ANALYSIS OF DRUGS IN BONE AT NC-OCME

10/11/2013

Pablo Neruda (1904-1973)

The laboratory utilized sensitive screening techniques for a wide variety of drugs and poisonous compounds in the bones and samples from the coffin of Pablo Neruda. The positive results were limited to expected therapeutic agents and naturally occurring compounds with low indices of toxicity.

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SPECIMENS:

The specimens received for analysis are documented in attachments 1 and 2. The specimens were accessioned (documented and assigned bar codes) according to standard laboratory procedure and stored in a limited access, video camera monitored evidence room.

SPECIMEN PREPARATION FOR DRUG SCREENING:

The skeletal remains that were submitted to the laboratory were without traces of soft tissue and had been cleaned by the SML in Santiago, Chile and allowed to dry before being catalogued, packaged and transported to the NC-OCME in Raleigh, NC, USA. In order to generate sufficient quantity of bone for analysis (2-5 g) the bones were grouped according to anatomic location. A listing of the bones used for drug screening, assay employed and internal standard utilized is detailed in Table 1. As the matrix could have an effect on the limit of detection (LOD) for each instrument and analyte, multiple standard additions were employed to determine the limit of detection (LOD). The middle femur (S130013441) was used for the limit of detection determinations (Tables 2-6).

The bones were initially crushed with a padded hammer and then ground into powder using an all-purpose household spice grinder. The grinder was cleaned by pressurized nitrogen between bone specimens to prevent cross contamination. Aliquots of bone were weighed into threaded glass 40 mL headspace vials, internal standard, standard addition compounds as detailed in Tables 7-10 and 12 mL methanol was added to each vial and the suspension allowed to reflux at 55 °C for 48-64 h. The methanolic solution was decanted into appropriately labeled test tubes and the remaining bone was rinsed with methanol (3 x 3mL). With each rinse the methanol was decanted and pooled with the original. The test tubes were then placed in a water bath set at 45 °C and dried under a gentle stream of nitrogen. The residue was then subjected to analysis according to the standard operating procedures of the NC-OCME for basic compounds, acidic/neutral compounds, benzodiazepines, cocaine and metabolites, and opiates/opioids and special non-routine analytes (Table XX).

SCREENING EXTRACTION AND ANALYSIS:

Basic Organic Compounds- The procedure has been previously described.[i] Briefly, 1 mL of DI water was added to the residue from the preparation as described above then made basic with

concentrated ammonium hydroxide and extracted into n-butyl chloride: ethyl ether (3:1). After back extraction with 1N sulfuric acid, concentrated ammonium hydroxide was added and the basic solution extracted with n-butyl acetate. The extracts were analyzed by electron impact gas chromatography mass spectrometry (GCMS) operated in the scan mode (Agilent Technologies, Inc. GC 6890 with MSD 5973) and identified by retention time and mass spectral library matching. Representative basic organic compounds detected by this method are listed in Table 2.

Acidic and Neutral Compounds- The procedure has been previously described. [ii] Briefly, 1 mL of DI water was added to the residue from the preparation as described above then made acidic by the addition of 1 mL of pH 5.0 potassium acid biphthalate-sodium hydroxide buffer. The specimens were loaded onto Chem Elut columns (Agilent Technologies, Inc.) and extracted with 2 x 6 mL of dichloromethane. The methylene chloride was evaporated to dryness under a gentle stream of nitrogen in a water bath at 45 °C and further purified by partitioning between hexane and acetonitrile. The extracts were analyzed by electron impact GCMS operated in the scan mode (Thermo-Scientific, Inc. GC Trace GC-ULTRA with MSD DSQ II) and identified by retention time and mass spectral library matching. Representative acidic and neutral organic compounds extracted by this method are listed in Table 3.

Benzodiazepines, cocaine metabolite, opiates/opioids and others- The residue from the preparation as described above was reconstituted with 200 µL of 0.1M phosphate buffer (pH 6.5) and analyzed by Liquid Chromatography/Ion Trap Mass Spectrometry (LC-ITMS, Thermo-Scientific , Inc. LC Accela with LXQ Ion Trap MS) and identified by their relative retention times and mass spectra. Representative organic compounds detected by his method are listed in Table 4.

Special non-routine organic compounds- The residue from the preparation as described above was reconstituted with 200 µL of methanol and analyzed by Liquid Chromatography/Triple Quadrupole Mass Spectrometry (LC-MSMS, Thermo-Scientific , Inc. LC Accela with TSQ Vantage MS) and identified by their relative retention times and mass spectra. Organic compounds detected by his method are listed in Tables 5 and 6.

RESULTS FROM SCREENING ANALYSES:

The LOD results conducted via standard addition are listed in tables 2-6. The drugs selected for the LOD experiments are representative of organic compounds found in many drug classes and poisons. It is expected that organic compounds with similar structure and pKa will exhibit comparable extraction efficiencies and detectability.

Compounds detected during screening in the specimens from Pablo Neruda are listed below. Screening results are considered presumptive positives and the results cannot be taken as unequivocal identification and confirmation of the compounds presence. The confirmation analysis of these compounds is discussed in a later section of this report.

- 4-aminoantipyrine (major metabolite of metamizol/dipyrone)
- Chlorotrianisene
- Methylanthranilate

CONFIRMATION ANALYSIS:

4-Aminoantipyrine:

An instrument method was created to confirm and quantitate 4-Aminoantipryine on the LC TSQ Vantage. Bone groups were chosen and combined for the purposes of standard additions as the quantity of specimens were limited (Table 11). Sample preparation of the decedent's bones for confirmation analysis followed the same techniques using 2g of bone for standards and blanks as listed above.

Following instrumental analysis the presence of 4-aminoanitpyrine was confirmed but it was discovered that because of the amount of decay in the bones, combining the bones from similar anatomic sites had a detrimental effect on the linearity of the standard addition for the quantification of 4-Aminoantipryine and the bones were deemed unsuitable for quantification. This problem was not foreseen from prior investigations.

Chlorotrianisene:

The laboratory was unable to procure a certified reference standard for this therapeutic agent. However, based on the MW of this compound, chromatographic behavior, and strong spectral library matches, it is believed that there is high likelihood that this drug was present in the bones.

Methylanthranilate:

From the screening of the decedent's bones, evidence of possible environmental contamination in the form of methylanthranilate was present. The laboratory was unable to procure a certified reference standard for this compound but was able to perform spectral and RT matching with methylanthranilate extracted from Grape Kool-Aid®. Since the source of this contamination was most likely from ground water, which had the ability to migrate in and out of the coffin, the following samples were chosen for testing: ground water from coffin (S130013442 and S130013443), sediment from coffin (S130013446 and S130013448), and sediment from under bone (S130013447). Two (2) grams of sample was used in the extraction via the previously described methods for organic bases by GC-MS, organic acids and neutrals via GC-MS, and Fassi.

The Fassi method has been previously described[iii]. Briefly, the samples were saturated with sodium sulfate and mixed to remove water from the sample. Ethyl Acetate was added until the salt mixture was covered and left to sit for 30 minutes with occasional stirring. The organic solution was poured off and dried under nitrogen at 40 °C and reconstituted with ethyl acetate.

Samples extracted by the organic bases method were injected on the Agilent GC-MS and samples extracted by organic acids/neutrals and Fassi methods were injected on the Thermo GC-MS. Inspection of the data did not reveal the presence of methylanthranilate that was found in the bone extracts. However, a peak consistent with chlorotrianisene was detected in the sediment recovered from under the bones in the coffin.

DISCUSSION:

There have been a number of studies examining drug distribution in bone and the general conclusion to the studies is that drugs and/or metabolites are not homogeneously distributed because of the diverse anatomic structure of a particular bone and structural variability between types of bone.[iv,v] Factors believed to influence the deposition of drugs in this matrix include whether drug exposure was acute or chronic, the rate of decomposition, distribution of the drug at the time of death, and chemical nature of the drug. These factors and their effects on the relative distribution of drugs in bones are poorly quantified.

In the case of Pablo Neruda, the laboratory was able to detect and confirm the presence of 4-aminoantipyrine which is a major metabolite of metamizol/dipyrone in all of the bone types tested. Dipyrone is used clinically as an analgesic and antipyretic. After administration, dipyrone is usually undetectable in plasma as it is rapidly hydrolyzed to 4-methylaminoantipyrine which is further metabolized via oxidation to 4-aminoantipyrine. Accidental dipyrone overdose is generally associated with mild symptoms that are easily resolved. Cases of intentional overdosage involve long clinical courses with symptoms that include obtundedness, metabolic acidosis, kidney failure, lactic acidosis, liver failure, seizures, coma and eventual cardiac and respiratory arrest. [vi, vii]

Two compounds uniformly detected in the bones but not independently verified by comparison with a certified analytical standard were methylanthranilate and chlorotrianisene. Because of low toxicity indices, neither compound is toxicologically relevant to this case but will be discussed for completeness. Methylanthranilate is a volatile organic compound present in high concentration in grapes and other fruits and is one of the first artificial flavor compounds to be described. The compound is utilized in perfumes, cosmetics, flavoring of soft drinks, and as a bird repellant. [viii] Chlorotrianisene is a synthetic estrogen that in the past has been used clinically for the treatment of menopause, lactation suppression and prostate cancer but is not currently in large scale use. Clinical trials investigating the use of chlorotrianisene in the treatment of prostate cancer were first recorded in 1952 and the use of the drug for this purpose continued into the 1980's. The primary purpose of its use in this context is to prolong the life of the patient and improve quality of life through suppression of androgen production in the prostate.[ix]

CONCLUSION:

The laboratory performed sensitive screening for a wide variety of drugs and poisonous compounds in the bones and samples from the coffin of Pablo Neruda. The positive results were limited to expected therapeutic agents and naturally occurring compounds with low indices of toxicity.

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Table 1: Bone groups and methods of analysis

Assay	Internal Standard (Concentration)	Bone Type (NC-OCME Identification #/ SML Identification #)
Organic bases analyzed by GC-MS (Liquid-Liquid Extraction)	Alphaprodine 1µg/L	• Rib (S130013428/1984410) • Right Coxal (S130013437/2000087) • Lumbar vertebrae (S130013435/2000086) • Middle Femur (S130013441/2000096) – with and without standard addition
Organic Acids/Neutrals by GC-MS (Extraction by Solid Support)	Mephobarbital 5µg/L	• Right Coxal (S130013437/2000087) • Lumbar vertebrae (S130013435/2000086) • Middle Femur (S130013441/2000096) – with and without standard addition
Special organic compounds analyzed by LC-ITMS	Alphaprodine 0.05 µg/L	• Lumbar vertebrae (S130013434/2000084) • Middle Femur (S130013441/2000096) – with and without standard addition
Special non routine organic compounds analyzed by LC- TSQ Vantage	Alphaprodine 1 µg/L (+ESI mode) Amobarbital 10 µg/L (-ESI mode)	• Lumbar vertebrae (130013434/2000084) • Right Coxal (S130013437/2000087) • Middle Femur (S130013441/2000096) – with and without standard addition

Table 2: Limit of Detection (LOD) for Organic Basic Compounds analyzed on Agilent GC- MS

Analyte	LOD Conc. (ng/g)		Analyte	LOD Conc. (ng/g)		Analyte	LOD Conc. (ng/g)
Amphetamine	69		MDEA*	0.38		Doxylamine	4.3
Methamphetamine	1.0		Meperidine	0.98		Orphenadrine	1.2
Memantine	0.36		Normeperidine	1.7		Levamisole	0.77
Nicotine	1.4		Norfluoxetine	220		Chlorpheniramine	0.75
MDA*	27		Fluoxetine	6.8		Brompheniramine	0.10
MDMA*	1.3		Fluvoxamine	0.070		Dextromethorphan	0.51
Tapentadol	0.72		Tramadol	20		Imipramine	0.51
Threo bupropion	46		Methadone	0.19		Desipramine	19
m-CPP*	0.64		Atropine	1.1		Bupivacaine	0.56
Lidocaine	1.2		Doxepin	0.46		Clomipramine	0.58
Venlafaxine	0.77		Nordoxepin	7.9		Loxapine	0.19
O-desmethylvenlafaxine	3.1		Desmethylsertraline	37		Amoxapine	0.60
Amitriptyline	2.6		Sertraline	0.79		Clozapine	0.21
Nortriptyline	160		Citalopram	0.22		Pseudoephedrine	190
Cyclobenzaprine	0.93		Hydrocodone	2.9		Triprolodine	0.74
Mirtazapine	0.24		Oxycodone	21		Trihexyphenidyl	5.7
Codeine	0.45		Paroxetine	7		Cyproheptadine	0.14
Diazepam	0.36		Metoclopramide	0.9		Chlorpromazine	0.73
Nordiazepam	1.2		Zolpidem	1.1		Haloperidol	17
Trazodone	12		Verapamil	0.13		Ketamine	0.16
Phentermine	0.70		Norverapamil	2.4		Thioridazine	100
PMA *	2.1		Propylhexedrine	0.96		Papaverine	1.3
PMMA*	6.7		Diphenhydramine	0.86		Propranolol	24
						Methylphenidate	1.1

* Abbreviations: MDA = 3,4-methylenedioxyamphetamine, MDMA = 3,4-methylenedioxy-*N*-methylamphetamine, m-CPP= meta- Chlorophenylpiperazine, PMMA = *para*-Methoxy-*N*-methylamphetamine, PMA = *para*-Methoxyamphetamine, MDEA = 3,4-methylenedioxy-*N*-ethylamphetamine

Table 3: Limit of Detection (LOD) for organic acidic and neutral compounds analyzed on Thermo GC-MS

Analyte	LOD Conc. (ng/g)	Analyte	LOD Conc. (ng/g)	Analyte	LOD Conc. (ng/g)
Butalbital	0.78	Levetiracetam	1.7	Phenylbutazone	42
Pentobarbital	0.66	Acetaminophen	19	Ibuprofen	NR @ 5 µg/g*
Phenobarbital	1.3	Meprobamate	1	Thiopental	4.7
Metaxalone	1	Carisoprodol	0.72		
Primidone	0.94	Theophylline	3.9		
Phenytoin	1	Topiramate	1.5		
Lamotrigine	3.7	Carbamazepine	0.56		

NR*= the analyte was not recovered at the spiked concentration.

Table 4: Limit of Detection (LOD) for Benzodiazepines, cocaine metabolite, opiates/opioids and others analyzed on Thermo LC-ITMS

Analyte	LOD Conc. (pg/g)	Analyte	LOD Conc. (pg/g)
Morphine	46	Gabapentin	NR @ 25 ng/g*
Oxymorphone	56	Pregabalin	NR @ 25 ng/g*
Hydromorphone	35	7NH2-Clonazepam	65
Codeine	150	Clonazepam	NR @1.25 ng/g*
Oxycodone	29	Midazolam	28
Hydrocodone	43	Oxazepam	NR @ 2.5 ng/g*
6-MAM	37	Temazepam	86
Fentanyl	2.0	Triazolam	12
Buprenorphine	25	Diazepam	NR @ 5 ng/g*
Methadone	30	Nordiazepam	NR @ 5 ng/g*
Benzoylecgonine	12	Lorazepam	NR @ 2.5 ng/g*
		Alprazolam	69

NR*= the analyte was not recovered at the spiked concentration.

Table 5: Limit of Detection (LOD) for Non routine organic compounds analyzed via (+) ESI mode on LC TSQ Vantage

Analyte	LOD Conc. (pg/g)
4-Aminoantipryine	2900
Apomorphine	4600
Colchicine	53
Cyproheptadine	19
Trihexyphenidyl	16

Table 6: Limit of Detection (LOD) for Non routine organic compounds analyzed via (-) ESI mode on LC TSQ Vantage

Analyte	LOD Conc. (ng/g)
5-Fluorouracil	130
Bromadiolone	0.12
Chlorpopamide	180
Gilbenclamide	7.1
Nifedipine	53
Warfarin	0.051

Table 7: Basic organic compound standard addition concentrations

Compound:	High Standard (µg/g)	Low Standard (µg/g)	Compound:	High Standard (µg/g)	Low Standard (µg/g)
Amphetamine	0.125	0.05	Sertraline	0.25	0.1
Methamphetamine	0.125	0.05	Citalopram	0.25	0.1
Memantine	0.25	0.1	Hydrocodone	0.25	0.1
Nicotine	0.25	0.1	Oxycodone	0.0625	0.025
MDA*	0.125	0.05	Paroxetine	0.25	0.1
MDMA	0.125	0.05	Metoclopramide	0.0625	0.025
Tapentadol	0.25	0.1	Zolpidem	0.05	0.02
Threo bupropion	0.25	0.1	Verapamil	0.25	0.1
m-CPP*	0.125	0.05	Norverapamil	0.25	0.1
Lidocaine	0.25	0.1	Propylhexedrine	0.125	0.05
Venlafaxine	0.25	0.1	Diphenhydramine	0.25	0.1
O-desmethylvenlafaxine	0.25	0.1	Doxylamine	0.25	0.1
Amitriptyline	0.25	0.1	Orphenadrine	0.25	0.1
Nortriptyline	0.25	0.1	Levamisole	0.25	0.1
Cyclobenzaprine	0.25	0.1	Chlorpheniramine	0.25	0.1
Mirtazapine	0.125	0.05	Brompheniramine	0.25	0.1
Codeine	0.25	0.1	Dextromethorphan	0.25	0.1
Diazepam	0.25	0.1	Imipramine	0.25	0.1
Nordiazepam	0.25	0.1	Desipramine	0.25	0.1
Trazodone	0.25	0.1	Bupivacaine	0.25	0.1
Phentermine	0.125	0.05	Clomipramine	0.25	0.1
PMA *	0.125	0.05	Loxapine	0.25	0.1
PMMA*	0.125	0.05	Amoxapine	0.25	0.1
MDEA*	0.125	0.05	Clozapine	0.125	0.05
Meperidine	0.25	0.1	Pseudoephedrine	0.25	0.0625
Normeperidine	0.25	0.1	Triprolodine	0.25	0.0625
Norfluoxetine	0.25	0.1	Trihexyphenidyl	0.25	0.0625
Fluoxetine	0.25	0.1	Cyproheptadine	0.25	0.0625
Fluvoxamine	0.25	0.1	Chlorpromazine	0.25	0.0625
Tramadol	0.25	0.1	Haloperidol	0.25	0.0625
Methadone	0.125	0.05	Ketamine	0.25	0.0625
Atropine	0.25	0.1	Thioridazine	0.25	0.0625
Doxepin	0.25	0.1	Papaverine	0.25	0.0625
Nordoxepin	0.25	0.1	Propranolol	0.25	0.0625
Desmethylsertraline	0.25	0.1	Methylphenidate	0.25	0.0625

* Abbreviations: MDA = 3,4-methylenedioxyamphetamine, MDMA = 3,4-methylenedioxy-*N*-methylamphetamine, m-CPP= meta- Chlorophenylpiperazine, PMMA = *para*-Methoxy-*N*-methylamphetamine, PMA = *para*-Methoxyamphetamine, MDEA = 3,4-methylenedioxy-*N*-ethylamphetamine

Table 8: Acidic and neutral organic compound standard addition concentrations

	High Standard		Low Standard
Compound:	**(µg/g)**		**(µg/g)**
Butalbital	5		2.5
Pentobarbital	5		2.5
Phenobarbital	5		2.5
Metaxalone	2.5		1.25
Primidone	5		2.5
Phenytoin	5		2.5
Lamotrigine	5		2.5
Levetiracetam	7.5		3.75
Acetaminophen	12.5		6.25
Meprobamate	5		2.5
Carisoprodol	5		2.5
Theophylline	5		2.5
Topiramate	5		2.5
Carbamazepine	5		2.5
Phenylbutazone	5		2.5
Ibuprofen	5		2.5
Thiopental	5		2.5

Table 9: Benzodiazepines, cocaine metabolite, opiates/opioids and other organic compound standard addition concentrations

Standard	(ng/g)		Standard	(ng/g)
Morphine	2.5		Pregabalin	25
Oxymorphone	1.25		7NH2-Clonazepam	5
Hydromorphone	1.25		Clonazepam	1.25
Codeine	5		Midazolam	2.5
Oxycodone	2.5		Oxazepam	2.5
Hydrocodone	2.5		Temazepam	5
6-MAM	0.5		Triazolam	0.5
Fentanyl	0.25		Diazepam	5
Buprenorphine	0.25		Nordiazepam	5
Methadone	2.5		Lorazepam	2.5
Benzoylecgonine	0.5		Alprazolam	1.25
Gabapentin	25			

Table 10: Special non- routine organic compounds standard addition concentrations

	High Standard	Mid level Standard	Low Standard
	(ng/g)	(ng/g)	(ng/g)
4-Aminoantipryine	2000	100	5
Apomorphine	200	10	0.5
Colchicine	200	10	0.5
Cyproheptadine	200	10	0.5
Trihexyphenidyl	200	10	0.5
5-Fluorouracil	10000	2500	1250
Bromadiolone	750	250	50
Chlorpopamide	37500	6250	1250
Gilbenclamide	2400	400	200
Nifedipine	2500	250	50
Warfarin	100	50	10

Table 11: Bone groups for confirmation

Bone group	Specimen Identification Number (NC-OCME #/ SML #)
Ribs	• S130013429/1984411 • S130013430/1984412 • S130013431/1984413
Coxal	• S130013437/2000087 • S130013438/2000079 • S130013439/2000090
Vertebrae	• S130013436/2000085
Femur	• S130013440/2000094 • S130013441/2000096