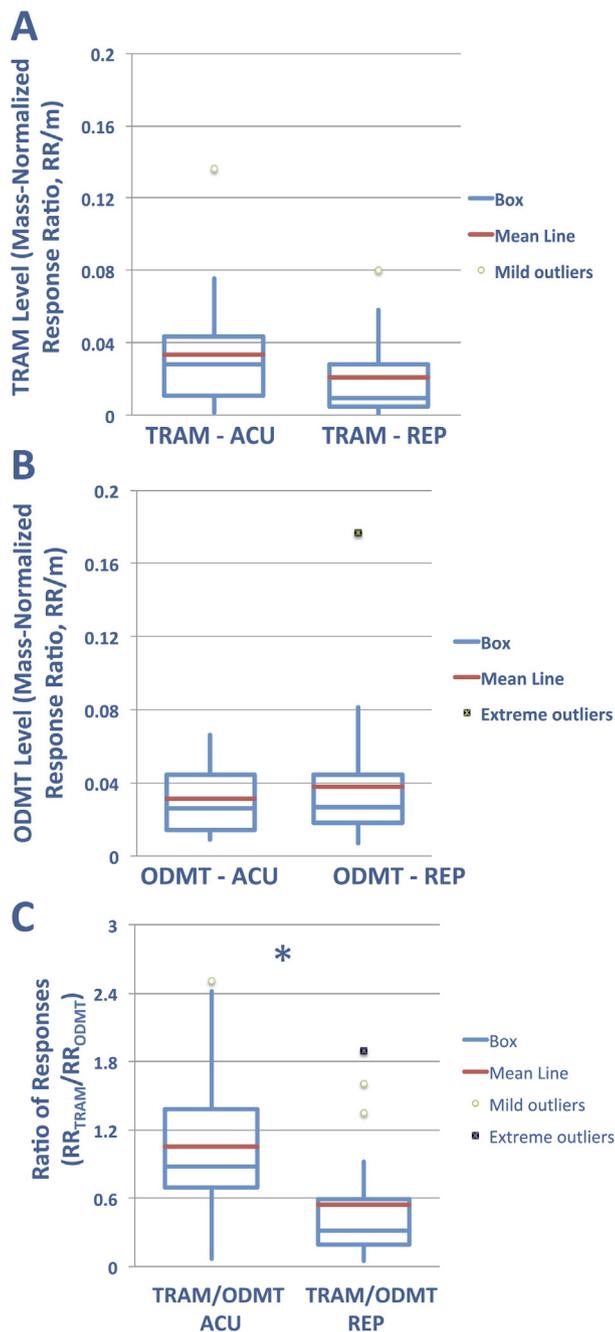


Fig. 2. Box and whisker plot showing levels of (A) TRAM and (B) ODMT, expressed as mass-normalized response ratios (RR/m) for all bone assayed (i.e., pooled samples), and (C) ratio of TRAM and ODMT levels (RR_{TRAM}/RR_{ODMT}) for all bone assayed. * Denotes significant difference in measures between ACU and REP exposure patterns (Mann–Whitney, $p < 0.05$).

1.1 $\mu\text{g/g}$ and 0.07 to 0.19 $\mu\text{g/g}$ in the ACU and REP groups, respectively.

3.4. Drug level correlation between plasma and bone

Pearson correlation factors were calculated between RR/m values for each bone type and plasma within ACU, REP, and as a combination of both exposures among all samples of that bone type. The correlation factors were determined for TRAM, ODMT, and RR_{TRAM}/RR_{ODMT} and are listed in Table 1. Calculated correlation factors ranged from strongly positive to strongly negative, with 15 of 54 correlations being negative.

4. Discussion

This study was done to examine the distribution of tramadol and its metabolite *O*-desmethyltramadol in skeletal tissues. As has been observed with other drugs [5–7], there was a wide variation in drug and metabolite levels in bone, both between animals and within an individual skeleton. The large inter-bone variability illustrated in Fig. 1 is likely the result of a number of phenomena, including structural heterogeneity of bone (relative amounts of cortical and trabecular bone), contact with marrow, vascularization, and contact with decompositional fluids. Further, it is clear from the data in Table 1, consistent with earlier work, that bone drug levels correlate poorly with blood drug levels, rendering prediction of toxicity based on bone drug level impossible.

Accordingly, this study was also one of a series that aims to investigate whether different patterns of drug exposure (e.g., acute large doses vs. smaller repeated doses) may be discriminated through toxicological analysis of skeletal remains. In earlier work [6,7], data has suggested the drug or metabolite levels themselves may not be significantly discriminating between the different exposure patterns examined. Interestingly, for the drugs examined thus far, measures of the relationship between drug and metabolite(s) have been observed to be more discriminating between the exposure patterns investigated. In cases where there are similarities in distribution between drug and drug metabolites to the various bones, measurements of the ratio of levels of parent drug to those of metabolite(s) may become more homogeneous than the analyte levels themselves, and better able to reflect differences in metabolite accumulation between different exposure patterns.

It is important to note that the work to date [6,7] has arbitrarily focused on the use of a single set of drug exposure patterns, wherein the acute dose administered was 3 times that of each dose in the REP group. It will become important in future work to assess the influence of various other exposure patterns, including different dose levels in acute and repeated administrations. Another important parameter that may influence the discriminatory power of any of the measures used thus far is the kinetics of drug and metabolite uptake into the skeletal tissues. Work

Table 1
Correlation factors (r) between RR/m values in blood and bone for TRAM, ODMT, and for the ratio of response RR_{TRAM}/RR_{ODMT} in blood and bone, for ACU and REP exposures.

Drug		Plasma vs skull	Plasma vs vertebra	Plasma vs pelvis	Plasma vs femur	Plasma vs rib	Plasma vs tibia
TRAM	ACU	0.311	0.150	0.365	0.521	0.543	0.380
	REP	−0.740	0.631	0.248	0.606	0.739	0.145
	Combined	0.404	0.450	0.330	0.429	0.572	0.566
ODMT	ACU	0.785	−0.122	0.689	0.336	0.385	0.363
	REP	−0.954	−0.642	0.258	−0.746	−0.917	−0.974
	Combined	−0.347	−0.059	0.020	0.027	0.226	−0.151
RR_{TRAM}/RR_{ODMT}	ACU	0.157	−0.448	−0.465	−0.632	−0.877	−0.822
	REP	0.184	0.701	0.538	0.317	0.986	0.491
	Combined	0.658	0.486	0.313	0.269	0.094	0.574

currently underway in our laboratory suggests that some compounds may partition extensively into bone tissue such that bone analyte levels may continue to rise while each analyte level has begun to decline in the blood. Such a scenario may influence the discriminatory power of the measures of the ratio of parent drug and metabolite levels in bone. This will be the subject of ongoing research in our laboratory.

5. Conclusions

This work has demonstrated that both TRAM and its primary metabolite ODMT may be detected in decomposed skeletal tissues following various exposure patterns. The data showed that while measurements of the levels of TRAM and ODMT in bone were not significantly different between exposure patterns examined (while those in plasma were), the ratio of TRAM and ODMT levels provided improvement in the discrimination between exposure patterns.

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