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Pharmacogenomics as an Aspect of Molecular Autopsy for Forensic Pathology/Toxicology: Does Genotyping *CYP 2D6* Serve as an Adjunct for Certifying Methadone Toxicity?*

ABSTRACT: Pharmacogenomics, applied as an aspect of molecular autopsy, may be used as an adjunct for certifying methadone fatalities. Methadone is metabolized by cytochrome P-450 (CYP) 1A2, 3A4, and 2D6. We hypothesized that methadone toxicity may be partially due to *CYP 2D6* *3, *4, and *5 variant alleles, resulting in poor drug metabolism. A retrospective analysis was performed on covariables and risk factors of 21 methadone cases from the Milwaukee County Medical Examiner's Office (1998–2000). PCR genotyping showed: one heterozygous for *2D6*3,* two homozygous for *2D6*4,* five heterozygous for *2D6*4,* and one heterozygous for both 2D6*3 and *4. This limited number of cases showed that the prevalence of poor metabolizer was higher but not significantly different from that of a control group ($n = 23$) ($P > 0.05$, Fisher Exact Test). Thus, *CYP 2D6* mutations may not yet be directly associated with methadone toxicity. However, pharmacogenomics, complementing other case findings, served as an adjunct in interpreting methadone toxicity of poor and intermediate metabolizers.

KEYWORDS: forensic science, forensic pathology, forensic toxicology, methadone toxicity, *CYP 2D6,* pharmacogenomics, molecular autopsy

Pharmacogenomics is a well-established scientific and an emerging clinical discipline. With the completion of the human genome project, pharmacogenomics is increasingly applied to drug therapy and discovery (1–14). Routine clinical pharmacogenomics as a standard of care is rapidly emerging, with estimates ranging from a few years to 2020 (3). However, in the treatment of AIDS and some cancers, pharmacogenomics have already become the standard of care. Recently, the U.S. Food and Drug Administration approved HIV genotyping for the optimization of antiretroviral therapy (15).

Since drug dosage/concentrations and response are dependent, in part, on the genomics of the patient's drug metabolizing enzymes, transporters, and receptors, a rational drug regimen may be designed by initially identifying patient's genotype. This information may be incorporated for stratifying patients in clinical trials and routine therapy for optimizing therapeutic response and minimizing drug toxicity. Thus, it may now be possible to individualize drug therapy with respect to the genotype-encoding drug metabolizing enzymes such as cytochrome P-450 (CYP)(16,17). One group of the drug metabolizing enzymes, CYP 2D6 (debrisoquine/sparteine hydroxylase), is encoded by a polymorphic gene. Mutations may be characterized by single nucleotide polymorphisms (SNPs), gene deletion, gene duplications, and others. Depending on variant alleles, individuals may demonstrate phenotypes of poor, intermediate, extensive (normal), or ultra-extensive (rapid) metabolizers (18). A recent study further confirmed the complex mechanisms that regulate the variable expression and function of hepatic CYP 2D6 (19). Poor drug metabolizers might be more susceptible to toxicity as in the clinical use of methadone for the treatment of drug addiction and pain. The corresponding genotype may be identified by molecular techniques.

Potential application of molecular diagnosis may be conceptualized as molecular autopsy in forensic pathology—toxicology. Postmortem molecular analysis of *SCN5A* defects was recently advocated as a genetic marker for sudden infant death syndrome (20). Another study showed genotyping *CYP 2D6* as a supplementary tool for forensic toxicology (21). The results indicated that high drug concentration was not commonly related to the poor metabolizer, and the toxicity of extensive metabolizers was frequently associated with drug interactions. In performing these investigations, the molecular techniques such as PCR allow rapid diagnostics to be performed on a cost-effective and timely basis (7–12,22,23). Recently, real-time PCR as well as DNA chip technology offer technically feasible and reliable genotyping. These "enabling" technologies complement the existing traditional immunodiagnostics, other clinical diagnostics, and analytical toxicological tests. They provide the biotechnological basis for understanding adverse drug reactions (24–28).

In 1994, adverse drug reactions were ranked between the fourth and sixth leading cause of death in United States (24). The 2000 annual report of the American Association of Poison Control Centers showed that analgesics, including acetaminophen, aspirin, opioid,

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methadone, and others, were the leading cause of death (29). Methadone toxicity has been well documented, especially during the initial treatment period (30). Toxicity may also be due to methadone abuse and poor drug metabolism, as in the cases of *CYP 2D6* deficiency (31,32). Thus, postmortem methadone concentrations should be interpreted with medical history, death scene investigation, and autopsy findings (33).

In this study, we hypothesized that methadone fatality as an adverse drug reaction may be attributed, in part, to the *CYP 2D6* variant alleles encoding for enzymes with resultant poor metabolism of methadone and other drugs. In this preliminary study, we initially performed a retrospective review of methadone cases as part of the opioids deaths between 1998 and 2000 of the Milwaukee County Medical Examiners' Office (MCMEO); we established the prevalence of *CYP 2D6* variant alleles of these cases by using PCRs, and then we assessed the appropriateness using it as an aspect of "molecular autopsy," an independent variable, and/or as an adjunct in addition to other pertinent case information to certify the cause of death.

Materials and Methods

Case Selection

The study protocol was approved by the Institutional Review Board of the Medical College of Wisconsin—Froedtert Memorial Lutheran Hospital. The inclusion criteria were: the time period between 1998 to 2000, nonhomicide cases, and cases certified with methadone intoxication. Exclusion criteria included: homicide, deaths due to gunshot wounds, carbon monoxide poisoning, and fire deaths. Further case history review focused on: postmortem intervals, medical history, suicidal ideation, previous suicide attempt, concomitant drug administration with potential drug-drug interactions, autopsy findings, medication history as a basis for acute versus chronic ingestion, and death scene investigations. In establishing the *CYP 2D6* prevalence of a "control" group, 23 volunteers not medicated with methadone were also genotyped.

Autopsy

The Milwaukee County Medical Examiner's Office performed complete autopsies on all drug deaths considered sudden, unexpected, or suspicious. A complete autopsy included dissection of the thoracic, abdominal, cranial, and neck compartments. Routine samples of subclavian blood were attempted upon initial admission to the office. Samples obtained during the autopsy included: vitreous fluid, bile, urine, peripheral blood—iliac vein, aorta, or heart blood in the absence of peripheral blood, a portion of liver, representative gastric contents, pulled head hair, and a dried blood sample for DNA analysis. The blood samples were preserved in 2% sodium fluoride to inhibit decomposition of drugs such as cocaine. All samples were refrigerated immediately following the autopsy.

Toxicological Analysis

Methadone in blood was initially identified by using a solid phase extraction (SPE) method, followed by GC/MS analysis. Promazine, at a concentration of 1.0 mg/L, was used as an internal standard and added to the blood specimen prior to extraction by using a 300-mg Clean Screen® co-polymeric solid phase extraction column (United Chemical Technologies Inc., Bristol, PA). Initial identification and subsequent confirmation were achieved with a Hewlett-Packard (HP) 5890 series II GC equipped with a 5972 mass selective detector (MSD). The MSD was operated in full scan electron impact ionization mode. The GC/MS was fitted with either an HP-5 (Agilent Technologies, Palo Alto, CA) or DB-5 MS (J&W Scientific, Folsom, CA) capillary column with the following dimensions: 15m × 0.25-mm ID and 0.25-μm film thickness. The oven temperature was programmed at 100°C for 1.0 min, increased to 280°C at 15°C/min, and held for 7 min (total analysis time of 20 min). The injector and detector temperatures were 200 and 300°C, respectively. Identification and confirmation of methadone was based on retention time at 9.8 min and mass spectral match with drug identification library, showing prominent ion peaks at m/z = 72, 165, 223, 294, 309.

Methadone in blood was quantified by GC/FID. Standards were prepared by adding to drug-free blood methadone to concentrations ranging from 0.100 to 1.00 mg/L. Promazine, at a concentration of 1.0 mg/L, was used as an internal standard and added to the blood specimens and controls prior to extraction as described above. Blood extracts were analyzed on a Hewlett-Packard Model 5890 GC with splitless injection, dual columns, and dual flame ionization detectors. Chromatographic separation was achieved using DB-5 and DB-17 capillary columns (J&W Scientific, Folsom, CA) with the following dimensions: 15 m × 0.25-mm ID and 0.25-μm film thickness. Chromatographic conditions used were described above.

Genotyping Protocols

*CYP 2D6*3 and *4 by Conventional PCRs (34–36)*

Bon et al.'s PCR protocols were modified (37). DNA extracts were amplified for specific sequences of *CYP450 2D6 *3* and *4* mutations, followed by digestion with restriction endonucleases Msp1 and Mva1, respectively. Resulting fragments were visualized by electrophoresis followed by ethidium bromide staining. Poor metabolizers (PM), homozygous for the *4 mutation, were identified by a single 355 bp band, and PM homozygous for the *3 mutation showed 168, 82, and 20 bp bands.

*CYP 2D6*4 by Realtime PCR (38)*

Using a pair of hybridization probes labeled with fluorescein and LC red 640, a one-step amplification and Fluorescent Melting Curve Analysis (FMCA) could be achieved in about 45 min. LC red 640 labeled probe formed an A:C mismatch with the mutant-type allele. Fluorescence resonance energy transfer occurred as a result of hybridization of the fluorophore pair to its complementary DNA sequence. FMCA illustrated the mutant allele by lowering the melting temperature (Tm = 52.2°C) compared to that of the wild type (Tm = 64.2°C) during melting curve analysis. The heterozygote exhibited the characteristic *Tm*s of both the homozygote and the wild type.

*CYP 2D6*3 by Realtime PCR (39)*

Primer and probe sets were designed for this mutation. The amplified product was identified and analyzed in the same manner as *CYP 2D6*4.* The hybridization probes were labeled with fluorescein and LC red 705, respectively. The results from the FMCA indicated the *Tm*s of the wild type and variants alleles to be 69 and 65°C, respectively.

*CYP 2D6*5 by Long PCR (40–42)*

The protocol was adapted with several key modifications from Hersberger et al. (40). Briefly, after DNA extraction, amplification

was performed with Amplitag Gold and G46E enzyme (Roche Molecular Systems, Alameda, CA), which allowed the use of uracil-N-glycosylase and uracil for decontamination. Amplification was performed for 35 cycles, followed by analysis by 0.8% agarose gel with ethidium bromide staining. Wild type showed a 5-kb band, homozygous, a 3.2-kb band, and heterozygous two bands corresponding to 5 and 3.2 kb.

Statistical Analysis

Fisher's Exact test and frequency tables were calculated with SAS statistical software (The SAS Institute, Cary, NC). T-test and descriptive means were calculated using MINITAB (Minitab, Inc.).

Results

This retrospective study showed that methadone was identified in 39 of the 282 opioid deaths between 1998 to 2000, with 21 listing methadone as one of the drugs in the cause of death. The total case for this period was 11,093 with 3,946 autopsies performed. Table 1 shows the genotype, the toxicology results including methadone concentrations, and the cause and manner of death. There were 16 males, 5 females, 17 whites, 2 blacks, 2 Hispanics. The volunteer control group consisted of 9 males, 14 females, 19 whites, 1 Asian, 1 Indian, and 2 blacks. The age range and average of the poor metabolizers were: 41 to 51 and 45, respectively, and for extensive metabolizers, 26 to 63 and 39, respectively. Methadone concentrations for three poor metabolizers ranged from 0.1 to 1.4 mg/L (0.32 to 4.5 μM) with an average of 0.733 mg/L (2.37 μM), while those of the 18 extensive including intermediate metabolizers ranged from 0.15 to 2.4 mg/L (0.48 to 7.76 μM) with an average of 0.54 mg/L (1.75 μM). There is no significant difference in methadone concentrations for both groups ($P > 0.05$, t-test). By re-grouping poor and intermediate metabolizers together and comparing them to extensive metabolizers, no significant difference was evident ($P > 0.05$, t-test).

With the exception of Case 4, alcohol and other drugs were also identified. Alcohol was detected in seven cases with concentration range of 0.05 to 0.27 gm/dL and as the only other drug identified in Cases 10 and 12. Other drugs identified in the remaining 18 cases included, in order of prevalence, seven each for diazepam and other benzodiazepines, six cocaine, five propoxyphene, three amitriptyline, two heroin, two morphine, and one each for acetaminophen, cannabinoids, diphenydramine, doxepin, meperidine, oxycodone, tramadol, and venlafaxine. Certifications listed the following—the cause of death, 19 mixed drug overdose, 2 non-mixed drug overdose, and manner of death: 15 accidents, 4 undetermined, 1 natural, and 1 suicide.

Table 2 lists the *CYP 2D6* allelic prevalence of these 21 MCMEO cases compared to those of the control group ($n = 23$) not medicated with methadone. Genotyping of the volunteer group showed: zero for *CYP 2D6*3*, one homozygous and three heterozygous for *CYP 2D6*4*, one heterozygous for CYP 2D6 *5, and one heterozygous for *CYP 2D6*4/*5*.

Within the MCMEO cases, the prevalence of the poor metabolizers, 14.3% were higher than, but not significantly different from that of the control at 8.6% ($P > 0.05$, Fisher Exact Test). Based on genotyped results with the predicted phenotypes of poor, intermediate (7,18) extensive metabolizers and methadone concentrations, Table 3 lists selected findings of covariables from case and medical history. For the poor metabolizers, seven covariables did not differ significantly from those of extensive metabolizers ($P > 0.05$, Fisher Exact Test). For assessing the role of pharmacogenomics as an aspect of molecular autopsy—an adjunct for death certification, Cases 1 to 9 are reviewed as follows:

Case 1

With a history of alcoholism and prescription drug abuse including taking his wife's medications, the decedent was found by his wife to be unresponsive and was pronounced dead at about 8 AM, Monday. The night before, he vomited and snored in his sleep. He had a history of back pain and sleep apnea and made a previous suicidal gesture.

Case 2

The decedent, six months pregnant and diagnosed with heart murmur and rheumatoid arthritis, was intoxicated during New Year's Eve. Snoring in her sleep, her husband found the decedent lying on the floor the following morning. Blood was noted in her nares. Death was pronounced at 12:50 PM, less than 12 h after last seen by her husband. The decedent was medicated with methadone for her arthritis and amitriptyline for her depression. Medication history accounted for ingestion of nine 50-mg tablets (450 mg) of amitriptyline within a 17-day period, and possibly two to three daily 95-mg doses of methadone during New Year's Eve. The decedent, with drug-seeking behavior and drug and alcohol abuse, had an overdose suicide attempt several years ago.

Case 3

Last seen alive at 7:00 AM Sunday, the decedent was found by his girlfriend, lying supine in the living room at 8:00 AM, with a bottle of methadone nearby. In addition to alcohol, cocaine, and cannabinoids, he had been addicted to heroin for the past 25 years and was maintained on methadone. He also had Hepatitis C and cirrhosis of the liver. On Friday, the decedent went to a methadone clinic with his girlfriend. In the clinic, he ingested one dose and kept one dose for the following day. Decedent also obtained illicit drugs from a drug dealer nearby.

Case 4

Reportedly "wired" during the weekend with labored breathing, the decedent complained of chest and back pain. In addition to ingesting Tylenol 3 and Nyquil, he admitted previously to having taken his wife's methadone without incident. Having fallen asleep at about 3:30 AM Monday, he was found unresponsive by his wife later that morning. Upon admission to the hospital, his alcohol was 0.011 mg/dL. Recently, he was treated successfully for testicular cancer.

Case 5

The decedent, with a history of alcohol and drug abuse, was drunk after one beer at a Friday night party in December. Having left the party, he was found unresponsive on the lawn the following morning.

Case 6

The decedent, with a history of alcohol and drug abuse, was found lying on the floor with no sign of trauma and mild decomposition. He appeared intoxicated five days before. Recently, he was depressed about his girlfriend's pregnancy.

TABLE 1—*Methadone cases—genotyping and toxicolgical data, and death certifications.*

Case	Age	Sex	Race	Genotype *3/*4/*5	Methadone	Sample	EtOH	Toxicology	COD	MOD
					POOR METABOLIZERS					
1	43	M	W	WT/HM/WT	0.1	Il	ND	Propoxphene–0.2 Diazepam 1.63 Nordiazepam–1.22	MDO Methadone Propoxyphene Diazepam	Accident
2	41	F	W	WT/HM/WT	0.7	Il	ND	Amitriptyline–1.50 Nortriptyline–2.20 Diazepam–0.19 N-desmethyl diazepam–0.13	MDO Amitriptyline Diazepam Methadone OSC—Drug alcohol abuse Rheumatic heart disease	Accident
3	51	M	W	HT/HT/WT	1.4	Sc	ND	Cocaine–ND Benzoylecgonine–0.871 Propoxyphene–0.32 Diazepam–0.12	MDO Cocaine Diazepam Propoxyphene OSC—End stage alcohol liver disease	Accident
					INTERMEDIATE METABOLIZERS WITH ONE VARIANT ALLELE (2D6*3 or *4)					
4	30	M	W	WT/HT/WT	0.19	AM Bl	ND	None (Antemortem blood)	Methadone OD OSC—dysplastic coronary arteries	Accident
5	43	M	W	WT/HT/WT	0.2	Sc	0.08	Oxycodone–1.5	MDO Methadone alcohol, oxycodone	Undet.
6	42	M	W	WT/HT/WT	0.23	Il	0.07	Cocaine–0.02 Benzoylecgonine–1.002 Cocaethylene–0.024 Cannabinoid detected in urine	MDO Cocaine Methadone	Accident
7	36	M	W	HT/WT/WT	0.28	Il	ND	Diazepam–0.04 Nordiazepam–0.10 Lorazepam–0.07 Temazepam–0.15	MDO Methadone, diazepam lorazepam temazepam	Accident
8	60	M	W	WT/HT/WT	0.5	Il	0.07	Venlafaxine–0.19	Sudden cardiac death associated with Myocardial bridging OSC—Acute methadone ingestion	Natural
9	36	F	W	WT/HT/WT	0.63	Il	ND	Propoxyphene–2.06 Alprazolam–0.42 Diphenhydramine–0.17	MDO Alprazolam Methadone Propoxyphene	Accident
					EXTENSIVE METABOLIZERS					
10	29	M	His.	WT/WT/WT	0.15	Il	0.18	N.D.	Positional asphyxia MDO	Undet.
11	47	M	W	WT/WT/WT	0.19	Il	0.08	Cocaine–0.033 BE–0.396 Morphine, total–0.613 Morphine, unconjugated–0.555	MDO Cocaine Methadone Morphine—heroin	Accident
12	35	M	W	WT/WT/WT	0.21	Il	0.27	N.D.	Sudden death associated with alcohol and Methadone OSC—alcoholism	Accident

continues

TABLE 1—*Continued.*

Case	Age	Sex	Race	Genotype *3/*4/*5	Methadone	Sample	EtOH	Toxicology	COD	MOD
13	27	M	W	WT/WT/WT	0.22	Sc	ND	Morphine, total–4.2 (bile) Morphine, unconj.,–0.018 Clonazepam–N.D 7-amino-clonazepam–0.012	MDO Methadone & morphine	Accident
14	41	M	W	WT/WT/WT	0.25	Sc	ND	Coccaine–0.005 Be–0.288 Tramadol–0.22 N-desmethyl diazepam–0.16	MDO Cocaine, tramadol methadone diazepam	Accident
15	20	M	W	WT/WT/WT	0.26	Il	ND	Propoxyphene–0.26 Cocaine–0.033 BE–4.222 CE–0.039 Diazepam–0.16 N-desmethyl diazepam–0.06 Alprazolam–0.01	MDO Cocaine, methadone propoxyphene diazepam alprazolam	Undet
16	44	M	W	WT/WT/WT	0.51	Sc	0.06	Doxepin–0.72 N-desemthyl doxepin–0.07 Clonazepam–0.007 7-amino clonazepam–0.097 Alprazolam–0.03	Multiple toxicity Alcohol, clonazepam methadone, alprazolam OSC—alcohol abuse drug abuse, Hepatitis B and C chronic pancreatitis Left ventricular hypertrophy	Accident
17	46	M	His.	WT/WT/WT	0.61	Sc	ND	Morphine, total, 0.104 (bile) Morphine, unconj. (AM)–0.030 Promethazine–0.22 Propoxyphene–0.21 Meperidine–1.2	MDO Meperidine morphine methadone promethazine propoxyphene oxycodone	Undet.
18	39	F	W	WT/WT/WT	0.93	Sc	ND	Diazepam–0.12 Desmethyldiazepam–0.10 Propoxyphene–0.82 Acetaminophen–17.7	MDO Clonazepam? Methadone OSC-Depression	Suicide
19	33	F	W	WT/WT/WT	0.91	Il	ND	Amtriptyline–0.63 Nortriptyline–N.D. Diazepam–0.07 Desmethyldiazepam –0.12 Lithium–0.7 meq/L	Methadone toxicity OSC—Hepatitis Drug Abuse	Accident
20	50	M	B	WT/WT/WT	1.1	Sc	ND	Heroin (6-MAM –0.015) Cocaine–0.074 BE–0.351 Morphine, total–0.249 Morphine, unconjugated–0.125	MDO Cocaine Heroin Methadone	Accident
21	48	F	B	WT/WT/WT	2.4	BI	0.05	Amitriptyline–0.55 Nortriptyline–0.39	MDO Methadone Amitriptyline OSC—Hypertension	Accident

NOTE: OSC = other significant conditions, ND = non detected, IL = iliac, SC = subclavian, and MDO = mixed drug overdose. COD = causes of death; MOD = manner of death; Undet. = undetermined. Methadone concentration, gm/L, alcohol, gm/dL.
am = antemortem

TABLE 2—*Prevalence of CYP 2D6 variant and wild-type alleles.*

CYP 2D6 Alleles	Classification	Control (n = 23)	MCMEO* (n = 21)
*1/*1	WT	82.70%	71.40%
*3/*1	IM	0	2.40%
*4/*1	IM	6.50%	11.90%
*5/*1	IM	2.20%	0
*3/*3	PM	0	0
*4/*4	PM	4.30%	9.50%
*5/*5	PM	0	0
*3/*4	PM	0	4.80%
*4/*5	PM	4.30%	0

* No significant difference in the prevalence of Poor Metabolizers between groups (Fisher's test, $p = 0.334$)

MCMEO = Milwaukee County Medical Examiners' Office.

WT = wild-type metabolizer, IM = Intermediate metabolizer, PM = Poor metabolizer.

TABLE 3—*Selected findings of case and medical history.*

Case	Type	WE/H*	Opioids Naïve*	Acute-Chronic*	Alcohol*	Alcoholic*	Suicide Attempts*	Depression*	DAU/Pres	Fallani's Postmortem Intervals
1	Poor	Y	N	Acute	ND	Y	Y	N	Pres	I
2	Poor	Y	N	Acute	ND	Y	Y	N	Pres	I
3	Poor	Y	N	Acute	ND	Y	N	N	DAU/Pres	I
4	Intermediate	Y	Y	Acute	ND	NK	N	N	Pres	I
5	Intermediate	Y	Y	Acute	Yes	Y	N	N	DAU	I
6	Intermediate	NK	Y	NK	Yes	Y	N	Y	DAU	IV
7	Intermediate	Y	N	Chronic	ND	N	N	N	Pres	I
8	Intermediate	NK	N	Acute	ND	Y	N	Y	NK	IV
9	Intermediate	Y	N	Chronic	ND	N	Y	Y	Pres	I
10	Extensive	Y	Y	Acute	Yes	Y	N	N	DAU	I
11	Extensive	N	N	Chronic	Yes	N	N	N	DAU	IV
12	Extensive	Y	Y	Acute	Yes	Y	N	Y	NK	II
13	Extensive	Y	Y	Acute	ND	Y	N	Y	DAU/Pres	III
14	Extensive	N	Y	Acute	ND	Y	Y	Y	DAU/Pres	I
15	Extensive	Y	NK	NK	ND	Y	NK	NK	DAU	II
16	Extensive	Y	N	Chronic	Yes	Y	Y	Y	DAU	II
17	Extensive	N	NK	NK	NK	NK	NK	NK	DAU	I
18	Extensive	Y	N	Chronic	ND	Y	Y	Y	DAU	I
19	Extensive	Y	N	Acute	ND	Y	Y	Y	DAU	I
20	Extensive	N	N	Chronic	ND	N	N	N	DAU	I
21	Extensive	Y	Y	Acute	Yes	Y	N	Y	DAU	IV

* Fisher's Exact test-$P > 0.05$, no significant different results were found between two groups—a. poor metabolizers, and b. intermediate and extensive.
DAU = drugs of abuse, Pres = prescription drugs abuse, WE/H = weekend and holiday, NK = unknown, N = no, Y = yes, ND = nondetected.

Case 7

The decedent, with a history of sarcoma and prescription drug abuse, was found lying on the side of the mattress with no sign of trauma on a Friday night. He was last seen alive earlier that day.

Case 8

The decedent was found mildly decomposed, with blood from his nares, sitting on the floor with an arm on a couch and his head hanging down. He was last seen alive a week before. A postal worker with a history of depression, alcoholism, financial problems, and constant back and leg pain was treated with neuromuscular stimulator and medications. Two to three weeks ago, he was prescribed with methadone.

Case 9

The decedent complained of being cold and fell asleep on the living room floor on Saturday night. Later that night, her boyfriend found her pulseless, nonbreathing, and lying on her right side on the living room floor. Congestion was noted on her face. She was a polysubstance abuser with a personality disorder. One year ago, she was admitted to a mental health hospital for attempted suicidal gesture by overdose. She had been medicated with a high dose methadone. Following a recent miscarriage, she developed depression. Both her physician and brother suggested suicide to be unlikely. Autopsy did not identify any anatomic cause of death.

Cases 10 to 21, with wild-type *CYP 2D6*, showed multiple drug intoxication, except Cases 10 and 12 with alcohol only.

Discussions

According to the 2000 annual report of the American Association of Poison Control Center (29), analgesic including aspirin, acetaminophen, methadone and others, was the leading cause of death. In a recent study, the potential role of pharmacogenomics was assessed in lowering adverse drug reactions (28). About 59% of the 27 drugs associated with adverse drug reactions are metabolized by enzymes encoded by polymorphic genes such as *CYP 2D6.* In that study, the drugs identified with CYP 2D6 enzymes included diltiazem, fluoxetine, imipramine, metoprolol, nortriptyline, and theophylline. Methadone, chosen for this preliminary study, is also metabolized by CYP 2D6, but was not included in that group. This may be due to case selection criteria. However, in a previously published report by Caplehorn and Drummer (30), patients were more susceptible to methadone toxicity during the first two-week treatment period, with a risk of 6.7 times more likely than street drug use and 98 times that of maintenance therapy. Blood concentrations were not well correlated to the dose. Fatality occurred with methadone concentrations as low as 0.05 mg/L (0.16 μM), as compared to those of deaths during: the first two weeks, 0.84 mg/L (2.71 μM), maintenance therapy, 0.69 mg/L (2.23 μM), and acute overdose from diverted syrup, 0.33 mg/L (1.07 μM). From another report of ten fatalities, the average fatal methadone concentration was 1.0 mg/L (3.23 μM) with a range of 0.4 to 1.8 mg/L (1.29 to 5.82 μM) (43,44). For tolerant patients on maintenance daily doses of 100 to 200 mg, the average peak concentration was 0.83 mg/L (2.68 μM). As a result of the overlapping concentrations observed in maintenance therapy and fatality, Karch suggested that methadone concentration alone would not be predictive of fatality (33). Thus, certification of methadone toxicity should be interpreted with autopsy findings, medical history, and death scene investigation. Covariables include: iatrogenic toxicity, inadequate dose titration, inexperienced therapist, opioid naïve response, metabolic difference, and unreliable self-report (30). Metabolic differences of methadone may be attributable, in part, to *CYP 2D6* mutations. This study assessed the potential application of pharmacogenomics for explaining drug toxicity in forensic toxicology—pathology. Interpretation of the postmortem methadone concentrations would be based on the current understanding of the clinical pharmacology, selected aspects of *CYP 2D6* pharmacogenomics, and two interrelated areas of forensic toxicology—postmortem drug redistribution and sample sites.

After oral administration, methadone peaks at about 4 h, followed by elimination with half-life ranging from 15 to 55 h. Methadone is metabolized by CYP 1A2, 2D6, and 3A4. Currently, there is a lack of clinical evidence on the role of recently identified polymorphisms of *CYP 1A2* and *3A4* (18). However, methadone interactions with concomitant drugs might be mediated by these two CYPs. With the exception of the well-established alcohol-methadone interaction resulting in increased sedation (45), there are few reports on other drug interactions with methadone (46). Further, Eap and Beauman (47) showed that for the usually administered racemic methadone, chiral selectivity is exhibited by the enantiomer, R-methadone, contributing to the majority of the pharmacological activity. R-methadone metabolism displays bimodal distribution, with *p*-hydroxylation preferentially metabolized by the polymorphic CYP 2D6. Methadone is also metabolized by *N*-demethylation, mediated by CYP 3A4 (48,49) and subsequent cyclization to 2-ethylidene-1,5-dimethyl-3, 3-diphenylpyrrolidine, and to 2-ethyl-5-methyl-3,3-diphenylpyrroline. Currently, the role of the hydroxylated and conjugated metabolites remains unestablished.

Since *CYP 2D6* is highly polymorphic, the optimization of methadone therapy may be achieved through dosage adjustment, possibly lowering the dose for patients with *CYP 2D6* deficiency (9). This approach has been recently recommended for antidepressant therapy (18). For patients identified as homozygous, dose reduction of 30 to 80% is recommended. For patients identified as heterozygous, currently, the phenotypic expression may be identified as an intermediate or extensive (normal) drug metabolizer with reduced enzyme activity (8,17,50). Intermediate metabolizers, a subset of 10 to 15% of Caucasians, demonstrated severely impaired but residual in vivo function (51). Dose reduction ranged from 10 to 30%. Further, racial difference shows that only 1% Asian as poor metabolizers of CYP 2D6, as compared to 5 to 10% of Causcasians (46).

In addition to pharmacogenetics, two covariables are considered: blood sampling sites and postmortem intervals. According to a review by Prouty and Anderson (52,53), postmortem methadone concentrations are site-dependent, as shown by an average heart/femoral blood concentration ratio of 1.1, with a range of 0.8 to 1.4 in five cases. In order to minimize this covariable, peripheral, iliac, or subclavian blood, as shown in Table 1, would be preferred for methadone quantitations. In addition, postmortem drug redistribution, according to Fallani (54), may be characterized by four periods: I, II, III, and IV, corresponding to time periods of $<$24 h, 24 to 48 h, 48 to 72 h, and $>$72 h, respectively. In Periods III and IV, peripheral blood, less subject to redistribution, is preferred for toxicological analysis. Currently, there is a lack of further data to validate methadone postmortem concentrations with the Fallani's intervals. Table 1 lists the cases with the corresponding genotyping results, which differ substantially from the previous findings by Druid et al. (21). In both groups of that study, poor metabolizers were not identified. These different findings might be due to case inclusion criteria such as high metabolic ratios, sample size, and the personal features (55,56). In further interpretation of methadone intoxications, selected covariables are reviewed further in some of the nine cases of poor and intermediate metabolizers and briefly in extensive metabolizers.

Poor Metabolizers

The decedent in Case 1 had a history of prescription drug abuse, including taking his wife's diazepam. The high concentrations of diazepam and *n*-desmethyl diazepam, along with therapeutic concentrations of methadone and propoxyphene, resulted in mixed drug toxicity. Thus, the contribution of CYP 2D6 poor phenotype was not significant. For Case 2, the high amitriptyline (1.5 mg/L) (5.41 μM) and nortriptyline (2.2 mg/L) (8.37 μM) concentrations were in fatal/toxic ranges (44). These high tricylic and methadone concentrations were not due to postmortem drug redistribution as evident by the source of iliac blood and the short postmortem interval of about 24 h. *CYP 2D6*4* homozygousity resulted in deficient CYP 2D6 metabolism of methadone and amitriptyline/nortriptyline, leading to the lack of hydroxylations of methadone, amitriptyline, and nortriptyline. Consequently, parent drug concentrations were elevated. Further, chronic toxicity in combination with genetic deficiency may lead to poisoning (57). According to the report, the decedent was snoring before death, an indication of pulmonary edema developed as a result of respiratory depression caused by methadone toxicity. In sum, the mixed drug toxicity of these elevated drug concentrations were probably due to the combination of acute drug overdose and the lowered metabolism by genetic predisposition. The Case 3 history indicated acute ingestion of methadone. With iliac blood for analysis and the postmortem interval of about 24 h, high methadone of 1.4 mg/L (4.52 μM) was not a result of post-

mortem redistribution. The decedent was identified as both *CYP 2D6*3* and **4* heterozygous, corresponding to the predicted poor metabolizer of methadone (18). Other drugs/metabolites included propoxyphene, cocaine and metabolites, diazepam, and nordiazepam. The autopsy also revealed end stage liver disease, which contributed to impaired drug metabolism. Thus, methadone and cocaine abuse, with methadone toxicity mediated by poor phenotype, resulted in mixed drug toxicity.

Intermediate Metabolizers

Having taken his wife's methadone, the decedent of Case 4 might be regarded as opioid naïve. The autopsy also identified dysplastic coronary arteries. His hospital admission alcohol was 0.011 mg/dL, possibly due to the recent ingestion of Nyquil. Being an intermediate metabolizer, methadone concentration might have been elevated due to reduced metabolism. Further, methadone-alcohol led to increased sedation, a well-established drug interaction (45). The decedent of Case 5 had a history of alcohol and drug abuse along with a fatal concentration of oxycodone, also metabolized by CYP 2D6. The high oxycodone concentration might be due to a combination of: acute oxycodone ingestion, impaired metabolism due to heterozygous *CYP 2D6*4*—encoded enzyme deficiency, and CYP 2D6 enzyme inhibition by methadone. However, a study on phenytoin showed that enzyme inhibition is subordinate to poor drug metabolism by enzymes encoded by variant alleles (58,59). Alcohol and methadone with high oxycodone resulted in his sedation. The cause of death was mixed drug overdose. However, the decedent was found outside on a cold December night; thus, the manner of death was certified as undetermined. The decedent of Case 6 had a history of drug abuse—cocaine and cannabinoid. Methadone and alcohol resulted in increased sedation. The presence of both cocaine and cocaethylene, a pharmacologically active cocaine metabolite, formed in the presence of alcohol, was indicative of acute ingestion of cocaine and alcohol. Further, the postmortem interval was Period IV, with possible drug redistribution (54). Thus, the methadone concentration at the time of death might be less than 0.23 mg/L (0.74 μM). The Case 7 history was unremarkable other than the decedent's reduced methadone metabolism and prescription drug abuse. The decedent of Case 8 was found mildly decomposed, with death having occurred possibly as long as seven days ago, corresponding to Period IV with possible methadone redistribution. Thus the methadone concentration at the time of death might be less than 0.50 mg/L (1.62 μM). Since he was recently reinitiated in methadone therapy and being an intermediate metabolizer, he might have been more susceptible to methadone toxicity. In addition, other toxicological findings identified alcohol and therapeutic concentration of venlafaxine. Methadone and alcohol co-ingestion would result in enhanced sedative effect. Since autopsy reviewed myocardial bridging possibly resulting in sudden death, the cause of death listed toxicological findings as other significant conditions. For Case 9, both methadone and propoxyphene were in toxic ranges. Since propoxyphene is a CYP 3A4 inhibitor, methadone *N*-demethylation might be decreased. The high methadone concentration was not attributed to postmortem intervals.

Genotype of Cases 10 to 21 were identified as *CYP 2D6* wild-type, extensive metabolizers without CYP 2D6 deficiency, and they were certified as a mixed drug overdose. In Case 17, methadone inhibited the metabolism of meperidine, a CYP 2D6 substrate. Cases 16 and 19 showed high concentrations of parent tricyclic, indicative of acute ingestion and possible inhibition of CYP 2D6 enzyme by methadone (57,60,61). A previous report showed that such interaction resulted in elevated desipramine due to overdose and/or inhibition of 2-hydroxylation (60). For the above two cases, methadone inhibited the hydroxylations of doxepin and amitriptyline. Further, the high metabolic ratios implied acute ingestion.

In assessing the appropriateness of using genotyping *CYP 2D6* as an aspect of "molecular autopsy"—an independent variable for certifying methadone toxicity, Table 2 shows a higher allelic prevalence of poor metabolizers of the MCMEO methadone group than that of the control, but the difference is not statistically significant. In order to assess this association, a larger sample size of at least 500 would be needed in future studies. However, as an adjunct for interpretation of methadone toxicity, genotyping was helpful in certifying methadone as one of the drug of the mixed drug overdose in Cases 2, 3, 7, and 8, in combination with alcohol in Case 4, in combination with alcohol and cocaine in Case 6, in Case 5 as contributing to mixed drug toxicity with alcohol and fatal concentration of oxycodone, and in Case 9 to fatal concentration of propoxyphene. In assessing the effect of genotype/phenotype on other covariables, Table 3 showed no significant differences of the two groups—poor and extensive metabolizers, probably due to limited sample size. However, a review of covariables showed a trend of a higher percent of deaths among alcoholic and illicit and/or prescription drug abusers, and a higher incidence during weekend and holidays. This latter finding was due to the availability and abuse of unsupervised doses, evident in Cases 1, 2, and 3 of the poor metabolizers who were not opioid naïve.

With this preliminary study of 21 cases, the complexity of the cases' medical and prescription history precluded the clear conclusion of a simple and direct causal relationship of methadone mortality as a sole, dependent variable on *CYP 2D6* encoded enzyme deficiency. Thus, the cause and manner of death were not altered as a result of the genotyping. Similar to the study by Druid et al., mixed drug toxicity was the predominant finding (21). Different from that study, poor metabolizers were identified with a higher percent but statistically insignificant from that of a control group. While alcohol and methadone interaction was well documented (45), there are few published and established reports on other drug interactions with methadone such as those encountered in this study (46). In interpreting the drug interaction, it would be important to consider CYP 450 enzyme inhibition and induction as being subordinate to the *CYP 2D6* genotype effect on enzyme activity and pharmacokinetics, as shown by the effect of CYP 2C9 genotype on phenytoin metabolism (58,59). Further, the contribution of other CYPs to drug metabolism and interaction would be important. All these entered into the interpretation on the relevance of genotype/phenotype in mixed drug overdose.

Conclusions

Genotyping of *CYP 2D6*3, *4,* and **5* homozygotes or heterozygotes as an aspect of molecular autopsy may serve as an useful adjunct for certifying methadone mortality, serving to complement, clarify, and confirm other forensic findings such as death scene investigations, toxicology reports, and autopsy. However, genotyping in this study did not alter the previously established certifications of cause and manner of the 21 methadone deaths. Even though the prevalence of poor metabolizers in methadone fatality was higher than the general population reported in the literature and the control group investigated in this study, the findings did not establish a statistically significant, direct relationship of *CYP 2D6* genotype and the resultant enzymes deficiencies as con-

tributing an independent variable to methadone fatality. In order to truly assess the role of poor drug metabolism as a result of *CYP 2D6* variant alleles, a future study with a larger sample size and similar studies with other drugs such as oxycodone and antidepressants would be helpful. From this preliminary study and other future forensic pathology-toxicology investigations, the applications of pharmacogenomics as an aspect of molecular autopsy may possibly provide a rational approach for understanding the application of pharmacogenomics for certifying drug toxicities. Further, these findings might enhance/justify the clinical application of pharmacogenomics for drug therapies such as in treatments of cancer, AIDS, pain, and drug addiction, and for interpreting and minimizing adverse drug reactions.

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