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Determination of tramadol and its main metabolite in pericardial fluid by dispersive liquid–liquid microextraction combined with gas chromatography–mass spectrometry

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ABSTRACT

Tramadol is an opioid used to treat mild to moderate pain. It has a dual mechanism of action, being not only a pure, non-selective agonist of μ-opioid receptors, but also an inhibitor of neurotransmitter reuptake. In addition, tramadol's main metabolite, O-desmethyltramadol (O-DMT), is also active and contributes to tramadol's effects through the same mechanisms. The use of tramadol became relevant because of its lower rate of respiratory depression as a side effect compared to other opioids and its low potential for abuse. The belief that tramadol is a safer opioid has led to its widespread use, but also to its misuse and abuse. Therefore, the number of deaths reported to forensic toxicology laboratories due to tramadol abuse/poisoning, in combination or not with other substances of abuse, has increased. For this reason, methods for the determination of tramadol in alternative forensic specimens, such as pericardial fluid, which are useful post-mortem clinical specimens, are needed. A correlation between the concentration of several compounds in this fluid and in blood has already been established. Thus, pericardial fluid has been proposed as an alternative forensic sample and is of considerable use when blood cannot be obtained or is affected by post-mortem redistribution. A novel sample pretreatment method based on dispersive liquid–liquid microextraction (DLLME) was developed for the first time for the isolation of tramadol and its metabolite O-DMT from pericardial fluid. Acetone was used as a dispersant and chloroform as an extractant. Determinations were performed by gas chromatography-mass spectrometry (GC–MS) and method validation was performed according to FDA validation guidance. Results showed target linearity in the range 0.05–5.0 μg mL^{-1} for tramadol and 0.3–5.0 μg mL^{-1} for O-DMT with limits of detection (LOD) of 0.02 μg mL^{-1} and 0.1 μg mL^{-1} for tramadol and O-DMT, respectively.

1. Introduction

Tramadol is a centrally acting opioid analgesic used for the treatment of mild to moderate pain [1,2]. Its mechanism of action is dual. Tramadol is a weak and pure agonist of μ-opioid receptors (MOR) and inhibits neurotransmitter reuptake, which increases serotonin release and decreases norepinephrine reuptake [1,3]. Structurally, tramadol is a 4-phenylpiperidine with a 3-methoxy group (Fig. 1), a feature related to its weaker binding to the MOR [4].

Tramadol has rapid absorption kinetics and a half-life of 5 to 6 h. In contrast, its only active metabolite, O-desmethyltramadol (O-DMT), is characterised by a significantly longer half-life (from 7 to 9 h). Tramadol is excreted slowly, mainly in the urine. 70 % undergoes hepatic metabolism including oxidation, N- and O-demethylation and conjugation. The active metabolite, O-DMT, is formed by the action of the enzyme CYP2D6. Genetic differences in this enzyme between individuals affect the way the drug works and its potential side effects and toxicity [1,3–5].

Although tramadol is considered less likely to cause side effects or addiction than other opioids, it still carries risks. Overdose symptoms

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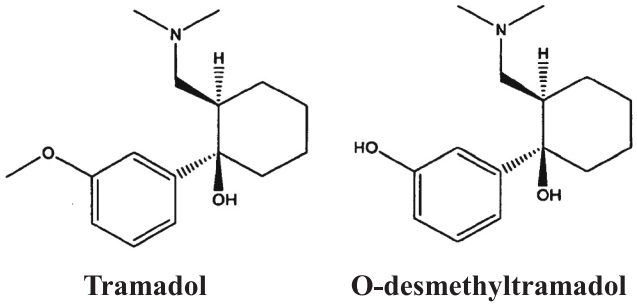

Fig. 1. Chemical structures of tramadol and O-desmethyltramadol metabolite.

are similar to those of other opioids. The perception of tramadol as a safer option has contributed to its misuse and abuse, leading to an increase in related deaths [1,3,4,6].

Blood analysis is essential in forensic toxicology as it allows us to know the toxicological state of the deceased at the time of death. However, its collection is sometimes complicated or impossible due to various factors such as exsanguination or advanced decomposition. For this reason, it is necessary to look for another biological sample that provides complementary information. Pericardial fluid (PF) has been proposed as a promising alternative forensic sample to cadaveric blood because it does not undergo haemolysis and is less affected by post-mortem redistribution (PMR), which facilitates sample preservation. In addition, the comprehensive interpretation of the PMR phenomenon is quite complex and is more difficult for targets such as tramadol due to the moderate volume of distribution of this drug. Therefore, recent studies have focused on PF and have shown good correlations between PF and blood concentrations for many xenobiotics [7–12].

Sample preparation is a key step in complex biological samples to avoid interferences and matrix effects. In toxicology, the most used extraction techniques include liquid–liquid extraction (LLE) and solid-phase extraction (SPE). These conventional methods require large amounts of solvent and often involve multiple steps, resulting in long extraction times [13]. Dispersive liquid–liquid microextraction (DLLME) has emerged as a more environmentally friendly alternative to traditional extraction techniques such as LLE and has been used extensively in toxicological studies [13,14]. The technique uses a ternary system of solvents: a dispersant miscible with the aqueous phase and an organic extractant that combines with the sample to form an emulsion. This mixture promotes phase contact, accelerates equilibration and improves extraction efficiency. After centrifugation, a concentrated droplet of organic phase is formed, facilitating efficient analysis. This technique minimises the use of organic solvents and sample volume [13–16], making it particularly advantageous when working with limited amounts of pericardial fluid. In addition, DLLME also provides a rapid and interference-reducing extraction procedure.

The purpose of this research was to develop a gas chromatography-mass spectrometry (GC–MS) method for the identification and measurement of tramadol and its major metabolite (O-DMT) in PF. Due to the widespread prescription of tramadol, the determination of tramadol is increasingly requested, especially in post-mortem studies. Simple and rapid methods for sample pretreatment are therefore needed in toxicology laboratories to cope with the large number of clinical samples to be analysed, and DLLME is an attractive technique for this purpose. To the best of our knowledge, the current research is the first to focus on the application of DLLME for PF and tramadol, and the procedure was optimised by minimising the amount of chlorinated solvent (in this case to 75 μL). Finally, the method was validated according to FDA guidelines [17].

2. Material and methods

2.1. Chemical reagents and standards

Gradient grade acetone, chloroform, methanol, and carbon tetrachloride, sodium chloride, sodium carbonate, sodium bicarbonate, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Ammonia (25 %v/v in water) was obtained from Panreac (Barcelona, Spain). Tramadol, O-DMT, and tramadol ^{13}C-D$_3$ were purchased from Cerilliant (Round Rock, TX, USA). Ultrapure water was processed through a Milli-Q water system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

Chromatographic analyses were performed using a 7890B gas chromatograph from Agilent Technologies (Santa Clara, CA, USA) with an electron impact ionization at 70 eV interfaced to a 5977B mass selector detector (MSD), also from Agilent Technologies. The selected column was a HP-5MS capillary column (30 m × 250 μm i.d., 0.5 μm film thickness; Agilent Technologies) with helium as carrier gas (1 mL min^{-1}). The injector temperature was set at 280 °C and a purge time of 2 min was used. Samples were injected in the splitless mode. The following temperature programme was used: the initial temperature of the column was held constant at 100 °C for 1 min and then ramped up at 40 °C min^{-1} to 220 °C and held at this temperature for 10 min. The temperature was then increased to 280 °C for 5 min to clean the column. The observed retention time was 9.95 min for tramadol and 11.35 min for O-DMT, and the total run time was 16.50 min. The MSD was maintained at 300 °C, the ion source at 230 °C and the quadrupole at 150 °C. The SCAN mode, scanning from 50 to 550 amu, was used initially to evaluate the tramadol and O-DMT standards, obtaining the retention time and mass spectra of each compound. For each analyte, quantifier and qualifier ions were selected based on their abundance and mass-to-charge ratio (m/z). Once the compounds were identified, the selected ion monitoring (SIM) mode was selected to increase the sensitivity of the method. The selected ions are listed in Table 1 together with their retention times.

2.3. Sample collection

Blank PF samples obtained from autopsies were used to perform the validation procedure. The selection of the blank samples was based on previous peripheral blood and urine analyses of the subjects, which were negative for tramadol and other drugs. The selected PF from deceased subjects were therefore mixed and stored in a freezer at −18 °C.

2.4. Sample preparation

PF (500 μL) was spiked with 5 μL tramadol-^{13}C-D$_3$ (100 μg ml^{-1}) and mixed with 10 mg sodium chloride in a conical glass tube. Then 500 μL of aqueous ammonia solution (pH 12) was added to maximise analyte extraction in the neutral state. The following steps follow the optimised DLLME procedure (Fig. 2): 250 μL of acetone and 75 μL of chloroform were chosen as dispersing and extracting solvents, respectively. Both solvents were rapidly injected into the sample solution, resulting in an

Table 1
Retention times and characteristic ions for tramadol, O-DMT and internal standard.

	Retention time/ min	Quantifier ion, m/z	Qualifier ions, m/z
Tramadol	9.95	263	218, 135
O-DMT	11.35	249	121, 93
Tramadol-^{13}C-D$_3$	9.90	139	267, 222

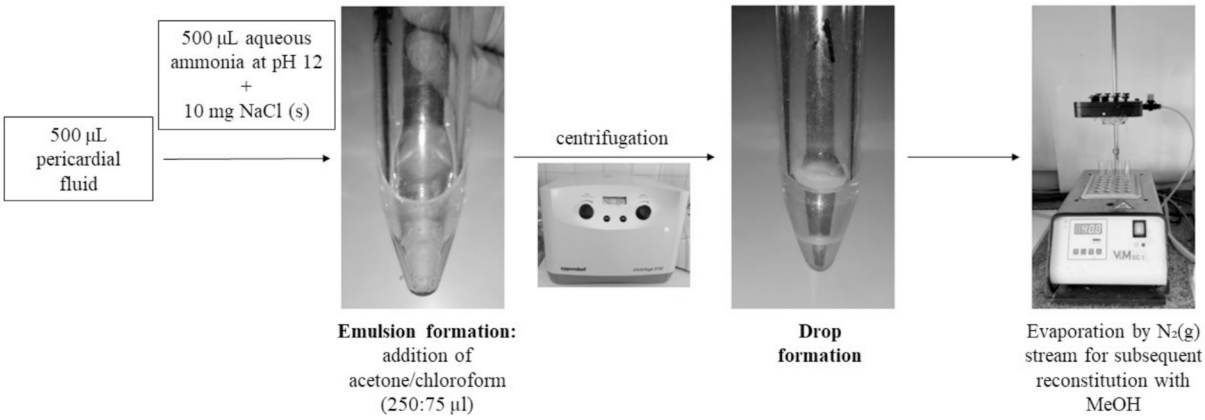

Fig. 2. Work-flow of the optimized DLLME procedure.

emulsion. The mixture was then centrifuged to form an organic droplet which was collected from the bottom using a 100 µL syringe and then transferred to a glass tube. The organic solvent was evaporated to dryness using a stream of N_2 and a heated aluminium block at 40 °C (VLM GmbH, Bielefeld, Germany). The dried residue was reconstituted in 40 µL of methanol before injecting a 2 µL aliquot into the GC–MS system.

2.5. Method validation

The method was fully validated according to the FDA Guideline for Bioanalytical Method Validation [17]. After testing the method for selectivity, linearity, sensitivity, precision, bias and recovery, the applicability of the method for the quantitative determination of tramadol and its metabolite was investigated.

Drug-free control PF, spiked with standards to achieve the selected concentration range, was used to generate standard addition curves after performing the described DLLME method. These curves were obtained by fitting the ratio of analyte peak areas to internal standard peak areas as a function of concentration.

The sensitivity of the method was determined by calculating the limit of detection (LOD) and limit of quantification (LOQ) using an empirical approach involving the analysis of several PF samples with progressively lower analyte concentrations. The limit of detection (LOD) was established as the lowest concentration that the equipment can detect, giving a response of at least three times the signal-to-noise ratio (Equation (1). The LOQ represents the lowest concentration on the calibration curve that could be quantified with the required bias and precision (Equation (2). In addition, the upper limit of quantification (ULOQ), which is determined according to the specific requirements of the analytical approach, was also evaluated. Thus, the ULOQ was established following an evaluation of the therapeutic, toxic and lethal concentration thresholds of tramadol [18,19].

$$Y_{LOD} = \frac{Signal}{noise} ratio \geq 3 \quad (1)$$

$$Y_{LOQ} = \frac{Signal}{noise} ratio \geq 10 \quad (2)$$

Selectivity is the ability of an analytical method to measure a specific analyte in a complex mixture without interference from other substances. This parameter could be justified by PF blanks of different origin, confirming the absence of signals that significantly interfere with the signals of the analytes.

Bias (error) is a measure of the closeness of agreement between the average results obtained by a particular method and the established reference value. Precision is defined as the degree of agreement between results obtained from a repeated series of analyses on a homogeneous sample under predefined conditions. Inter-day and intra-day evaluations were performed to determine both parameters. Negative PF samples fortified with tramadol and O-DMT at three concentrations were analysed for inter-day precision and bias. This involved daily analysis of five replicates for each concentration level, including the LOQ, the ULOQ and an intermediate level.

Recovery was quantified as the ratio between the experimental concentration of the analytes determined after analysis and the true analyte concentration. This parameter represents the efficiency of the extraction method and was evaluated by analysing PF blanks fortified with several target concentrations (high, medium and low) in quintuplicate within several days.

3. Results and discussion

This study presents a method for the determination of tramadol and its main metabolite in PF, an alternative forensic sample, together with an environmentally friendly sample pretreatment. To our knowledge, there are no methods in the scientific literature for the determination of tramadol and O-DMT in PF using DLLME. Therefore, the results obtained in this work could not be directly compared with those found in the scientific literature.

3.1. DLLME optimization

Optimisation of several critical parameters is essential in DLLME to improve extraction efficiency. Therefore, parameters such as type and ratio of extractant and dispersant, pH and ionic strength (salting-up) were evaluated. Extraction time after injection of extractant/dispersant has previously been shown to have a minimal effect on extraction efficiency due to the rapid rate of diffusion and equilibration [15]. Therefore, extraction time was not considered for optimisation. The combination of the sample with the extractant/dispersant mixture was centrifuged immediately after injection of the solvent mixture.

As the PF volume was small, the sample volume was kept constant at 500 µL, which is consistent with the conventional sample volumes used in DLLME. The reported applications of DLLME for tramadol extraction from plasma, urine, and vitreous humour have proposed sample volumes within the 1–5 mL range [11,20–24].

3.1.1. Selection of the disperser and the extractant solvents

The choice of an appropriate extractant is crucial to optimise analyte extraction. Typically, solvents that are denser than water, often halogenated organic solvents, are used. Conversely, the dispersant must have the ability to facilitate emulsification and demonstrate solubility in both phases [15].

An initial screening was carried out to identify the most suitable

dispersing/extracting solvent combination. In all cases, 500 μL of dispersants (acetone and acetonitrile) and 40 μL of extractants (chloroform and carbon tetrachloride) were used at pH 12. The final evaluation was performed by pre-treating PF samples spiked with tramadol and O-DMT at 1.0 μg mL^{-1} with those extractant/dispersant combinations that led to droplet formation. The results of these experiments are plotted in Fig. 3 and show that the combination of acetone as dispersant and chloroform as extractant gave the highest target signals.

3.1.2. Evaluation of solvents volume ratio

The ratio of the volume of dispersant to the volume of extractant is a critical factor in achieving high extraction efficiency. To avoid adverse effects on dispersion, it is important that the proportion of extractant is kept lower than that of dispersant [15]. Based on the findings in the scientific literature, different dispersant/extractant volume ratios (500:40, 250:20, 500:150 and 250:75) were systematically compared in terms of signal intensity. The evaluation showed that the disperser/extractant ratio of 500:150 gave the highest signal intensity. However, the 250:75 disperser/extractant ratio was selected as it gave a slightly reduced response using half the volume of solvent. This slight difference in signal intensity is considered acceptable in the context of toxicology where strict requirements for extremely low LODs are not necessary.

3.1.3. Effect of pH

pH is a critical parameter to ensure proper extraction of analytes in their neutral state. The pH must be kept close to the isoelectric point of the analytes to facilitate efficient transfer from the aqueous solution to the organic phase [15]. Tramadol and O-DMT are alkaline in nature with pK_a values of 9.13 and 9.12 for tramadol and O-DMT respectively. Various alkaline solutions were therefore evaluated, including the disodium carbonate/sodium hydrogen carbonate buffer at pH 9.5, aqueous ammonium at pH 12 and 0.5 M aqueous sodium hydroxide at pH 14. The results showed that the most favourable response was obtained by using 500 μL of aqueous ammonium at pH 12 (Fig. 4A). This choice ensured an optimal pH environment for effective target extraction.

3.1.4. Effect of the ionic strength

The presence of salts increases the ionic strength of the aqueous phase solution, thereby facilitating extraction. However, caution must be exercised when using salts in DLLME as their effect may not be consistently beneficial to the extraction process due to potential physicochemical changes in the aqueous phase [15]. The amount of sodium chloride was tested in the range of 0–30 mg, with an optimum response at 10 mg (Fig. 4B).

3.2. Method validation

Method validation was performed following the FDA Bioanalytical Methods Validation Guide [17].

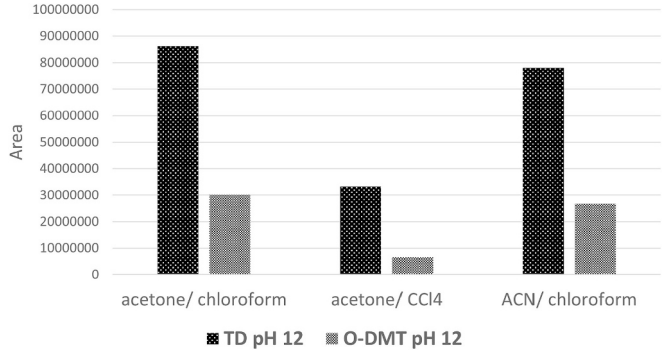

Fig. 3. Tramadol and O-DMT response (peak area) for several dispersant/extractant mixtures.

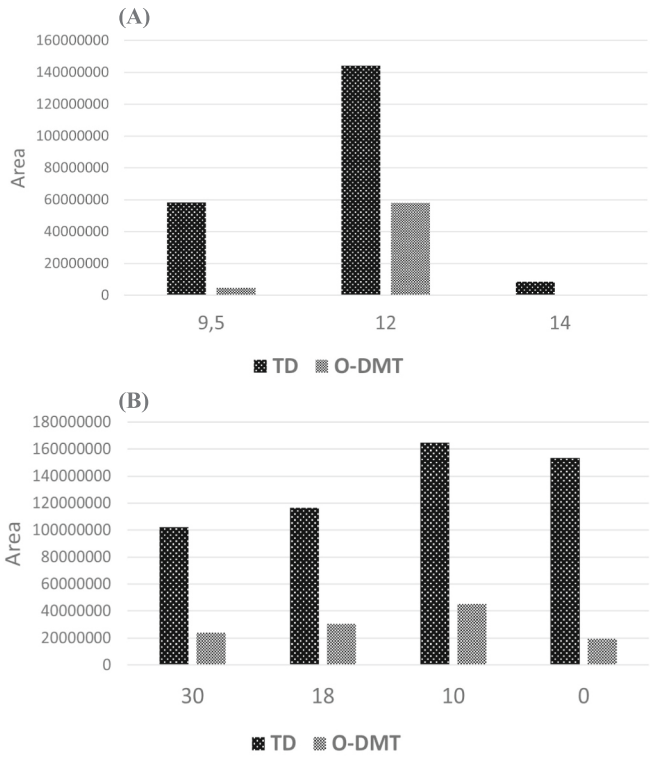

Fig. 4. Tramadol and O-DMT response (peak area) for several pHs (A) and for several mounts of NaCl (B).

The selectivity study was performed by applying the optimized procedure to 6 blank pericardial fluid samples from 6 different autopsies. In all cases, no interferences were found that could affect the determination of the analytes of interest.

The obtained regression lines and LODs of the method are summarised in Table 2, with LODs of 0.02 μg mL^{-1} and 0.1 μg mL^{-1} for tramadol and O-DMT, respectively. The linear range was obtained between the LOQ and the ULOQ. For tramadol the range was 0.05–5 μg mL^{-1} and for O-DMT from 0.3 to 5 μg mL^{-1}. A linear response was observed with good correlation coefficients, higher than 0.99 for both analytes in the investigated range.

Regarding intra- and inter-day precision, bias and recovery (Table 3), precision and bias were found to be less than 15 %, and for concentration close to the LOQ these quality parameters were less than 20 %. Average recovery also met the FDA requirements, and they were found to range from 99 to 116 % for tramadol and within the 88–108 % range for O-DMT.

3.3. Comparison with other analytical methodologies

Literature regarding tramadol in PF is scarce and O-DMT metabolite is not assessed in most of reported methods. Therefore, comparison of DLLME and GC–MS for the determination of tramadol have been focused on other forensic samples such as urine and blood (Table 4). The reported LOQs of 2.6×10^{-4} and 2.57×10^{-3} μg mL^{-1} when using GC–MS detection [11,20] and from 9×10^{-4} to 1.6×10^{-2} μg mL^{-1} for HPLC analysis [21–23] (Table 4). The LOQs achieved with the proposed method are lower than those reported for other forensic samples but as listed in Table 4, other forensic specimens such as serum and urine are available in a high amount whereas the volume of PF is limited. The proposed method offers therefore the advantage small sample and solvent volumes requirements. It should be noted that comparisons are not possible for O-DMT due to the lack of studies using DLLME for the isolation of this target. Therefore, the proposed method has a notable

Table 2
Calibration, LOD, LOQ, and ULOQ for tramadol and O-DMT.

Sample	Intercept	Bias	Slope	Bias	LOD[a]	LOQ[a]	ULOQ[a]
Tramadol	0.017079	0.015	0.296505	0.008	0.02	0.05	5
O-DMT	0.0829451	0.007	0.890584	0.003	0.1	0.3	5

[a] expressed as μg mL^{-1}.

Table 3
Intra-day and inter-day precision (RSD), bias (ME) and recovery of the method.

	Intra-day assay			Inter-day assay		
Concentration (μg mL^{-1})	ME (%)	RSD (%)	Recovery (%)	ME (%)	RSD (%)	Recovery (%)
Tramadol						
0.05	10.9	13	116	8.1	15	108
0.5	0.46	11	99	0.07	12	100
5	7.2	11	107	0.16	6	100
O-DMT						
0.3	8.0	14	88	9.7	12	90
1	7.6	9	106	9.5	14	109
5	2.3	12	98	5.6	11	106

advantage in that it allows the simultaneous quantification of both tramadol and its primary metabolite.

Other sample pre-treatments such as liquid–liquid extraction (LLE) and solid phase extraction (SPE) for isolating tramadol and O-DMT from oral fluid, plasma and urine have been also proposed, implying sample volumes within the 500–2000 μL range [25–31]. These methods have been found to yield LODs and LOQs in a wide range, from 0.001 to 0.03 μg mL^{-1} for tramadol and from 0.025 to 0.01 μg mL^{-1} for O-DMT, which are consistent with those achieved by the proposed method. A further comparison of the proposed methodology with other microextraction techniques when using GC–MS for quantification (Table 5) has shown similar capabilities (LOD and LOQ) than the proposed method but, generally they also required larger volumes of sample and reagents for extraction [32–37]. As listed in Table 5, Only headspace solid-phase microextraction (HS-SPME) [35], microextraction by packed sorbent (MEPS) [36], and DLLME combined with magnetic solid phase extraction (MSPE) using functionalized nanocomposites [37] have been found to offer lower LOD and LOQ than the proposed method (in case of MEPS, detection was performed by gas chromatography tandem mass spectrometry, GC–MS/MS). Other nanomaterials, such as carbon nanotubes [38,39], nickel oxide nanoparticles [40] and molecularly imprinted polymers [41], have been also used for developing SPE procedures for further HPLC analysis.

Finally, advanced solvent-liquid phase microextraction by using deep eutectic and switchable hydrophilicity solvents [42,43] as green analytical sample treatment has been also proposed for treating forensic specimens when assessing tramadol.

3.4. Application to forensic cases

The developed method was used to analyse eight PF samples obtained from the Forensic Toxicology Service (Institute of Forensic Sciences of Santiago de Compostela), corresponding to different cases including suicide, accident and natural deaths. Information on the cause of death and the presence of other drugs of abuse is given in Table 6. Five cases were positive for tramadol, but only three cases were positive for O-DMT (Fig. 5 shows the chromatogram of case number 8, positive for both analytes). Case 6 also stands out, showing a very high content of tramadol (118 μg mL^{-1}) and O-DMT (4.65 μg mL^{-1}), values obtained after a high dilution of the extract. As the validation of the method was not studied taking into account high dilution rates, the results for case 6 are reported in Table 6 as being higher than the ULOQ of the method. Due to the unknown doses prescribed in each case and the time of tramadol consumption, it is not possible to explain the differences in tramadol concentrations found in the different cases. In addition, the small number of current cases does not allow a correlation to be made between tramadol levels in PF and tramadol blood levels (correlations between blood and PF levels have been previously reported for other drugs [7–11]). The LODs of the method were found to be satisfactory, although in some cases the amount of target was less than the LOQ. However, this low amount of drug was not an influential variable in the cause of death.

4. Conclusions

The purpose of this paper is to present the results of a method validation for the quantification of tramadol and O-DMT. This study presents a novel approach for the detection of these two analytes in PF by exploring the use of DLLME as an environmentally sustainable

Table 4
Comparison of proposed methods for the assessment of tramadol in forensic samples by DLLME and GC–MS and HPLC-UV/FD.

Sample	Technique[a]	Sample volume/ mL	Dispersant/extractant	Extractant volume/ μL	Dispersant volume/ μL	LOQ/μg mL^{-1}	Ref.
Urine	GC–MS	5.0	Ethanol/Carbon tetrachloride	30	500	0.0257	[11]
Blood and urine	GC–MS	5.0	Ethanol/Tetracarbon chloride	30	1000	0.00026	[20]
Vitreous humour	HPLC-UV	1.0	Methanol/Chloroform	300	1000	0.016	[21]
Vitreous humour	HPLC-UV	1.0	Methanol/Chloroform	100	500	0.015[b]	[22]
Urine	HPLC-FD	5.0	Acetone/Chloroform + ethyl acetate	70 + 30	600	0.00090	[23]
Urine	HPLC-MS/MS	0.5	Ethyl acetate/Chloroform	–[c]	–[c]	0.0027	[24]
PF	GC–MS	0.5	Acetone/Chloroform	75	250	0.05	Proposed method

[a] GC–MS, gas chromatography – mass spectrometry; HPLC-UV, high performance liquid chromatography – ultraviolet detection; HPLC-FD, high performance liquid chromatography – fluorescence detection.
[b] LOD.
[c] not given.

Table 5
Comparison of proposed methods for the assessment of tramadol in forensic samples by micro-extraction techniques and GC–MS.

Sample	Sample volume/ mL	Extraction procedure[a]	LOD/μg mL^{-1}	LOQ/μg mL^{-1}	Ref.
Blood and urine	1	DSPE	0.01–1.5	0.01–1.5	[32]
Plasma, saliva and urine	2	UA-MSPE	0.45–0.8	–[b]	[33]
Serum	1	UA-DMSPE	8×10^{-4}	0.0025	[34]
Plasma	0.5	HS-SPME	2×10^{-4}	0.001	[35]
Urine	0.25	MEPS	–[b]	0.001[c]	[36]
Urine	4.0	DLLME-MSPE	7.3×10^{-5}	1.1×10^{-4}	[37][d]
PF	0.5	DLLME	0.02	0.05	Proposed method

[a] HF-LPME, hollow fiber liquid-phase microextraction; UA-MSPE, ultrasound-assisted micro solid-phase extraction; HS-SPME, headspace solid-phase micro-extraction; UA-DMSPE, ultrasound-assisted dispersive micro solid-phase extraction; DSPE, dispersive solid-phase extraction; MSPE, dispersive magnetic solid-phase extraction; MEPS, Microextraction by packed sorbent.
[b] not given.
[c] LLOQ.
[d] GC–MS/MS.

alternative to traditional LLE. In addition, this investigation highlights the potential utility of PF as an alternative forensic sample, particularly in scenarios where obtaining suitable blood samples proves challenging. However, it is important to note that despite previous research in this area, a definitive correlation with blood content could not be established, primarily due to the limited number of cases. Therefore, further research is needed to investigate the correlation between tramadol and O-DMT in PF and blood.

Chloroform and acetone were found to be ideal extraction and dispersion solvents, respectively. Successful separation and identification of tramadol and O-DMT was achieved by GC–MS. The proposed method was validated according to FDA guidelines and showed limits of quantification of 0.05 and 0.3 μg mL^{-1} for tramadol and O-DMT, respectively. Precision and accuracy were confirmed with errors below 20 % and recoveries ranging from 87 % to 117 %. These results indicate that the method is highly selective, sensitive and accurate, making it suitable for forensic applications. The developed method was used to analyse eight FP samples. Five cases were positive for tramadol, but only three were positive for O-DMT. Future research aims to extend the application of this technique to demonstrate the potential of PF as an alternative sample in toxicology. In addition, efforts will focus on optimising the DLLME method to improve its performance and minimise the use of halogenated organic solvents, thereby addressing its current limitations.

5. Institutional Review Board Statement

The approval of the Ethics Committee of Galicia was not required because the toxicological data used in this work do not allow the identification of the subjects.

Table 6
Results of applying the method to real cases.

N°	Gender[a]	Age	Cause of death	Other drugs (μg mL^{-1})[b]	[Tramadol] (μg mL^{-1})	[O-DMT] (μg mL^{-1})
1	F	47	Natural	Gabapentin (12.8), Alprazolam (0.180) Paroxetine (0.05)	< LOQ	< LOQ
2	F	60	Natural	Paracetamol (1.80)	1.30	< LOQ
3	M	55	Natural	–	0.104	< LOQ
4	M	87	Accidental choking	–	1.14	< LOQ
5	F	97	Accidental fall	–	< LOQ	–
6	F	75	Suicide intoxication	Venlafaxine (0.7)Paracetamol (363) , Lorazepam (0.180)	>ULOQ	>ULOQ
7	M	83	Suicide choking	–	< LOQ	< LOQ
8	M	63	Natural	–	1.23	0.384

[a] F, female; M, male.
[b] other drugs (concentration in brackets).

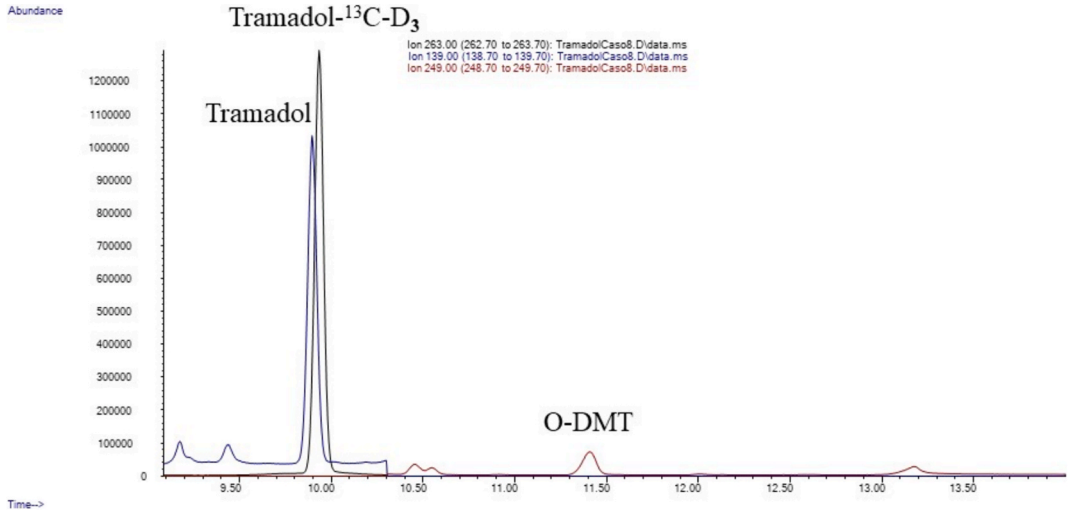

Fig. 5. Chromatogram of real case number 8 (positive for Tramadol and O-DMT).

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CRediT authorship contribution statement

Laura Blanco-García: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Pamela Cabarcos:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Iván Álvarez-Freire:** Validation, Software, Formal analysis, Data curation. **María Jesús Tabernero:** Validation, Data curation. **Pilar Bermejo-Barrera:** Software, Resources, Funding acquisition. **Antonio Moreda-Piñeiro:** Writing – review & editing, Visualization, Supervision, Resources, Methodology. **Ana María Bermejo:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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