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Lisbon, 30th September of 2015

Dear Prof. Drummer,

We are submitting our paper “Sequencing CYP2D6 for the detection of poor-metabolizers in post-mortem blood samples with tramadol” for peer review and subsequent publication in the Forensic Science International, as a contribution for the special issue “TIAFT 2015” in the form of an Original Research Article.

We further state that this paper reports original work and is not under consideration for publication elsewhere.

Yours sincerely,

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Este e-mail pretende ser amigo do ambiente. Pondere antes de o imprimir!
Sequencing CYP2D6 for the detection of poor-metabolizers in post-mortem blood samples with tramadol

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Highlights:

- Validation of a Sanger sequencing method to detect null alleles of CYP2D6 in post-mortem samples
- Application to 100 samples of forensic cases with tramadol
- Poor-metabolizers correlated with the post-mortem concentrations of tramadol and metabolites
- CYP2D6 inhibitors must be included in the toxicological screening in cases with tramadol
Original Research Article

Sequencing CYP2D6 for the detection of poor-metabolizers in post-mortem blood samples with tramadol

Abstract
Tramadol concentrations and analgesic effect are dependent on the CYP2D6 enzymatic activity. It is well known that some genetic polymorphisms are responsible for the variability in the expression of this enzyme and in the individual drug response. The detection of allelic variants described as non-functional can be useful to explain some circumstances of death in the study of post-mortem cases with tramadol. A Sanger sequencing methodology was developed for the detection of genetic variants that cause absent or reduced CYP2D6 activity, such as *3, *4, *6, *8, *10 and *12 alleles. This methodology, as well as the GC/MS method for the detection and quantification of tramadol and its main metabolites in blood samples was fully validated in accordance with international guidelines. Both methodologies were successfully applied to 100 post-mortem blood samples and the relation between toxicological and genetic results evaluated. Tramadol metabolism, expressed as its metabolites concentration ratio (N-desmethyltramadol/O-desmethyltramadol), has been shown to be correlated with the poor-metabolizer phenotype based on genetic characterization. It was also demonstrated the importance of enzyme inhibitors identification in toxicological analysis. According to our knowledge, this is the first study where a CYP2D6 sequencing methodology is validated and applied to post-mortem samples, in Portugal. The developed methodology allows the data collection of post-mortem cases, which is of primordial importance to enhance the application of these genetic tools to forensic toxicology and pathology.

Keywords: pharmacogenetics; CYP2D6; poor metabolizers; tramadol; post-mortem; forensic toxicology
1. Introduction

Tramadol is a centrally acting opioid analgesic commonly prescribed for treatment of postoperative, dental, cancer, neuropathic and acute musculoskeletal pain control, with high clinical efficacy, low incidence of adverse effects and low abuse potential. Tramadol is administrated in a racemic mixture and undergoes extensive phase I and II metabolism to 23 metabolites, mostly excreted in the urine. The main metabolites resulting from the phase I metabolism are O-desmethytramadol (ODT), catalyzed by CYP2D6 enzyme, and N-desmethytramadol (NDT), catalyzed by CYP3A4 and CYP2B6 enzymes. Tramadol acts as a norepinephrine and serotonergic re-uptake inhibitor, possesses low affinity for µ opioid receptors and no affinity for δ or κ opioid receptors. The main opioid analgesic effect is attributed to ODT because it has approximately 300 times more affinity to µ-opioid receptors than the parent compound [1–3].

Post-mortem concentrations of tramadol are difficult to compare with reference values of therapeutic and toxic levels. There are variables as post-mortem redistribution, variations on the sample collection site and the time between the administration and the death that can influence the post-mortem concentrations. Tolerance in chronic users, drug interactions and individual genetic factors are also specific aspects of each case that must be considered in the interpretation of toxicological results. Genotyping can be a useful tool to post-mortem toxicology to explain some unexpected concentrations of tramadol and parent/metabolite ratios.

1.1. CYP2D6

CYP2D6 enzyme is coded by a gene with the same name that is located on the human chromosome 22 (22q13.1) and is part of a cluster with 2 pseudogenes, CYP2D7 and CYP2D8. These three genes have a high genetic homology but can be distinguished by some well characterized sequence variants that are responsible for the incapacity of the pseudogenes to produce a functional enzyme [4]. CYP2D6 gene is highly polymorphic and more than 20 allelic variants have been already correlated with the enzyme inactivation, usually called null alleles (http://www.cypalleles.ki.se/cyp2d6.htm), some of them with high prevalence in Caucasian population. When these genetic variants are present the metabolic activity is compromised. The characteristics of CYP2D6 gene locus, possible genotypes and phenotypes, as well as the difficulties of the genetic
analysis and interpretation were extensively reviewed by Andrea Gaedigk in 2013 [4].

1.2. Genotype and metabolism

The metabolic capability can be distinguished in four different groups: poor (PM), intermediate (IM), extensive (EM) and ultra-rapid metabolizers (UM). Poor metabolizers have very low metabolic capacity and higher metabolic ratios. Genetically they are characterized by the presence of 2 null alleles and 5 to 10% of the European population are considered to be PM [5]. The alleles CYP2D6 *3, *4, *5 and *6 are responsible for 93-98% of the PM [6]. The relationship between CYP2D6 genetic variation and enzymatic activity has already been studied by many authors and recently reviewed by Zanger and Schwab [5].

In tramadol positive cases, the relation between low metabolism phenotype and PM genotype of CYP2D6 has already been demonstrated by several studies: PM have a lower concentration of ODT, the main active metabolite, reducing the opioid analgesic effect as well as the opioid related adverse effects [2,7–13]. The half-life and concentration of tramadol can be higher in PMs [11] and the alternative metabolic ways can be stimulated. CYP2B6 and CYP3A4 enzymes catalyze the biotransformation of tramadol to N-desmethyltramadol (NDT), an inactive metabolite. The inhibition of the CYP2D6 metabolism can conduce to an increment of the concentration of NDT [1,7,10].

High blood concentrations of tramadol, due to accumulation or to increasing dosage, can lead to adverse reactions, not directly related with the opioid depression of the central nervous system, but specially with the inhibition of serotonin and norepinephrine reuptake [1].

1.3. Forensic application

Forensic pharmacogenetics is a relatively new and growing area of research [9,14–16]. The application of genotyping methodology to post-mortem forensic cases is dependent on the level of the DNA degradation, the existence of reliable methodologies that can be applied to routine analysis and on the gene characteristics. The interpretation of the results depends on the knowledge based on scientific research with statistical coverage, reason why further studies and the compilation of post-mortem data are needed to fully understand the relation between toxicological and genetic results. This information is important to enable the use of genotyping in the evaluation of some cases, specially when the concentration of drugs and metabolites can be considered suspect of an acute
To aid the interpretation of unexpected high concentrations of tramadol in post-mortem cases, it is important to have a method capable to detect genetic variants of CYP2D6 responsible for the enzyme inactivation. The purpose of this study was to develop and validate a Sanger sequencing method to detect the more prevalent null variants in post-mortem blood samples, using the technology usually existent in the forensic genetics laboratories without further costs.

2. Materials and methods

2.1. Samples

100 post-mortem peripherical blood samples, positive for tramadol, were selected from forensic toxicological cases that were analyzed between 2012 and 2015 in the south branch of the National Institute of Legal Medicine and Forensic Science (INMLCF), in Portugal. In the selected cases, 56 were male and the mean age of the subjects was 65 years old (range: 30 to 93). The probable cause of death mentioned in the toxicological request was: violent traumasism (34 cases); intoxication (10 cases); natural death (16 cases) and unknown (40 cases).

2.2. CYP2D6 genetic analysis

Blood spots were collected in Whatman® FTA cards according to manufacturer’s recommendations. DNA was extracted using Chelex100® method [17] and quantified by Real-Time PCR using the Quantifiler® Trio DNA Quantification kit from Applied Biosystems (AB) [18], according to the standard protocol.

The PCR amplification method was modified and optimized from Levo et al [7] and Hersberger et al [6]. For each sample, three fragments of 200bp, 437bp and 736bp were directly amplified, to detect the main null alleles CYP2D6 *3 (2549delA); CYP2D6 *4 (100C>T and 1846G>A) and CYP2D6 *6 (1707delT), but also other variants located in these fragments, such as CYP2D6 *8 (1758G>T), *10 (100C>T), *12 (124 G>A), *14 (1758G>A), *15 (137_138insT), *40 (1863_1864 ins), *43 (77G>A), *44 (82C>T), *47 (73C>T), *49 (1611T>A) and *50 (1720A>C). PCR was prepared to a final volume of 25 µL, using Multiplex PCR Master Mix 2x (QIAGEN); dimethylsulfoxide 5% (DMSO); 200nM of primers and approximately 5 ng of DNA. Thermocycling conditions were adjusted to obtain the better results for each fragment (Table 1). After
purification with ExoSAP-IT® (Affymetrix) the amplification was confirmed by SDS-PAGE Electrophoresis with Silver Staining in a Phastsystem (GE Healthcare).

The modified Sanger sequencing reaction was performed with BigDye Terminator v.3.1 Cycle Sequencing Kit of AB, using 4 μL of Better Buffer (Microzone); DMSO; 500nM of each of the PCR primers and 1μL of amplified template, to a final volume of 10 μL. Thermocycling conditions used are in Table 1. After purification with the BigDye XTerminator Purification Kit (AB), the sequencing products were analyzed by capillary electrophoresis in a Genetic Analyzer 3130 of AB.

| Table 1. Thermocycling conditions for the PCR and sequencing methods. |
|-----------------------|------------------|------------------|------------------|
|                      | [173 to 264]     | [1299 to 2035]   | [2369 to 2569]   |
| Initial denaturation | 95°C/15 min 40   | 95°C/15 min 40   | 95°C/15 min 35   |
| Cycles               |                  |                  |                  |
| denaturation         | 94°C/30s         | 94°C/30s         | 94°C/30s         |
| annealing            | 55°C/30s         | 55°C/30s         | 57°C/30s         |
| extension            | 72°C/30s         | 72°C/30s         | 72°C/30s         |
| Final extension      | 72°C/7 min       | 72°C/7 min       | 72°C/7 min       |
|                      |                  |                  |                  |
| Initial denaturation | 95°C/3 min 35    | 95°C/3 min 35    | 95°C/3 min 35    |
| Cycles               |                  |                  |                  |
| denaturation         | 95°C/20s         | 95°C/20s         | 95°C/20s         |
| annealing            | 55°C/20s         | 55°C/20s         | 57°C/20s         |

The results were verified using the Sequencing Analysis v.5.2 software. The sequence alignment was done according to the Human Cytochrome P450 Allele Nomenclature Committee (http://www.cypalleles.ki.se) and the allelic variants were detected comparing the sequences obtained with the reference sequence (entry M33388.1 at Genbank) using SeqScape v.3 software.

2.3. Validation of the genetic method

The method was validated according to the general SWGDAM guidelines, with a special approach for Sanger sequencing methodology [21], and fulfilling the ENFSI recommended minimum criteria for the following parameters: specificity, accuracy, repeatability, reproducibility and sensitivity [19–23]. Reference materials were selected from the Coriell Cell Repositories (National Institute of General Medical Sciences) based on genotypic characterization with the more prevalent variants (ref: NA17226; NA17280 and NA17300) that included: 100C>T; 1707 del T; 1846G>A, 2549delA [24]. The genotypes were correctly assessed and the
peak balance ratios of heterozygote alleles were above 60%. These samples were used as positive controls in the analysis. All the sequences obtained were well aligned with the reference sequence. NCBI’s BLAST (basic local alignment search tool) analysis for sequence similarity was also used in the primers and in the sequences of 3 different samples to evaluate the specificity and to check for homology to other genes or pseudogenes that may interfere with the analysis. The search was made using “Standard Nucleotide BLAST“, in the “nucleotide collection nr/nt; human (taxid:9606)”, with Megablast [29]. The 18 sequences verified had matches of 99-100% with the “Homo sapiens CYP2D6 (CYP2D6) gene, complete cds, Sequence ID: gb|JF307778.1|Length: 6587”, depending on the variants of each sample. The search using NCBI Genomes (chromosome) database only match with the Homo sapiens Chromossome 22 Primary Assembly with 100% identity.

The Limit of Detection was determined by performing dilution experiments of a high-quality genomic DNA with a known concentration for the following final concentrations: 10, 1, 0.5, 0.1, 0.05, 0.01ng/uL. Signal/Noise and variant identification were the parameters evaluated. The minimum concentration at which was possible to correctly identify the genotype in all the fragments was 0.1ng/µL. The limits of detection of the smaller fragments were lower (0.05ng/µL for the fragment with 437bp and 0.01ng/µL for the fragment with 200bp), as expected. Nevertheless one postmortem sample with a DNA concentration of 0.06ng/µL was successfully analyzed.

The precision of the method was verified by repeatability and reproducibility experiments. To test the repeatability, three replicates of five samples with different genotypes were simultaneously analyzed, only varying the location in the thermocycling equipment and in the sequencing plate. With 2 sequences for each of the 3 fragments, a total of 30 replicates for each variant were evaluated, with 100% of success.

To test the reproducibility, 15 samples were analyzed in three different days using the same technique, including the normal variables of the routine work in the lab, such as room temperature, reagents and equipments. With 2 sequences for each of the 3 fragments, a total of 90 replicates in each variant were evaluated for quality and the correct variant identification. Results are given in the Table 2.

Table 2. Reproducibility results of the genetic method validation
### Allele Frequencies

| Variant | Allele |
|---------|--------|
|        | *4, *10 | *12 | *6 | *8 | *4 | *3 |
| Day 1  | 15/15 | 15/15 | 15/15 | 15/15 | 14/14 | 15/15 |
| Day 2  | 15/15 | 15/15 | 14/14 | 14/14 | 14/14 | 15/15 |
| Day 3  | 15/15 | 15/15 | 15/15 | 15/15 | 15/15 | 15/15 |

1 – Failure in the amplification of the fragment. 2 – Variant not sequenced.

Most fragments were consistently amplified and sequenced, with only two failures: one in the sequence (day1) and one in the amplification (day2). The validation was made with routine post-mortem samples. The reported failures are in the larger fragment (with 736bp) and the 2 samples with problems had a degradation index of 1.3 and 1.6, which are above 1 (the cut-off of the Quantifiler trio kit).

The quality of the sequences was evaluated with the following criteria, as referenced in the Userguide for DNA Sequencing by Capillary Electrophoresis of the Applied Biosystems: Signal > 50, Signal/noise > 25 and Sample score between 20 and 50.

Selecting 5 samples of one day of the reproducibility study, the values were calculated for 30 sequences and fulfilled the criteria: minimum Signal was 278, minimum signal/noise was 120 and minimum Sample score was 23.

#### 2.4. Toxicological analysis

All the samples were analyzed by a general toxicological screening for pharmaceutical drugs as antidepressants, antipsychotics, opioids and others. The screening comprised the more prescribed compounds that are known to inhibit CYP2D6 enzymatic action, such as fluoxetine, paroxetine, sertraline, citalopram, haloperidol, methadone or ticlopidine [5,25]. The confirmation analysis of tramadol and its metabolites, O-desmethyltramadol (ODT) and N-desmethyltramadol (NDT) was done in 500 μL of peripherical blood stored at -10°C in test tubes containing 1% of sodium fluoride.

Blood samples were prepared by solid phase extraction using Oasis® HLB 3cc 60 mg cartridges (Waters) and GC-MS analysis was performed using an Agilent 6890 Gas Chromatograph equipped with a HP-5MS (30mx0.25mmx0.25mm) capillary column and a 5973 Mass Detector. The injector was set a 280°C and the injection (1 μL) was made in split mode with 10:1 split ratio. The oven temperature was held at 150°C for 1 min, increased to 290°C at a rate of 5°C/min with a final hold time of 8 min. Data was acquired using selected ion monitoring mode (Error! Reference source not found.).

Using a positive control prepared and analyzed simultaneously to the samples, the
identification criteria for positivity was: retention time within 2% or ±0.1 min; the presence of 3 ionic fragments per compound with S/N > 3; the maximum allowed tolerances for the relative ion intensities were as required by the World Anti-Doping Agency [26].

2.5. Validation of the analytical method
The method was fully validated according to international parameters. Experiments were conducted as described in the SWGTOX guidelines in terms of selectivity, interference studies, recovery, limit of detection, limit of quantification, linearity and calibration model, repeatability, reproducibility, accuracy and carryover [27,28]. All validation experiments were conducted using fortified samples of blank post-mortem blood using LGC and Lipomed standards.

Selectivity was evaluated by analyzing 40 blank samples pooled. Two aliquots of each of the 10 pools were prepared: one was analyzed as blank and the other was spiked with all the analytes (100 ng/mL). The chromatograms were compared, the identification criteria applied and the existence of interferences by matrix constituents was checked in the blank chromatograms. The method proved to be selective, fulfilled the criteria for all the samples and without interferences. For the recovery studies, six replicates were prepared at three concentrations (150, 500 and 850 ng/mL), three of them were spiked before extraction and the others 3 after. The internal standard was only added after the extraction procedure. The obtained peak area ratios were compared and the results are in the Table 3. Five calibration curves were measured over a period of 15 days, using seven levels of spiked blood samples in the working range (between 50 and 1000 ng/mL) and three independent controls were prepared each day with the concentrations of 150, 500 and 850 ng/mL. The calibration model was chosen as explained by Almeida et al [28] using as criteria the correlation coefficient higher than 0.99 and the best calibrators’ accuracy (obtained by back calculating their concentrations). The method was linear over the working range using a weighting factor of 1/x^2. Repeatability (within-day precision) was determined by analyzing six spiked samples at the low, medium and high concentration levels simultaneously. The accuracy and the precision were determined by the calculation of BIAS and the coefficient of variation (% CV), using the concentration obtained for the triplicates of controls (see Table 3). The limit of detection (LOD) was determined by the analysis of blood samples spiked with decreasing amounts of the analytes, being the lowest concentration that
fulfilled the identification criteria, with the signal/noise of all the peaks above 3, in the
replicates (Table 3). The limit of quantitation (LOQ) was validated by analyzing six
replicates of spiked samples with a concentration of 50ng/mL (the first point of the
calibration curve) and verifying the coefficient of variation (<10%). Dilution of the
sample was tested for 1:2 and 1:5 using 10 real samples, covering a concentration range
between 50ng/mL to 5000ng/mL. The main results of the method validation are
summarized in Table 3 Error! Reference source not found..

Table 3. Summary of the main results obtained in the validation of the confirmation method for
tramadol (TMD), N-demethyltramadol (NDT) and O-demethyltramadol (ODT).

| Compound   | RT (min) | Ions (m/z) | Mean Recovery | LOD (ng/mL) | CV | BIAS |
|------------|----------|------------|---------------|-------------|----|------|
| NDT        | 12.1     | 58, 135, 263 | 88%           | 10          | 11% | 2.0% |
| ODT        | 12.5     | 249, 188, 135 | 95%           | 12.5        | 5.2% | 1.0% |
| TMD        | 13.4     | 58, 249, 121  | 93%           | 10          | 6.6% | 0.7% |

2.6. Statistical analysis

The genetic and toxicological results were graphically and statistically compared, using
SPSS 17.0 software. The distribution of the results was tested for normality with
Kolmogorov-Smirnova test and the hypothesis was rejected. Non-parametric tests were
then used and the statistical differences between the medians of the genotype groups
were calculated using the Mann–Whitney test with 95% of confidence interval.

3. Results and discussion

Post-mortem blood samples were studied searching for CYP2D6 genetic variants
responsible for the enzyme inactivation, and the results obtained were then compared
with the concentration of tramadol and its main metabolites: NDT and ODT.

3.1. Genetic Results

Among the 100 post-mortem samples analyzed in this study, amplification failed only in
3 samples, which is comparable to other studies [9]. The DNA degradation was
probably the main limitant factor as was demonstrated by the degradation index (DI)
obtained with the Quantifiler trio kit. The degradation index of these samples was 4.2,
6.5 and 12.1 whereas it was under 1.6 for all the others samples.
The sequencing methodology allowed the detection of 4 different alleles: CYP2D6*3 (2549delA); CYP2D6*4 (100C>T and 1846G>A); CYP2D6*6 (1707delT) and CYP2D6*10 (100C>T). Sanger sequencing methodology can’t detect the copy number variation (CNV) of the gene, so it was not possible to identify the gene complete deletion (allele *5). Nevertheless, in cases with allele *5 the PM phenotype assignment is not necessarily compromised. In heterozygotes, this methodology will assign the individual as if he was homozygote for the other allele: if it is null, the individual will be designated as PM; if it’s functional, he will not. On the other hand, in *5/*5 homozygotes, there will be no amplification product because there is no gene. So, when the amplification fails, there may be two main explanations: the low quantity or the degradation of the DNA in the sample, which can be evaluated using the degradation index given by Quantifiler trio kit; or maybe the individual is homozygote for the CYP2D6*5 allele, and this genotype should then be confirmed by a suitable method.

The allele with the higher prevalence was CYP2D6 *4, with a frequency of 19.6%. The allele *10 was detected with a prevalence of 16.5%, which is higher than the expected based on large European population studies and according to CYP2D6 Allelic Variation Summary Table in http://www.cypalleles.ki.se/cyp2d6.htm [30,31] but is in accordance with a recent study for Portuguese population [32]. CYP2D6*10 isn’t a null allele but it can decrease the enzymatic activity and change the substrate specificity of the enzyme [13,33–35]. Only one allele *3 and one allele *6 have been detected. All the alleles that hadn’t any variant in the studied fragments were considered as wild type (WT).

The genotype distribution is presented in the Table 4.

Table 4. Genotype distribution for the 97 post-mortem samples.

| Genotype   | n  | Prevalence |
|------------|----|------------|
| *4/*4      | 5  | 5,1%       |
| *3/*4      | 1  | 1,0%       |
| *4/*10     | 15 | 15,5%      |
| *10/*10    | 1  | 1,0%       |
| *4/WT      | 12 | 12,4%      |
| *6/WT      | 1  | 1,0%       |
| *10/WT     | 15 | 15,5%      |
| WT         | 47 | 48,5%      |
Six persons were predicted as poor metabolizers (PM) according to their CYP2D6 genotype: 5 individuals were found as homozygotes for the allele *4 and one was heterozygote with one allele *3 and one allele *4.

3.2. Toxicology results
Among the post-mortem samples selected for this study, half presented tramadol concentration below 800 ng/mL, within the therapeutic range according to the published reference tables [36,37]. 27 of these cases had a negative result for at least one of the metabolites: 4 of them in the second group (tramadol concentrations above 800 ng/mL).

Descriptive statistics of the concentration of tramadol and metabolites are presented in Table 5.

### Table 5. Descriptive statistics for the concentration of tramadol (TMD), N-desmethytramadol (NDT) and O-desmethytramadol (ODT) in 100 post-mortem blood samples.

|                  | TMD < 800ng/mL | TMD > 800ng/mL |
|------------------|----------------|----------------|
| **mean**         | 439            | 3070           |
| **median**       | 421            | 1514           |
| **max**          | 789            | 34000*         |
| **min**          | 94             | 813            |

|                  | TMD < 800ng/mL | TMD > 800ng/mL |
|------------------|----------------|----------------|
| **mean**         | 312            | 497            |
| **median**       | 77             | 161            |
| **max**          | 464            | 3863           |
| **min**          | 114            | 3127           |

* values < 50 ng/mL and > 5000 ng/mL were obtained by extrapolation of de calibration curves.

3.3. Correlation results
The genetic and toxicological results were correlated. Cases where was not possible to confirm the presence of at least one of the metabolites were excluded, remaining a total of 73 cases. The concentration ratio of TMD and its metabolites was plotted in a boxplot graphic with a decimal logarithmic scale and the samples were grouped in two categories according to the genotypes: PM and others. A third group of cases with positive result to inhibitors compounds, such as fluoxetine, paroxetine, sertraline, citalopram, ticlopidine and methadone [25,38,39] was also plotted (INIB). Graphics are presented in Fig. 1.
The metabolic ratios TMD/ODT or TMD/NDT used by other authors [7,9], as well as the concentration ratio NDT/ODT were tested. The best correlation was obtained using NDT/ODT ratio, as is shown in the graphics presented in Fig. 1. This observation can be explained by the complementarity of the two tramadol metabolic pathways [1,7,10]. In the presence of high substrate concentrations, low CYP2D6 concentrations or when this enzyme is inhibited, a metabolic switch in favor of enhanced N-demethylation can be observed. On the other hand, the possible involvement of CYP2D6 in the elimination process of NDT may explain the increase in its concentration. So, in these cases the ratio between the two metabolites will be higher, allowing to differentiate the PM phenotype and the possible presence of a CYP2D6 inhibitor.

Using the concentration ratio NDT/ODT, the poor metabolizers (PM) are completely separated from the others (INIB and Others), with a NDT/ODT concentration ratio
above 7. The INIB group has a wide concentration ratio interval, but more than 3 quarters are between 2.5 and 7. In these cases, regardless of genotype, the interaction of the inhibitors leads to a different metabolic behavior. These results are in accordance with other previous studies [15,38,40,41]. The medians of the groups were statistically compared using a Mann-Whitney test with a level of significance of 0.05 and proved to be significantly different using the NDT/ODT ratio.

In post-mortem cases, the information about the administration is oftentimes unknown, like the time, dosage, route and the time until death [9]. The concentration range is very wide for both the parent compound and the metabolites and is not correct to compare it with the results obtained in clinical studies. A high TMD/ODT does not necessarily means that there is a deficient metabolization. Many factors can explain it, like co-medication, existence of pathologies, or if the death occurred right after the administration. NDT/ODT ratio can be useful to reduce the impact of those unknown variables, as the degree of metabolization at the time of death. Further evaluation of these data might be important and should be considered in future studies.

3.4. Case Results

In this study, six individuals were found to be poor-metabolizers (PM). All the available information concerning these six cases is presented in the Table 6.

Table 6. Case information: Age, gender, probable cause of death, toxicological findings and genotype of the 6 PM cases.

| CASE | age | gender | Probable cause of death | TMD (ng/mL) | NDT (ng/mL) | ODT (ng/mL) | Other substances (ng/mL) | Ethanol (g/L) | GEN |
|------|-----|--------|-------------------------|-------------|-------------|-------------|--------------------------|---------------|-----|
| 1    | 66  | male   | Unknown                 | 1137        | 1479        | 161         | Pethidine (12) Sertraline (212) Benzodiazepines (< therap) | negative     | *4/*3 |
| 2    | 64  | male   | Unknown                 | > 5000      | 1338        | 167         | Ticlopidine (401) Trazodone (156) Bromazepam (54) Flurazepam (291) | 0.13          | *4/*4 |
| 3    | 71  | male   | Natural (Neoplasy)      | 2130        | 1502        | 50          | negative                 | negative     | *4/*4 |
The first three cases have tramadol concentrations higher than the therapeutic range according to reference tables [36,37] but the ODT concentrations are comparatively low, considering the data published by Grond et Sablotzki and Stamer et al [1, 11, 12]. Namely in the case number 3 the ODT concentration is comparable to the obtained as a therapeutic concentration in the pharmacokinetics studies. In this particular case, the individual was a cancer patient. The absence of a functional genetic variant for CYP2D6 can explain the concentrations found in post-mortem peripheral blood. The higher concentration of tramadol may be due to accumulation or to an increment of the dosage, which can be related with a decrease in the opioid analgesic effect associated with the lower ODT concentration. However, we cannot exclude the possibility of the lack of analgesic effect be due to the development of tolerance in a chronic user. Only one pill was found at the stomach content and the cause of death was determined as natural.

Predicting the analgesic effect of tramadol based on the pharmacogenetics results is tempting but there are multiple factors, some of them still unknown, that can influence the interpretation [42,43]. Additional fundamental research and collection of routine data is still needed before using pharmacogenetics results as evidence in court. However, in particular post-mortem cases, these approaches, together with all the autopsy findings and clinical information, can be very useful in the investigation of cause of death.

4. Conclusions

This study proved that Sanger sequencing methodology can be successfully applied to the detection of genetic polymorphisms at CYP2D6 in post-mortem blood samples. The method proved to be specific, accurate, with a good precision and limit of detection for the null variants analyzed.
The results showed a good correlation between the PM genotype and the toxicology results of the tramadol metabolic ratio NDT/ODT, appearing to be an alternative parameter in the evaluation of the degree of metabolization of tramadol in post-mortem cases.

The presence of enzymatic inhibitors affects significantly the degree of metabolization, which can be seen in the results obtained. By this reason, is very important to include that compounds in the toxicology screening.

The detection of allelic variants described as non-functional can be useful to explain some circumstances of death in the study of tramadol positive cases and the results obtained demonstrate the importance of this genetic tool to forensic toxicology and pathology.

Sanger sequencing methodology applied in this study can also be applied to cases with other substances with the same metabolic pathway (CYP2D6), such as codeine, antidepressants and neuroleptics.

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A. Sajantila, J.U. Palo, I. Ojanperä, C. Davis, B. Budowle, Pharmacogenetics in medico-legal context., Forensic Sci. Int. 203 (2010) 44–52. doi:10.1016/j.forsciint.2010.09.011.
We would like to thank both reviewers for the deep appreciation of our manuscript and for all the suggestions. We tried to fulfill all the requirements and we will answer point-by-point to the comments.

Reviewer #1:

1. The introduction of this manuscript oversimplifies the metabolism and pharmacological properties of tramadol. The metabolism of tramadol is complex and mediated by several polymorphic cytochrome P450 enzymes [Gong Li et al. Pharmacogenetics and genomics (2014)].

* Secondly, tramadol analgesia is mediated not only by the polymorphic mu opioid receptor but also through modulation of norepinephrine and serotonergic re-uptake.

A: I have reformulated the introduction as suggested.

* Thirdly, tramadol is not always given in controlled doses (as stated), but commonly prescribed in outpatient settings. Therefore, the assumption that higher than expected tramadol doses would arise from malpractice or neglect is short-sighted and should probably be removed from the manuscript altogether as it is outside the scope of this work.

A: Removed as suggested.

2. Please provide a reference to support the statement (page 3) that PM of tramadol have reduced analgesic effects with tramadol and reduced adverse effects with tramadol.

A: Done.

3. The postmortem concentrations should not, in general, be compared to those in clinical studies/therapeutic studies. Please discuss at length the various issues with postmortem findings (post-mortem redistribution, variances in drug collection sites (femoral blood, etc), death interval) as they pertain to the population in this study, and as they pertain to tramadol specifically.

A: We have added the information required.

4. The methods section should clearly state which concomitant medications were considered in this study as CYP2D6 substrates. Furthermore, not all CYP2D6 substrates are "inhibitors". Please refer to the page by David Flockhart in Indiana University for correct classification of medication as CYP2D6 substrate or inhibitor.

A: The analytical method has the most common medication, including anti-depressants, antipsychotic and other opioids. For the INIB group we have focused in the substances that are reported as inhibitors (also in the Flockhart chart). The outliers of the “others” group on the NDT/ODT graphic are, curiously,
positive for compounds that are also substrates of CYP2D6, as venlafaxine and nortriptiline. Done as suggested.

5. There are over 100 allelic variants of CYP2D6. In this study, the author's method only categorizes 4 of these variants. The authors then subscribe individuals to either poor, intermediate, or extensive metabolizers based on only 4 variants and make several assumptions on their tested population to rationalize this approach. As their testing approach is not the gold standard in pharmacogenetic testing, given its limited scope of testing, they need to compare their methodology with a platform that tests for the majority of CYP2D6 alleles in order to justify these assumptions. A false positive and false negative rate of attributing an individual to the poor metabolizer phenotype based on only 4 variants needs to be provided.

A: We have clarified in the manuscript the aims of our work. The focus was to detect the PM. We have tried to explain better the exceptions. We changed also the assignment of the genotypes based in the variants searched to restrict it to the PM.
On the other hand, we described in more detail the validation of the method. It is not possible for us to compare our results with other methodology as suggested, but we had used reference materials to evaluate the accuracy of the method.
We would like to thank both reviewers for the deep appreciation of our manuscript and for all the suggestions. We tried to fulfill all the requirements and we will answer point-by-point to the comments.

Reviewer #2:

1. The present manuscript describes a Sanger sequencing method for detection of some CYP2D6 polymorphisms causing a reduced or absent enzyme function, as well as a GC/MS method for the quantitation of tramadol, O-desmethyltramadol and N-desmethyltramadol. The methods were applied to 100 tramadol positive post-mortem samples. The purpose of doing that is however not clearly stated. Several papers on CYP2D6 sequencing methods and quantitative tramadol methods have been published previously. Furthermore, many of those are better described in terms of validation and performance and are using techniques able to detect also the CYP2D6 ultra-rapid metabolizers and quantifying the enantiomers of tramadol and its metabolites. If the authors clearly state the aim of identifying poor metabolizers and further describe and discuss the six cases being poor metabolizers the present manuscript could however add on to the current knowledge within this field.

A: We have clarified in the manuscript the aims of our work.

2. Abstract:
   * "A Sanger sequencing methodology was developed for the detection of genetic variants that cause absence of CYP2D6 activity, such as *3, *4, *6, *8, *10 and *12 alleles". As mentioned later in the manuscript *10 causes a decreased function of the enzyme, not a total absence. Consider to reformulate, for example "…genetic variants that cause absent or reduced CYP2D6 activity".

A: Done.

   * Why is not all alleles possible to identify with the present method mentioned?

A: The amplification of the entire gene is very difficult (> 5000pb), specially in post-mortem samples with high degradation index. On the other hand, Sanger sequencing only allows the analysis of amplicons with a maximum of 1000 pb. To detect copy number variation is necessary to use other methods, with other equipments such as real-time PCR or platforms as AmpliChip CYP450 test from Roche. The method that we have used is simple, rapid, low cost and available in forensic genetics labs and can detect the main variants. We had clarified it on the manuscript as required.

3. Introduction:
   * The aim of the study is not clearly stated. Why was the study conducted? What was the research question? Any hypothesis?

A: We have clarified in the manuscript the aims and scope of our work.
* In the first paragraph it is written that "The information about therapeutic concentrations of ODT is scarce". There are however several publications covering both tramadol and ODT concentrations.

A: The sentence was removed. There are several publications with clinical studies but the toxic or lethal concentrations aren’t well defined, to our knowledge.

* UMs are usually referred to as ultra-rapid metabolizers but in this paper the term ultra-metabolizers are used instead. Consider changing that.

A: Changed

* I also suggest that explanations of abbreviations, for example "poor metabolizer (PM)", are given the first time the word is written. Subsequently only the abbreviation is used.

A: Changed

4. Materials and methods:

* If the toxicological analysis has been published previously a reference referring to that publication could be added to section 2.2. If there is no previous publication the sample preparation and validation could be further described. The validation parameters investigated are given but no information on how the experiments were conducted. What chemicals, reagents and reference compounds were used? What quality control levels were used? How many replicates were utilized? What kind of blank blood was used? What were the predetermined acceptance criteria for each validation parameter? Please clarify in the manuscript.

* It is also important that results are given for all the validation parameters investigated, and they could with advantage be given in the results and discussion section.

* It is stated that the coefficient of variation is under 11% for the three compounds. Clarify what parameters that are referred to.

* Was dilution integrity part of the validation? Since calibration ranges between 0.05-1 mg/L and some samples have significantly higher concentrations I assume some dilution was made? Please clarify in the manuscript.

A: we described in more detail the validation of the method.

* What transitions were used in SIM mode? Add those to the manuscript.

A: added as requested.

* The two first comments for this section are applicable also to the validation of the sequencing method. There is no information on how the validation experiments were conducted and results are scarce. "The quality of the sequences was considered good", in terms of what? Accuracy is an especially important parameter when it comes to Sanger sequencing; how was the accuracy experiment conducted and what was the result of it? Please describe in the manuscript.

A: we described in more detail the validation of the method.
* Should pb be bp instead, bp for base pairs?

A: Changed

* Which polymorphisms were used for the identification of allele *8, *12, *14, *15, *40 etc., mentioned on page 5? Add those to the manuscript.

A: Done

* The thermocycling conditions used for each fragment could be described in the manuscript.

A: Done

5. Results and discussion:

* The first sentence on page 6 is in my opinion not necessary.

A: Removed

* Check the maximum concentration for NDT in table 1, should it be 5889?

A: Corrected

* It does not emerge from table 1 that 27 cases had metabolite concentrations below the detection limit (is the limit of detection and the limit of quantitation the same?), as it does in the text. Consider if it should since minimum values for the metabolites are presented in the table, and the table text says descriptive statistics for all the 100 cases.

A: Corrected.

Values \(< 50\ \text{ng/mL and} > 5000\ \text{ng/mL were obtained by extrapolation of de calibration curves.}

* Concerning the first and second sentence in section 3.2: What was the degradation index of the three samples that failed amplification in comparison to the degradation index of the other samples? That information would be of interest to add to the manuscript.

A: added as requested.

as suggested * If it is desirable to shorten the manuscript figure 1 and 2 could be deleted since the information is given in the text as well.

A: Removed

* In figure 2 and table 2 the text implies results for all the 100 post-mortem samples. However, since 3 samples failed amplification the results are only for 97 samples, right? Prevalences in table 2 as well as allele frequencies in figure 2 need to be slightly adjusted in case of calculating with 100 samples, which seems to be the case since the sum of prevalences in table 2 should be 100% but is only 97%.

A: Corrected.

* The allele frequency of the null allele *5 (which was not searched for) is higher than that of *3 and *6 (which was searched for) in a Caucasian population. Therefore consider to reformulate the following sentences on page 7 "All the alleles that hadn’t any variant in the studied fragments were considered as wild type. This assumption was considered acceptable because the prevalence of the other null alleles that weren’t searched for is very low in Caucasian population".
A: The sentence was removed and we have tried to explain better the exceptions, focusing in the identification of the PM, which is the main purpose of the study.

* In section 3.2 it is written "Since this method is not able to detect CNV events, the ultra-metabolizers were included in the group of EM. This assumption was considered acceptable once the final objective of the present work was to detect the PM". What was the purpose of constructing figure 3 if only PMs were important to find? Consider to reformulate.

A: We changed the assignment of the genotypes based in the variants searched to restrict it to the PM. Thank you for your suggestions.

* A further description of the INIB group would be valuable. Were those cases PM, IM or EM? What was the concentrations of the inhibitors? To be able to draw conclusions about the significance of the inhibitors a comparison between for example EMs with and without inhibitors seems more valuable than putting all cases with inhibitors in one group, regardless of genotype.

A: Since we had focused in the PM cases, we removed the EM and IM assignment. On the other hand, and unfortunately, the available information of the cases is scarce. We were not able to do this.

* Table 3 is in my opinion not necessary. It would be more interesting to compare all the measured values for each group, than just comparing medians between groups. Statistically significant differences based on all measured values for each group could be indicated in figure 3, a separate table is not necessary. The number of individuals that are given in table 3 could be given in the text below figure 3.

A: Done as suggested.

* Abbreviations are not explained in the text below figure 3 which would be good.

A: corrected as suggested.

* Why does the authors conclude that the NDT/ODT ratio is a better measure of TMD to ODT metabolism than the TMD/ODT ratio? The correlation seemed better for the NDT/ODT ratio, yes, although other factors than the CYP2D6 genotype might affect this ratio. A discussion concerning the impact of CYP2B6 and CYP3A4 genotype for the formation of NDT would be meaningful. If the authors think that the correlation between the TMD/ODT ratio and CYP2D6 genotype was less than expected a discussion concerning reasons for this would be highly valuable. Could for example the inability of the sequencing method to detect allele *5 and multiple copies of the gene have had an impact on the results? Or could the classification into IMs and EMs have had an impact? The present classification is not wrong although according to other definitions EMs have two functional alleles. The present EM group includes individuals with both one, two and multiple copies of CYP2D6 alleles.

A: We have tried to explain it better, especially for post-mortem cases.

* The information in table 4 is interesting, as well as the measured concentrations in table 5, although the concentration unit must be given in table 5. This information could however be compiled in one table, while DNA-concentration, degradation index and polymorphisms are left
out. The important thing is that all individuals are PMs and that information is given in the table text.

**A: Done as suggested.**

* The results of table 4 and 5 are only discussed with a few sentences on page 11, saying that in three cases the tramadol concentration was higher than the therapeutic range but ODT concentrations were acceptable. What does that mean, what are the conclusions drawn? Case 2 and 3 have "unknown" and "natural" stated as the cause of death, respectively, in spite of high tramadol concentrations. Was the tramadol concentrations not considered toxic (because of low ODT concentrations)? What were the circumstances of death? Any comments regarding the other cases? Please discuss the results in more detail.

* What substances were included in the toxicological screening? It could be interesting to describe case 3, 4 and 5 (without other substances present) in more detail.

* It could perhaps be of interest to show the concentrations and CYP2D6 genotypes for the 10 intoxication cases mentioned in section 2.1.

**A: Done as suggested, although we cannot discuss better the results because of the lack of information. The study of the autopsy results of these cases together with the pathologists would be indeed very interesting but it was not possible yet.**

**6. Conclusions:**

* "The Sanger sequencing method proved to be specific, accurate, with a good precision and limit of detection". As mentioned previously results for all validation parameters are not presented in the manuscript, for what reason it is difficult for the reader to be able to draw the same conclusion.

**A: We have reformulated the manuscript and included the validation parameters.**

* "The results of genotypes showed a good correlation with toxicology results of the tramadol metabolic ratio NDT/ODT, appearing to be a better parameter to know the degree of metabolization of TMD to ODT, than the usual metabolic ratio as TMD/ODT". See also the comments above in the "results and discussion" section. In my opinion this is not a correct conclusion.

**A: We have reformulated the results and discussion and also the conclusions in the manuscript.**

* "The presence of enzymatic inhibitors affects significantly the degree of metabolization" See also the comments above in the "results and discussion" section. It is known that some drugs acts as inhibitors of CYP2D6 and therefore affect the metabolism of tramadol. In my opinion it is however not satisfactory shown in this manuscript.

**A: We have reformulated the conclusions in the manuscript.**

* "The detection of allelic variants described as non-functional were useful to explain some circumstances of death in the study of tramadol positive cases and demonstrate the importance of this genetic tool to forensic toxicology and pathology". What circumstances were clarified in
this study? A more profound discussion regarding the results is desirable.

A: As said above, it was not possible yet. Nevertheless, the cases number 1 and 3 can be good examples of the future applications of this approach.

7. References:
   * Is reference 8, 12 and 13 complete? Where can one find them?
   A: Corrected
   * Where is reference 19 published?
   A: Corrected
   * I am not sure if user guides (reference 20 in this case) are appropriate to refer to in the reference list?
   A: Removed