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PAPER

TOXICOLOGY

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Postmortem Blood Concentrations of *R*- and *S*-Enantiomers of Methadone and EDDP in Drug Users: Influence of Co-Medication and P-glycoprotein Genotype

ABSTRACT: We investigated toxicological and pharmacogenetic factors that could influence methadone toxicity using postmortem samples. *R*- and *S*-methadone were measured in femoral blood from 90 postmortem cases, mainly drug users. The *R*-enantiomer concentrations significantly exceeded that of the *S*-enantiomers (Wilcoxon's test, $p < 0.001$). The samples were divided into four groups according to other drugs detected (methadone only, methadone and strong analgesics, methadone and benzodiazepines, or methadone and other drugs). There was no significant difference in any of the *R*-methadone/total methadone ratios among the four groups. The median *R/S* ratio was 1.38, which tends to be higher than that reported for the plasma of living subjects. In addition, we investigated whether small nucleotide polymorphisms in the *MDR1* gene that encode the drug transporter P-glycoprotein were associated with the concentrations of *R*- and *S*-methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine. No significant association was detected.

KEYWORDS: forensic science, *R/S*-methadone, postmortem blood, chiral liquid chromatography/mass spectrometry/mass spectrometry, P-glycoprotein, addiction, pharmacogenetics

Methadone is frequently used to treat persons who are dependent on opiates, and abuse of the drug is common. Thus, methadone is one of the most frequently detected drugs in postmortem cases involving drug addicts. Measurements of methadone concentrations in cases in which methadone poisoning is regarded as the cause of death generally show considerable overlap with measurements in cases where methadone is not regarded as the cause of death (1–3). This also applies to other opioids, such as morphine, and is generally ascribed to interindividual variations in tolerance to opioids (4). However, some additional factors should be considered. In most countries, methadone is administered as an equal (racemic) mixture of *R*- and *S*-enantiomers. Thus, routine measurements include both the *R*- and the *S*-enantiomers. The *R*-enantiomer has up to 50-times more analgesic activity than the *S*-enantiomer; therefore, independent determination of *R*- and *S*-enantiomers may be relevant (5). In recent years, several studies have concerned chiral measurement in plasma or serum of methadone concentrations in living subjects being treated with or abusing methadone (6–8). We developed a procedure for determining the concentrations of methadone enantiomers in postmortem blood samples and performed a preliminary study on postmortem concentrations of *R*- and *S*-methadone in a limited series (9,10). Here, to explore the relationship between *R*- and *S*-methadone concentrations in postmortem cases in more detail, we studied samples from a larger group of methadone-related deaths. We wanted to assess the degree of variation between concentrations of the active *R*-enantiomer and the total methadone concentration that is routinely measured in forensic laboratories. The possible influence of drug–drug interactions was assessed. We also measured the main metabolite, *R,S*-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP), to assess whether the presence of other drugs influenced the ratio between the parent compound and its metabolite.

In recent years, there has been increasing focus on the influence of pharmacogenetic factors on methadone dependence and toxicity. In particular, the role of polymorphisms in the *MDR1* (*ABCB1*) gene, which encodes the drug transporter *P*-glycoprotein (P-gp), has been evaluated with regard to methadone (11–14). P-gp is a transmembrane protein expressed in various tissues including the intestines, where it functions as an efflux pump to excrete drugs from the intracellular to the extracellular lumen. Methadone is a substrate for P-gp. Pumping methadone from the intestinal cells into the lumen limits the amount of methadone absorbed into the blood. Crettol et al. (15) found that P-gp exhibits a weak stereoselectivity in favor of the *S*-enantiomer. There are large interindividual differences in the amount of P-gp expressed in the intestine (up to 10-fold) (16). It is possible that at least some of the differences in P-gp expressed in the intestines are due to genetic polymorphisms in the *MDR1* gene. The impact of polymorphisms in the *MDR1* gene (especially the synonymous C to T transition at position 3435) on the absorption of P-gp substrates has been the

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topic of numerous studies, but the results are contradictory. Some studies suggest that genetic variants influence the expression or function of P-gp, while other investigations failed to verify any correlation (reviewed in 17–19). Thus, we also wanted to investigate whether genetic polymorphisms in the *MDR1* gene influence the amount and *R/S* ratio of methadone and EDDP.

Methadone metabolism is mostly done in the liver mediated by cytochrome P-450 (CYP) enzymes, primarily CYP3A4 and CYP2B6 and to a lesser extent CYP2C19 (20–22). It is possible that some of the interindividual differences in methadone tolerance are due to genetic polymorphisms in *CYP* genes. However, as no polymorphism related to the large difference in expression of *CYP3A4* has been detected and CYP2B6 mainly metabolizes the inactive (*S*)-enantiomer (20,22), it seems unlikely that much of the variability in methadone response can be explained by polymorphisms in CYP enzymes. However, it has been shown that for a cohort of patients treated with methadone, CYP2B6 slow metabolizers have longer QT intervals compared with those in normal metabolizers, most likely caused by stereoselective block of the cardiac potassium channel human *Ether-à-go-go* Related Gene (23). Here, we present data of measurements of *R*- and *S*-enantiomers of methadone and EDDP in 90 postmortem cases. We also assessed the influence of drug–drug interactions. In addition, we investigated if genetic polymorphisms in the *MDR1* gene influence the amount and *R/S* ratio of methadone and EDDP.

Materials and Methods

Samples

Ninety blood samples from routine autopsy cases in which methadone had been detected (69 males and 21 females, 19–63 years of age) were evaluated. The majority of the cases were addicts who had been in a methadone maintenance program or had abused methadone. In 77 cases, the cause of death was poisoning; in the 13 nonpoisoning cases, the cause of death was considered disease or trauma. Methadone poisoning, either alone (five cases) or in the presence of other psychoactive compounds such as morphine, benzodiazepines, and/or alcohol was the most frequent cause of death. In only a few cases, poisoning with other drugs such as morphine and amphetamine was judged primarily responsible for the death. In the nonpoisoning group, the causes of death were heart disease, subdural hematoma, alcoholic ketoacidosis, gastrointestinal ulceration, fall lesions, and suffocation.

Femoral blood was sampled at the start of the autopsy and stabilized with 100 mg sodium fluoride in a 10-mL container. The time from finding of the body to autopsy extended from 22 to 144 h (median 70 h). All samples were collected at the Department of Forensic Medicine, University of Copenhagen, Denmark over the course of *c*. 1 year (2006). Twenty-seven cases were previously included in a study on method development and postmortem sampling variation (9,10). The department serves the eastern part of Denmark, which has a population of about 2.5 million. To validate the genotyping procedure and to compare the genotyping results from the autopsy cases with the healthy Danish population, 200 living Danes (100 males and 100 females) were genotyped. The protocol was approved by the Danish Ethical Committee (KF-01-118/02).

Quantification of Methadone and EDDP

Blood concentrations of *R*- and *S*-enantiomers of methadone and EDDP were determined by chiral liquid chromatography/mass spectrometry/mass spectrometry, as previously described (9).

MDR1 Genotyping

Genomic DNA was extracted from 100 μL of peripheral blood using QIAamp DNA Blood Mini kit (Qiagen, Ballerup, Denmark) according to the manufacturer's protocol. The DNA was quantified by the Quantifiler Human DNA quantification kit (Applied Biosystems, Naerum, Denmark) using the ABI Prism 7900HT Sequence detecting system according to the manufacturer's protocol.

Seven single nucleotide polymorphisms (SNPs) in the *MDR1* gene were analyzed by multiplex polymerase chain reaction (PCR) followed by multiplex single base extension (SBE) reaction. The protocol was modified from Gwee et al. (24). To genotype samples containing degraded DNA, we redesigned the PCR primers so that the lengths of the PCR products ranged from 90 to 140 bp. In addition, we redesigned one SBE primer due to annealing between the SBE primers. The primer sequences are shown in Table 1.

Multiplex PCR was performed in a 25 μL volume containing from 0.2 to 30 ng DNA (the amount of DNA varied due to partial degradation of DNA in the samples), 1x buffer (Amplitaq Gold buffer; Applied Biosystems), 4.5 mM $MgCl_2$, 160 μM of each dNTP, 0.08–0.12 μM of each primer (Table 1), and 1 U Amplitaq Gold DNA polymerase (Applied Biosystems) in an Eppendorf Mastercycler gradient thermal cycler as follows: initial denaturation at 94°C followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by a final extension at 72°C for 10 min.

Purification of PCR products, the SBE reaction, and purification of the SBE reaction were performed according to the SNaPshot protocol (Applied Biosystems). A total of 0.3 μL of the purified SBE reaction was analyzed on an ABI Prism 3130XL Genetic Analyzer. All samples were genotyped in duplicate.

Results

Methadone Concentrations

The total methadone concentrations for the whole group ranged from 0.011 to 8.0 mg/kg (median: 0.62 mg/kg). The *R*-methadone concentrations ranged from 0.006 to 4.2 mg/kg (median:

TABLE 1—*Primer sequences.*

Location	Forward PCR Primer (5′–3′)	Reverse PCR Primer (5′–3′)	μM	Amplicon Size (bp)
Exon	TTAAATGCGAATCCCGAGAA	AAGTAGAGAAACGCGCATCA	0.08	119
Exon 1	ACAGCGCTTCGCTCTCTTT	CTCCGACTTTAGTGGAAAGACC	0.12	140
Exon 12	CACGGTCCTGGTAGATCTTGA	CATCAGCTGGACTGTTGTGC	0.08	110
Exon 21	TGCAATAGCAGGAGTTGTTGA	TCATATTTAGTTTGACTCACCTTCC	0.08	102
Exon 26	CATTGCTGAGAACATTGCCTA	GCATGTATGTTGGCCTCCTT	0.08	90
Exon 28	TGAGAGACATCATCAAGTGGAGA	CAGTTACAGTCCAAATGGGAAA	0.08	130
Redesigned SBE primer 1236 T/C	$(GACT)_7$TCCTGGTAGATCTTGAAGGG			

PCR, polymerase chain reaction; SBE, single base extension.

0.33 mg/kg) and *S*-methadone concentrations ranged from 0.001 to 3.8 mg/kg (median: 0.26 mg/kg). There was significantly more *R*-methadone than *S*-methadone in the samples (median difference: 0.068 mg/kg, Wilcoxon's test: $p < 0.001$). The *R/S* ratio ranged from 0.77 to 30 with a median of 1.4. No significant correlation between the total methadone level and the *R/S* ratio was observed.

The samples were divided into four groups according to the drugs detected: (i) methadone only, (ii) methadone and strong analgesics, (iii) methadone and benzodiazepines, or (iv) methadone and other drugs (the last group included samples in which methadone, other drugs, and a strong analgesic or benzodiazepine were present).

Figures 1–4 show plots of the total amounts of methadone detected, the *R*-methadone concentrations, the *R/S*-methadone ratios, and *R*-methadone/total methadone ratios in the four groups. No significant difference between the groups (Kruskal–Wallis test: $p > 0.05$) was detected. Figure 4 shows that the *R*-methadone/total methadone ratio varied considerably among the groups, but there was no systematic difference between the groups. Overall, the *R*-methadone/total methadone ratio ranged from 0.43 to 0.97, with a median value of 0.58. The 10- and 90-percentile values were 0.49 and 0.71, respectively. Thus, the active fraction constituted between 49% and 71% of the total methadone amount in 80% of the cases.

Methadone/EDDP Ratios

To assess the possible influence of drug–drug interactions, we calculated the *R*-methadone/*R*-EDDP ratios for the four groups (Fig. 5). The median values ranged from 7.0 to 9.7, and the range extended from 0.6 to 39. There was considerable overlap between the groups and there was no statistically significant difference between the groups (Kruskal–Wallis test: $p > 0.05$). Similar results were obtained for the *S*-methadone/*S*-EDDP ratio (Fig. 6). The range extended from 0.1 to 29 and the median values ranged from 3.7 to 7.5.

Methadone Concentrations in Relation to Cause of Death

The total methadone concentrations ranged from 0.011 to 8.0 mg/kg (median: 0.70 mg/kg) for the poisoning group and from

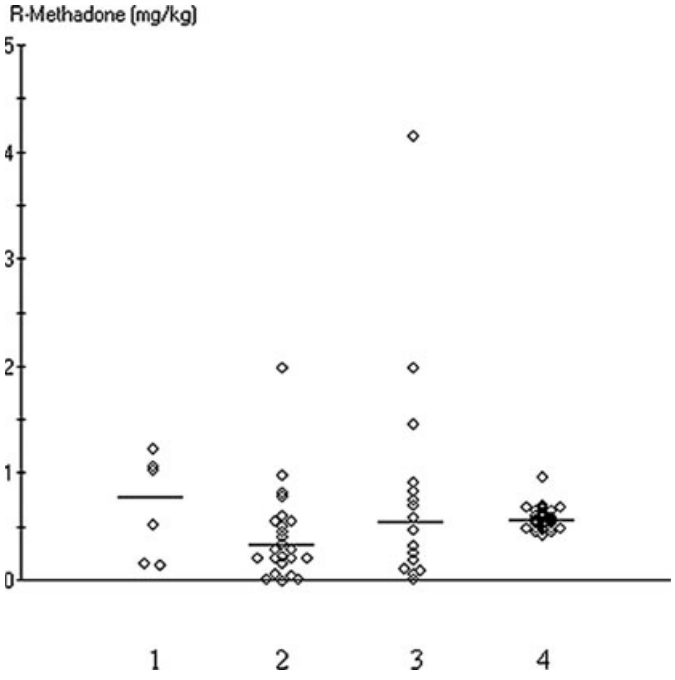

FIG. 2—R-*methadone concentrations in groups (1) methadone only, (2) methadone and strong analgesics, (3) methadone and benzodiazepines, and (4) methadone and other drugs. Median values are indicated by bars.

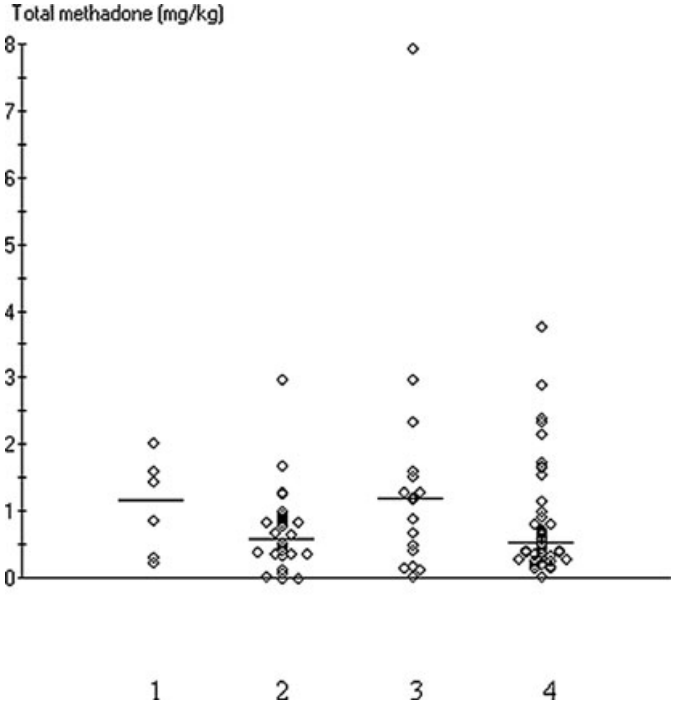

FIG. 1—*Total methadone concentrations in femoral blood from groups (1) methadone only, (2) methadone and strong analgesics, (3) methadone and benzodiazepines, and (4) methadone and other drugs. Median values are indicated by bars.*

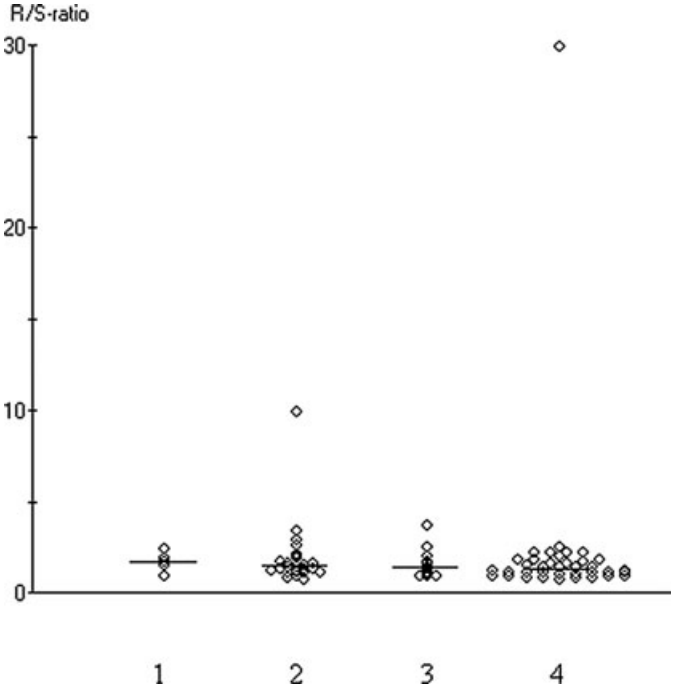

FIG. 3—R/S-*methadone ratios in groups (1) methadone only, (2) methadone and strong analgesics, (3) methadone and benzodiazepines, and (4) methadone and other drugs. Median values are indicated by bars.*

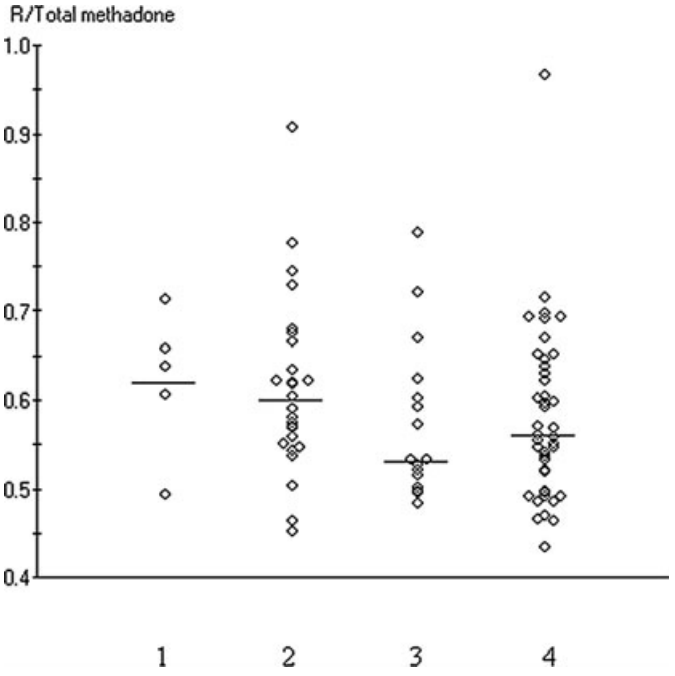

FIG. 4—R/*total methadone ratios in groups (1) methadone only, (2) methadone and strong analgesics, (3) methadone and benzodiazepines, and (4) methadone and other drugs. Median values are indicated by bars.*

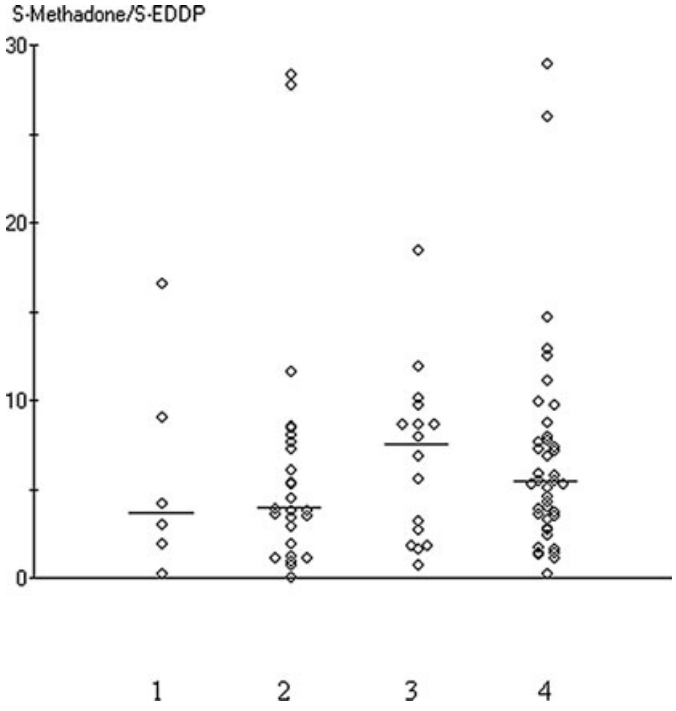

FIG. 6—S-*methadone/*S-*EDDP ratios in groups (1) methadone only, (2) methadone and strong analgesics, (3) methadone and benzodiazepines, and (4) methadone and other drugs. Median values are indicated by bars.*

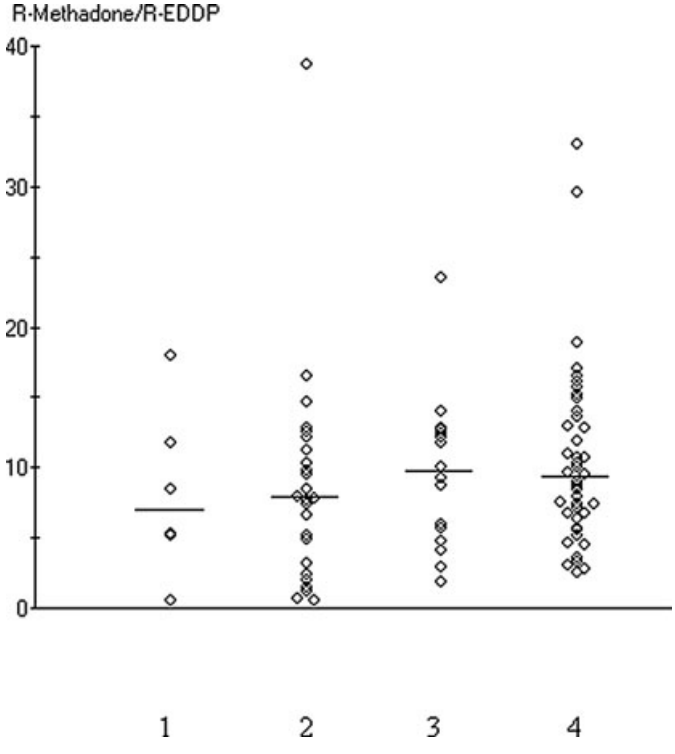

FIG. 5—R-*methadone/*R-*EDDP ratios in groups (1) methadone only, (2) methadone and strong analgesics, (3) methadone and benzodiazepines, and (4) methadone and other drugs. Median values are indicated by bars.*

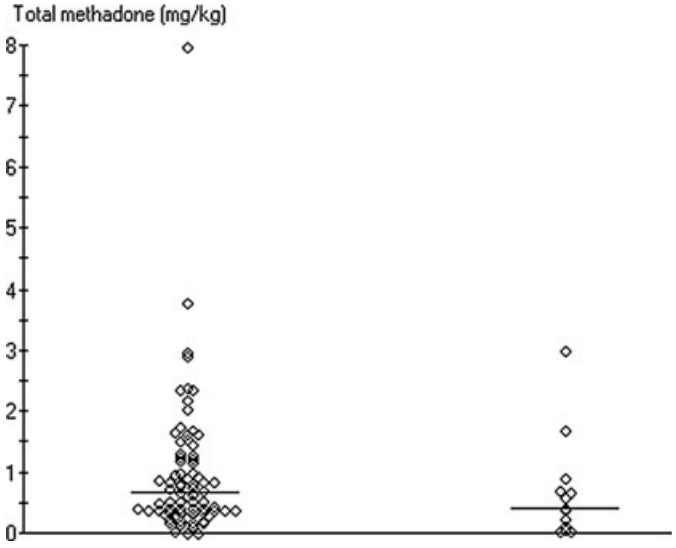

FIG. 7—*Total methadone concentrations in poisoning cases (left) versus nonpoisoning cases (right). Median values are indicated by bars.*

0.019 to 3.0 mg/kg (median: 0.41 mg/kg) for the nonpoisoning group (Fig. 7). There was no significant difference between the two groups (Mann–Whitney test: $p > 0.05$). Similar results were obtained for *R*-methadone (Fig. 8). Concentrations of *R*-methadone ranged from 0.006 to 4.2 (median: 0.35 mg/kg) for the poisoning group and from 0.015 to 2.0 (median: 0.28 mg/kg) for the nonpoisoning group. The medians did not differ significantly from one another. There was no significant difference in the *R*-methadone/total methadone ratio between the groups (results not shown).

MDR1 Polymorphisms

All samples were genotyped in duplicate. Seven samples had to be typed more than twice due to weak or inconclusive results. It

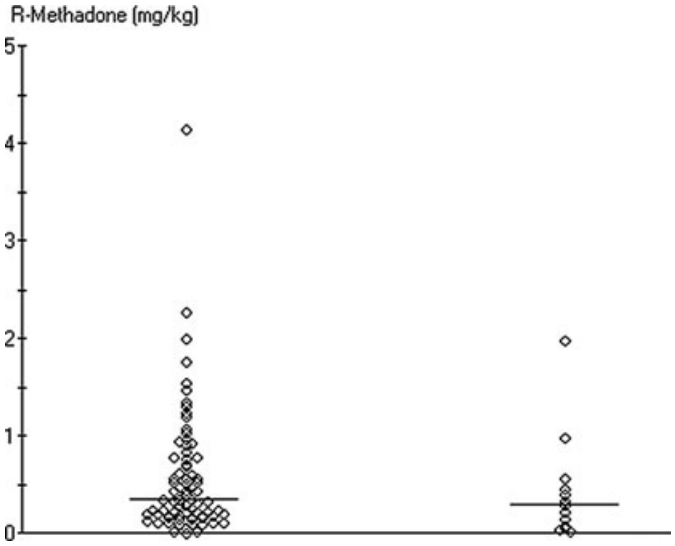

FIG. 8—R-*methadone concentrations in poisoning cases (left) and non-poisoning cases (right). Median values are indicated by bars.*

was not possible to genotype six of the postmortem samples, most likely due to degradation of the DNA (25). Concordant results were obtained for all other samples. Table 2 shows the distribution of *MDR1* genotypes in the living subjects and in the postmortem group. No significant deviation from Hardy–Weinberg (HW) proportions was found in the group of living subjects. In the postmortem group, the frequency of SNP 4036 A/G deviated from HW proportions ($p = 0.024$) due to an excess of homozygotes. However, after adjusting the *p*-value for multiple testing (Bonferroni's principle), no deviation from the HW proportions was observed. Table 3 shows the concentrations of methadone and EDDP in the postmortem femoral blood samples according to SNP 3435 genotype. No significant association was found (Kruskal–Wallis test: $p > 0.05$). Also, we found no significant association between the amounts of methadone and EDDP detected and the six other SNPs (data not shown). The distribution of the SNP 4036 A/G genotypes was significantly different between the healthy Danish population and the postmortem samples ($p = 0.047$, Fischer's exact test); however, after adjusting the *p*-values for multiple testing (sequential Bonferroni's principle), the two groups were not significantly different. There was no significant difference between the two groups with regard to all other *MDR1* SNPs investigated.

TABLE 2—*Frequencies of MDR1* genotypes.

Genotype	Healthy Danes ($n = 200$)	Postmortem Samples ($n = 84$)
−145 C/G		
CC	1	1
CG	–	–
GG	–	–
−129 T/C		
TT	0.91	0.89
TC	0.08	0.11
CC	0.01	–
−41 A/G		
AA	0.99	1
AG	0.01	–
GG	–	–
1236 C/T		
CC	0.35	0.32
TC	0.46	0.50
TT	0.19	0.18
2677 G/T/A		
GG	0.32	0.29
GT	0.43	0.52
GA	0.04	0.02
TT	0.19	0.17
TA	0.02	–
AA	–	–
3435 C/T		
CC	0.16	0.18
CT	0.48	0.50
TT	0.36	0.32
4036 A/G		
AA	0.82	0.75
AG	0.17	0.19
GG	0.01	0.06

Discussion

When racemic methadone is administered, the *R*-enantiomer is mainly responsible for the analgesic effects; therefore, there has been increasing focus on the relationship between the concentrations of *R*-methadone and total methadone. The disposition of methadone is stereoselective, with a longer half-life and a larger volume of distribution for *R*-methadone when compared with *S*-methadone (6). Methadone is metabolized primarily by the CYP enzymes 3A4, 2B6, and 2C19, and *in vitro* studies have confirmed the presence of stereoselectivity of the involved enzymes (21). Furthermore, methadone has been shown to be a substrate for P-gp and stereoselectivity may also be involved (15).

In vivo measurements of *R*- and *S*-methadone in plasma are made to monitor the therapeutic activity of methadone and to study the pharmacokinetics of the compound (6,26,27). Studies show that the *R/S* ratio varies considerably from subject to subject; therefore, measurements of the total amount of methadone in the plasma do not necessarily reflect the amount of active opioid present. The same may apply to postmortem measurements of methadone enantiomers. Here, as in our initial studies (9,10), we confirmed that the *R/S* ratio varied widely among individuals. *R*-methadone concentrations were significantly higher than *S*-methadone concentrations, resulting in a median *R/S* ratio of 1.38, higher than that found in the plasma living subjects in whom the mean or median ratios were less than or about unity (6,26,27).

Previously, we hypothesized that if death occurred preferentially in subjects with relatively higher amounts of the active methadone enantiomer in their blood, the median *R/S* ratio in postmortem samples would be higher than that in samples from living subjects. However, it is possible that a higher volume of distribution of *R*-methadone when compared with *S*-methadone contributes to a higher influx of *R*- when compared with *S*-methadone in the postmortem phase. Here, we found that the *R*-enantiomer constituted from 43% to 97% of the total amount of methadone present in postmortem blood samples. Thus, the measured total concentration of methadone in blood may not give a precise representation of the possible impact of the compound, providing instead only a rough indication.

Compared with plasma, the *R/S* ratio in blood may also depend on the distribution of the enantiomers between the plasma phase and erythrocytes. For total methadone, the concentration ratio between blood and plasma has been reported to be about 0.75 (28). Postmortem redistribution of methadone is generally considered to be displayed at a moderate degree. For a series of five cases, heart/femoral blood concentration ratios ranged from 0.8 to 1.4 with an average of 1.1 (29). Somewhat higher variations with ratios from 0.3 to 2.03 between subclavian and heart blood concentrations were reported by Levine et al. (30). The higher volume of

TABLE 3—*Concentrations of methadone and EDDP in postmortem femoral blood samples according to SNP 3435 genotype.*

	CC (n = 15)	CT (n = 42)	TT (n = 27)	p-Value
Total methadone (mg/kg)	0.70 (0.39–1.0)	1.1 (0.63–1.5)	0.88 (0.59–1.2)	0.74
R/S methadone	1.9 (0.64–3.2)	2.2 (0.86–3.6)	1.5 (1.4–1.7)	0.49
R/total methadone	0.58 (0.52–0.64)	0.60 (0.56–0.63)	0.59 (0.57–0.62)	0.49
Total EDDP (mg/kg)	0.14 (0.026–0.26)	0.20 (0.097–0.30)	0.19 (0.11–0.28)	0.49
R/S EDDP	0.80 (0.72–0.88)	1.1 (0.77–1.3)	1.0 (0.57–1.5)	0.41
R/EDDP total	0.44 (0.41–0.46)	0.47 (0.44–0.49)	0.46 (0.43–0.50)	0.46

SNP, single nucleotide polymorphism; EDDP, *R,S*-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium.

distribution of *R*-methadone compared with *S*-methadone may result in a higher influx of *R*- compared with *S*-methadone in the postmortem phase and may be of relevance when interpreting the postmortem *R/S* ratio of methadone.

Investigations of methadone-related deaths are often complicated by the presence of one or more other drugs. Some drugs influence the metabolism of methadone by inhibiting or inducing CYP enzymes, while others, like opioids and benzodiazepines, have an additive toxic effect, and some inhibit drug-transporting proteins. To assess whether drug–drug interactions influenced *R*- and *S*-methadone concentrations, we divided the samples into four groups depending on whether other drugs were detected. Pharmacokinetic interactions might be revealed by changes in the ratio between methadone and EDDP; however, we found no evidence that drug–drug interactions influenced the amounts of *R*- and *S*-methadone detected or the ratio between them.

We investigated the allelic frequencies of seven *MDR1* SNPs to evaluate whether the *MDR1* genotype was associated with variations in the concentrations of, or ratios between, *R*- and *S*-methadone. We found that the allelic frequencies fell within the range of frequencies previously reported for Caucasian populations closely related to Danes (31–33). In the Danish population, no significant deviation from HW proportions was found. However, it should be noted that in the postmortem group, the SNP 4036 A/G positioned in the 3′-untranslated region of *MDR1* deviated from HW proportions if the p-values were not adjusted for multiple testing. The most investigated *MDR1* SNP is 3435 C/T. This SNP was associated with decreased P-gp expression in one study (31). Coller et al. (11) found that *MDR1* polymorphisms influenced methadone dosage requirements in a group of individuals in a methadone maintenance program, whereas Crettol et al. (12,13) found no significant correlation between *MDR1* polymorphisms and methadone dosage requirements. In the study by Coller et al. (11), the mean daily methadone dose of the patients was about half of that in the study by Crettol et al. (13). In addition, Levran et al. (14) found significantly different *MDR1* genotype frequencies between patients receiving a high daily dose (>150 mg/day) and patients receiving a low daily dose (<150 mg/day) among a group of individuals in methadone maintenance treatment. It is possible that the polymorphisms in the *MDR1* gene had a larger impact in the group receiving a lower daily methadone dose than in the group receiving the higher daily methadone dose due to P-gp saturation by excess methadone. If P-gp is saturated, the impact of any functional disturbance would be relatively small. Here, we found no relationship between *MDR1* genotype and the amounts of methadone or EDDP detected in postmortem blood samples. It has been shown that P-gp exhibits weak stereoselectivity toward *S*-methadone (15); however, we found no correlation between the *MDR1* genotype and the *R/S* ratio. Our results indicate that the *MDR1* SNPs investigated here are not risk factors for methadone intoxication.

Accidental deaths caused by methadone may rely on various mechanisms. Important aspects may include variations in sensitivity, the accumulation of drug to a steady state level after initiation of therapy, and the co-occurrence of other sedative drugs, such as opioids, benzodiazepines, or alcohol (34,35). Although interindividual variations in the *R/S* ratio might also be important, the results in Fig. 8 show that with regard to the active component, *R*-methadone, there was considerable overlap between the concentrations present in the poisoning group and the group with cause of death related to other factors. Thus, chiral analysis of methadone concentrations in the blood does not provide information different from that obtained by measuring total methadone concentration. Therefore, our data do not support the routine measurement of methadone enantiomer concentrations in postmortem analysis.

Conclusion

In a postmortem series of 90 samples, we confirmed a tendency toward higher *R/S* ratios for methadone measured in femoral blood than previously reported for living subjects in plasma being treated with or abusing methadone. There was no correlation between the *R/S* ratio and the total amount of methadone. Co-medication with other drugs did not influence the *R/S* ratio. Concerning the pharmacogenetic aspects, we found no evidence of the *MDR1* genotype being of significance for methadone concentrations.

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