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Article

Assessing the usefulness of solid-phase microextraction (SPME) for methadone and its metabolites analysis in urine samples using HPLC/SPME fibre tips

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Abstract:

Solid-phase microextraction (SPME) has been introduced as a novel, simple and single- step technique of extracting methadone (MDN), 2-ethylidene-1,5-dimethyl-3,3- diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP) from urine samples. In this study, direct immersion SPME followed by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) was developed to identify methadone and its metabolites. The separation was performed on a Gemini C18 (octadecyl carbon chain) analytical column (150 x 2.0 mm, 5 µm) and detected by an ultraviolet (UV) detector at 210 nm. The factors influencing the SPME procedure, such as the sample's pH, fibre coating type, salt addition and desorption solvent type were optimised. The best conditions were obtained with a C18 coating at a pH of 11, NaCl 15% and ethyl acetate.

The recoveries of MDN, EDDP and EMDP under optimum conditions were 90.8%, 89.3% and 86.5 %, respectively. The calibration curves for urine samples showed good linearity under optimum conditions (R^2 range 0.9983–0.9988) in a concentration range of 0.025–4 µg/mL for the analytes, using hydrocodone as the internal standard. All samples were analyzed using validated method. The selectivity of this method was evaluated and provided clean chromatograms with no interference in the analysis. Finally, the results show that the novel SPME fibre tips have relatively high extraction efficiency for methadone and its metabolites.

Keywords:

SPME; MDN; EMDP; EDDP; Urine; forensic toxicology; HPLC-UV.

1. **Introduction**

One of the key requirements of forensic toxicology practitioners is identifying and detecting drugs and their associated metabolites. With an understanding of the drug-to- metabolite ratio, one can draw conclusions about the time and dose at which the drug was administered [1]. A significant development in recent years has been the emergence of knowledge relating to the metabolites associated with various drugs, which has benefited the practice of identifying parent drugs from human biological fluids [2].

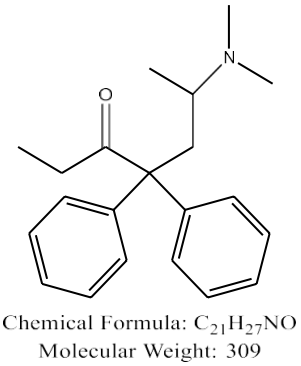

Figure 1: Chemical structure of methadone

The name 'methadone' derives from the fragment of its chemical name: 6-di**meth**yl**a**mino- 4, 4-**d**iphenylheptan-3-**one** [3]. It is a synthetic opioid analgesic used to relieve chronic pain in cancer patients and as a maintenance drug to control withdrawal symptoms in people undergoing treatment for opiate addiction. For humans, cytochrome P450 3A4 (CYP3A4) is implicated in the metabolization of methadone (MDN), particularly in the liver, where it gives rise to 2-Ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine (EDDP; 45–55%) and 2-Ethyl-5-methyl-3,3-diphenyl-1- pyrroline (EMDP; 10–15%), the two critical inactive metabolites [4]. This occurs as a consequence of the process of *N-demethylation* of the hydroxyl group.

Methadone was developed by German scientists in the late 1930s. It was approved by the U.S. Food and Drug Administration (FDA) in 1947 as a painkiller and, by 1950, oral methadone was used to treat the painful symptoms of patients withdrawing from opioids, especially heroin. These patients usually underwent methadone maintenance treatment (MMT) [5]. It is a comprehensive treatment programme that involves prescribing methadone long-term, as an alternative to the opioid on which the patient was dependent. The clinical use of this opioid is primarily in the form of an analgesic for individuals attempting to transition from heroin dependence. Although the benefits of MMT have been established, the potential negative consequences of long-term treatment with this drug on these patients have been accepted [6]. The percentage of institutions providing MMT treatments to their patients was up to 70% [7]. Globally, the number of these patients increased dramatically from 0.5 million in 1999 to 6 million in 2009 [8,9]. Nevertheless, studies have found that the relaxation effect this drug provides can lead to its misuse among these patients. Koob [10] defined this type of addiction as a chronic, relapsing brain disease characterised by compulsive drug-seeking and use, despite harmful consequences. Misuse of this drug after MMT has become a major concern for 75% of World Health Organisation members and should be minimised. Therefore, there is a need for proper monitoring of these patients by laboratory analysis. Moreover, questions have arisen regarding the efficacy of long-term methadone use in cancer treatment. Preclinical studies have demonstrated that methadone can also enhance apoptosis in cancer cells of varying origins [11,12]. This confirms the concept of using methadone as a chemosensitiser in the future treatment of cancer [11,13,14].

However, according to the National Cancer Institute in the U.S., long-term use of this drug in cancer patients has been identified as a promoter of addiction [14]. As a result of this addiction potential, identifying methadone has become increasingly demanded in forensic laboratories [15].

In this study, a solid-phase microextraction (SPME) technique combined with HPLC–UV was applied for the determination of methadone and its metabolites in a urine sample. The effect of various experimental parameters such as sample pH, fiber coating type , salt addition & desorption solvent type on the extraction performance of target analyte was explored and optimized using a multivariate optimization approach based on the design of experiments and response surface methodology. Finally, the optimized SPME method was used for the determination of MDN and its metabolites in a urine sample.

2. Experimental

2.1.Chemicals &ReagentspH (pH=7, 9, and 11)

Methadone, EDDP, EMDP standards, and hydrocodone (HDC) internal standards (IS) were purchased from Cerilliant (Texas, USA). For the HPLC analysis, HPLC grade acetonitrile (ACN), HPLC grade methanol (MeOH), HPLC grade acetone (AC), HPLC grade ethyl acetate (EtAC), HPLC grade isopropanol (IPA), Potassium hydroxide (KOH) analytical reagent, and disodium hydrogen orthophosphate anhydrous (Na_2HPO_4) analytical reagent and sodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) analytical reagent were supplied by VWR International, Ltd. (East Grinstead, UK). Ethanol (EtOH) biotechnology grade was supplied by Sigma-Aldrich (Liverpool, UK).

For the extraction procedure, Low adsorption 1.2-millilitre (mL) vials, SPMELC Tips, 45-µm octadecyl carbon chain (C18), and 65-µm

polydimethylsiloxane/divinylbenzene (PDMS/DVB) were purchased from Supelco Analytical, as was an IonSense 45-µm octadecyl carbon chain- solid-phase extraction (C18-SPE) unit, (Bellefonte, USA). A sample evaporator with nitrogen stream was purchased from Fisher Scientific (Loughborough, UK). HPLC vials (2 mL) and the HPLC instrument were purchased from Agilent Technologies, (Cheshire, UK). Finally, urine samples were purchased from Surine (Lenexa, USA) and were confirmed negative for all analytes.

2.2. Instrumentation

The 3D ChemStation system served as the LC software to collect data and determine peak integration (Revision B.04.02). The Agilent 1200 HPLC system was employed for the overall analysis. The system is comprised of a column oven and UV-DAD (G1315D) detector, a degassing unit, a quaternary pump (G1311A) with four solvent lines, and an auto sampler ALS (G1329A). With the oven set to 25 °C, a Gemini column measuring 150 x 2 mm (Phenomenex C18 110A 5 µm particle size) was used to isolate the methadone and its metabolites. The 2mm diameter had an effect on flow rate; thus, injection volume had to be carefully controlled. A volume of 20 µL was optimal, as that gave a flow rate of 0.2 mL/min (≤ 10%). In order to achieve maximum purity, and therefore identification, the DAD detector allowed for a wavelength range of 193 to 400 nm, while the wavelength of the UV signal was specified at 210 nm. The mobile phase, which was analysed over the course of 40 minutes and comprises solvent A (phosphate buffer [0.0125 M, pH 7.4]) and solvent B (acetonitrile), eluted at a flow rate of 0.2 mL/min in gradient mode.

2.3. Experimental Method

2.3.1. **Extraction procedure**

The SPME LC Tips were initially pre-conditioned by application of a 50:50 methanol: water solution for at least 20 minutes in order to dissolve the stationary phase in the fibre. The fibre appeared dark grey following exposure to the wetting solution within the sample vials.

2.3.2. **Optimization strategy**

For choosing the best extraction, SPME fibre coatings, optimum pH, and different solvents were evaluated, and the extraction recovery was calculated for each parameter. To achieve the optimum pH, extraction of methadone, and its metabolites from urine samples were performed at different pH (7, 9, and 11) using C18, C18-SPE, PDMS/DVB SPME fibre coatings with 1.2-mL low-adsorption vials, allowing 30 minutes for desorption to occur at an agitation speed of 1000 rpm. Samples were dried in nitrogen gas and reconstituted in 50 µL of mobile phase prior to analysis by HPLC-UV. The ionic strength and the addition of NaCl salt, ranging from 0% to 30 %, was examined. Methanol, acetonitrile, isopropanol, acetone, ethanol and ethyl acetate were used as extraction solvents. As a result, relative recovery was calculated by comparing the mean peak analyte areas in the extracted sample and unextracted sample, multiplied by 100 for each parameter.

2.4. Validation Method

All samples were analyzed using a validated method for the efficient extraction and detection of MDN, EDDP &EMDP from urine using HPLC-UV.

Linear range for unextracted samples were prepared at 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, and 4 µg/mL by combining 20 µL of the internal standard (IS) working solution, certain amounts of working solutions A and B and the mobile phase (MP) (phosphate buffer 0.0125 M, pH 7.4 + acetonitrile) in an HPLC vial in order to achieve the desired concentration.

3. Results and Discussion

3.1. Peak identification

Once the injection had been performed, it was the C18 reversed-phase column with a phosphate buffer (0.0125 M) of pH 7.4 that displayed the most obvious separation between injected substances readied in mobile phase for MDN, EDDP and EMDP.

The varied lengths of retention, along with the UV data, enabled these products to be identified. The retention time of each was as follows: MDN – 26.7 minutes, EDDP – 22.3 minutes and EMDP – 28.8 minutes (Figure 2). Moreover, the peaks for these analytes were narrow in shape with a good baseline. Therefore, the separation of these compounds was achieved. Finally, the proposed method was successfully applied to the detection of these drugs in urine samples.

Retention times of methadone and its metabolites with this method could be suitable and be considered not to take as long a time for drug analysis. When a sample from a patient using methadone for anti-heroin addiction is run, for example, and it has morphine, codeine, glucuronides, methadone and its

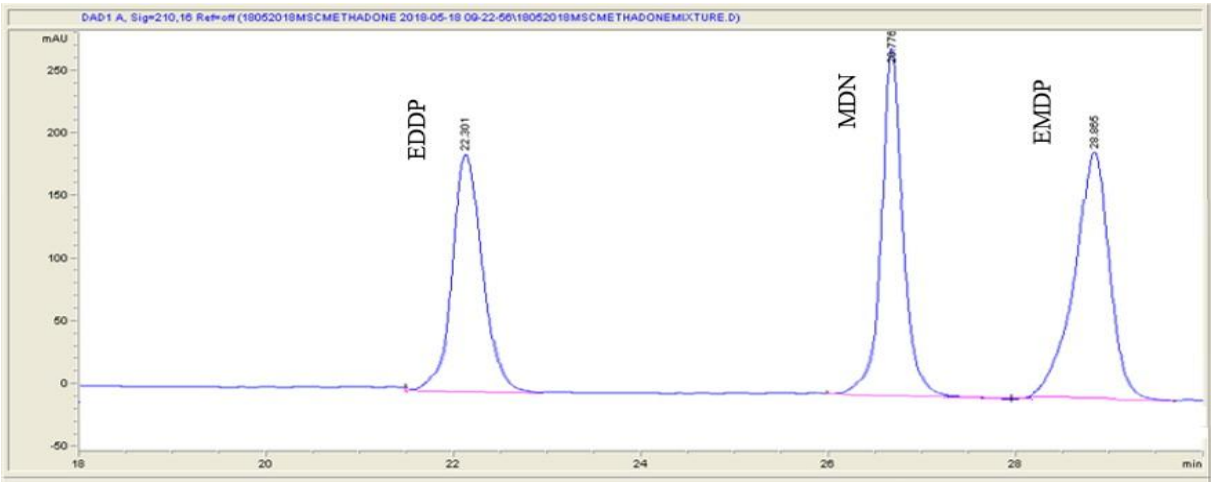

Figure 2: Reference HPLC chromatogram of mixture standard containing 10 µg/mL each EDDP, methadone, and EMDP, which was included to provide retention information for the expected compounds.

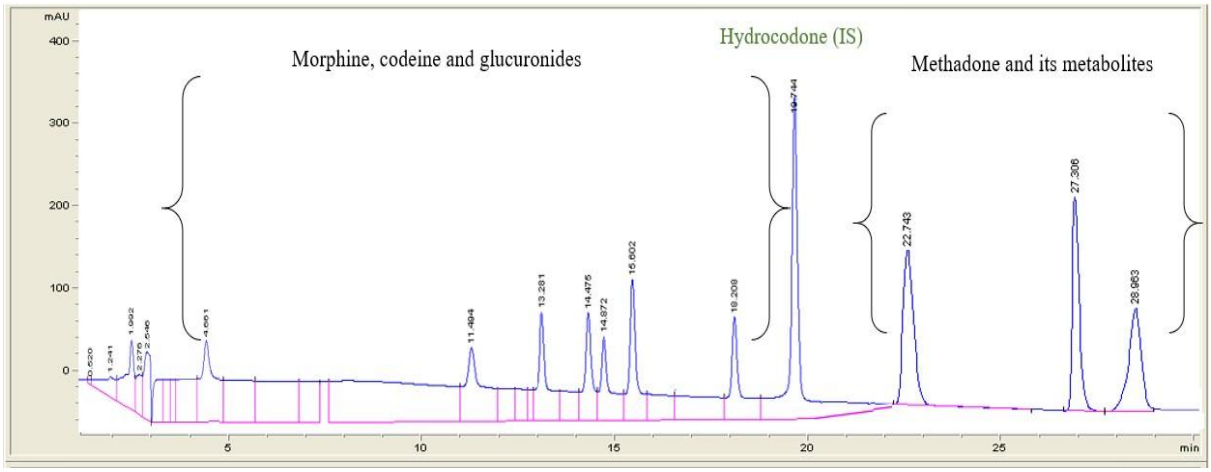

Figure 3: Chromatogram of 10 µg/mL of morphine, codeine, glucuronides, methadone and its metabolites and shows better separation between them.

metabolites, this method could detect all these compounds without interfering with their peaks. Moreover, all these compounds shared hydrocodone as an internal standard, excluding the possibility of the presence of components that could interfere with the purity of these peaks (Figure 3). Finally, the proposed method was successfully applied to the detection of these drugs in urine samples.

3.2. Validation

The validation parameters included: Linearity, limit of detection (LOD), Limit of Quantification (LoQ), recovery, accuracy, and precision were evaluation as shown in Table 1. By coupling with HPLC-DAD, an SPME technique was established for the detection of methadone and its metabolites in urine samples. The selectivity of the method was evaluated comparing the chromatographic profile of the urine sample (the blank) with a urine sample spiked with methadone and its metabolites under optimum conditions (Figure 2). It was found that there were no interferences in the analysis. This gave a good selectivity of the analytical method. These tests were conducted to demonstrate thereliability of the identification method, and all provided satisfactory results.

3.3. Effect of the sample pH and type of fibre coatings

In this study, the effect of sample pH on the extraction of MDN and its metabolites was studied through the pH range of 7–11. These differences in pH values were used to examine the influence of pH on extraction efficiency of these analytes when employing SPME LC tips. As shown in Figure 3, there is a direct correlation between increased pH value and increased recovery of MDN, EDDP and EMDP when using C18, C18-SPE and PDMS/DVB

Table 1. Method validation parameters and acceptance criteria for MDN, EDDP and EMDP quantification

Method Validation Parameter	MDN	EDDP	EMDP
RT(min)	26.7	22.3	**28.8**
Linear range (µg/mL)	0.025-4	0.025-4	**0.025-4**
Correlation coefficient (R^2)	0.9999	0.9946	**0.9952**
Regression equation	Y=0.7971X+0.0091	Y=1.0087X+0.0857	**Y=1.0208X+0.0196**
LoD	3	5	**6**
LoQ	8	9	**16**
Inter-day RSD (%)	11.5-18.6	11.2-17.1	**2.05-3.3**
Accuracy (%)	99.4-100	95.1-99.8	**98.6-99.7**
Extraction recovery (%)	**81.3-88.5**	**83.3-86.4**	**84.5-86.3**

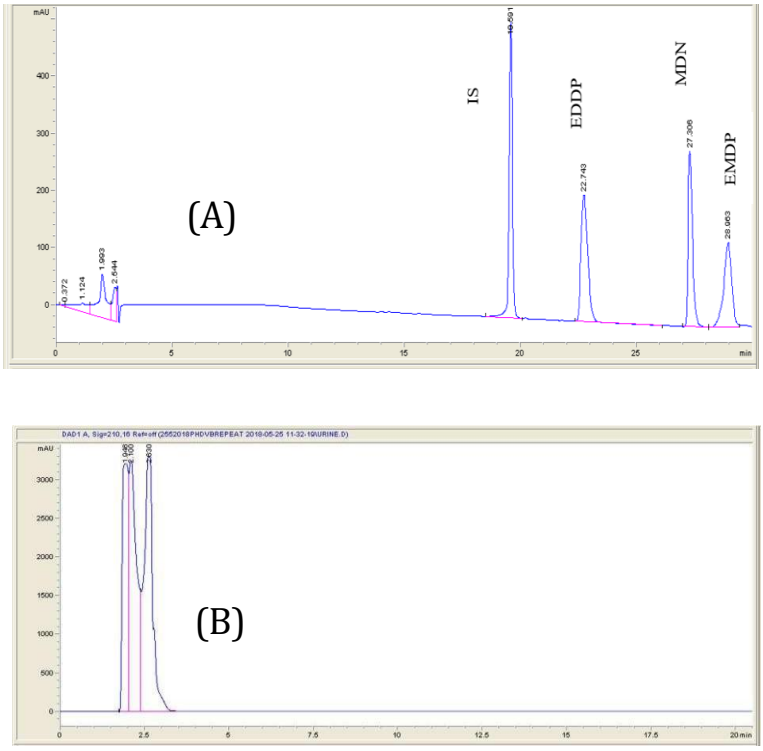

Figure 4: (A) Chromatogram for methadone and its metabolites (hydrocodone as internal standard; optimum conditions were applied) at concentration of 1 µg/mL in a urine sample. (B) Chromatogram for a blank urine sample.

coating, and all coatings show the best recovery at pH 11. When different thin-film SPME (C18, SPE-C18 and PDMS/DVB) fibres were compared in varying pH values, the highest recovery for these analytes was at pH 11, while the lowest level was at pH 7 for all fibres. This indicates that these analytes are hard to extract at pH 7 because they are still unionised in the urine sample, whereby they are easier to extract at a pH of 11 due to the ionisation change. The acid-base equilibrium for analytes containing functional groups and their ionic or neutral forms can be altered by changing the pH value. It can be observed from the structure (Figure 1), that MDN has polar oxygen and nitrogen groups.

To increase the affinity of the MDN for sorbent coating and enhance extraction efficiency, the pH of the solution should be controlled to keep MDN in its molecular form. Since MDN is a weak basic compound (pKa 8.25) at pHs lower than pKa, this analyte remains in its cationic form [16].

Ebrahimzadeh et al [17] suggested that, for total conversion of analytes to neutral forms, the pH should be at least two units above the pKa of a given basic

analyte (pKa + 2). Therefore, a pH of 11 was found to be the optimum condition for determining methadone and its metabolites by the SPME method.

There was a slight difference in recovery of all analytes when comparing C18 to SPE-C18 fibre coatings, and this was related to composition similarities of these two fibres, even though they were provided by different suppliers (C18 and SPE-C18; Supelco and Supelco IonSense, respectively). However, when compared to all fibres at pH 11, the C18-coated fibre obtained the highest recovery for MDN, EDDP and EMDP, at 35.8%, 22.9% and 13.3 %, respectively. All these analytes had the lowest recovery, reduced by 50%, when using a PDMS/DVB coating. In addition, the PDMS/DVB coating showed the lowest extraction efficiency for all analytes through the pH range of 7–11 than other fibre coatings. This poor a recovery can be caused by low disruption between the urine matrix interference and the stationary phase of the fibre on the target analyte. This is because the mode of action underlying the SPME technique is founded upon equilibrium partitioning of the compounds relative to the stationary phase of fibre and a sample. Thus, the stationary phase of PDMS/DVB fibre could affect the extraction efficiency of these analytes resulting in a low recovery of MDN, EDDP and EMDP (17.7%, 11.3% and 6.6 %, respectively). Therefore, the best extraction efficiency is obtained using C18 coating at a pH of 11, with which MDN and its metabolites in their neutral forms and further extractions were carried out.

A performance comparison at pH 11 with two different coatings, (A) C18 and (B) PDMS/DVB in Figure 4, indicates that C18 achieves higher recovery performance and better chromatographic results than PDMS/DVB coating for MDN, EDDP and EMDP. However, as shown in Figure 4 (B), all analytes exhibited a 50% lower recovery when using PDMS/DVB than in Figure 4 (A). This is due to the stationary phase of PDMS/DVB for extraction of moderately polar semi-volatile or volatile analytes from aqueous samples or headspaces, whereas C18 was used for extraction of polar and nonpolar analytes from aqueous samples [18]. Thus, the diffusion coefficient of these polar analytes in PDMS/DVB was lower than in C18, because the stationary phase of C18 fibre was designed for the extraction of these polar analytes.

3.4. Effect of salt addition

In this study, the effect of salt addition on extraction efficiency of methadone was examined by adding sodium chloride to urine samples at percentage levels of 0–30% (w/v). As Figure 5 shows, the addition of a salting agent (NaCl 15%) afforded much better extraction results than those obtained without salt, with the maximum recovery obtained for MDN, EDDP and EMDP as 56.3%, 55.1% and 82.3%, respectively. When the optimum conditions of pH and coating were compared with salt addition, C18 coating at a pH of 11 had a higher recovery for MDN and EDDP than EMDP, and EMDP showed a higher recovery in the presence of salt. Thus, EMDP was the most abundant component obtained through recovery with this parameter. These results can be explained by the fact that the presence of salt increases the ionic strength of the solution and decreases the solubilities of the organic analytes because of the salting-out phenomenon. A study has indicated that NaCl addition can influence extraction performance of methadone positively [19]. Ebrahimzadeh et al [17] claim that high NaCl concentrations may change the physical properties of the fibre and reduce the recovery of these analytes. Therefore, EMDP was sharply dropped to six-fold and MDN and EDDP reduced to a double of recoveries when a double concentration of this salt was used (NaCl 30%). In addition, no statistically substantial changes were observed when the salt was increased from 5% to 10% and from 20% to 25%. Considering these results for salt addition, NaCl 15% was selected as the optimum parameter and carried out in further extractions.

Another challenge in extraction efficiency performance is determining the concentration ofsalt that can extract the highest amount of target analytes, as a large salt concentration can lead to significant extra column dispersion during the separation process, causing peak broadening. This is depicted in Figure 6 (A), the concentration of salt was 30% and caused peak broadening for EMDP. To minimise the band-broadening effects, the concentration of NaCl must be strictly decreased. Therefore, the chromatogram in Figure 6 (B) showed that EMDP was improved in peak shape and increased six-fold in response when the concentration of NaCl was

reduced to half. Moreover, EMDP was significantly different in Figure 6 (B) from that obtained with a C18 coating at a pH of 11 in Figure 4 (A). This enhancement was achieved by the addition of NaCl 15% for the sample to increase six-fold in EMDP recovery.

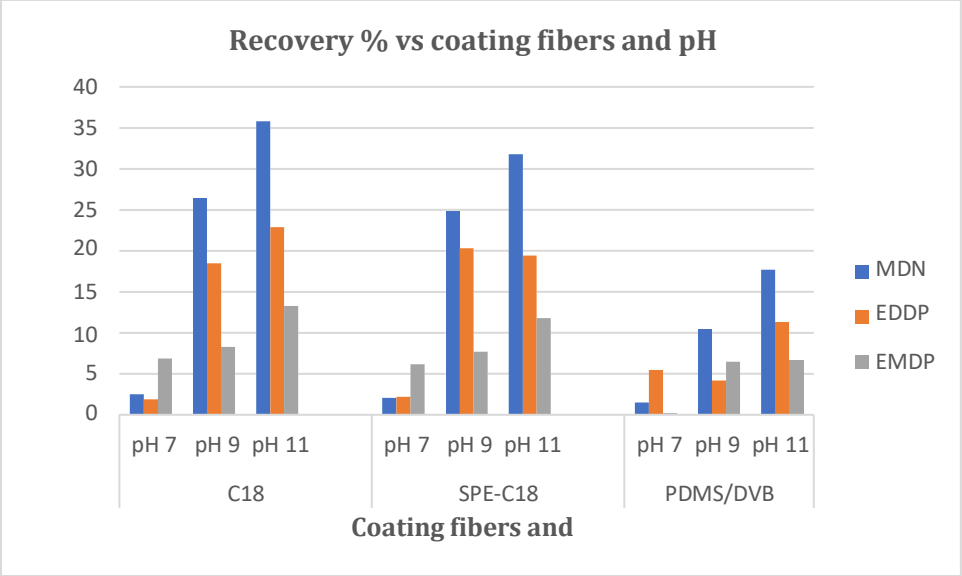

Figure 5: Bar chart of the effect of sample pH on the recovery of MDN, EDDP and EMDP using C18, SPE-C18 and PDMS/DVB SPME Tips in urine sample.

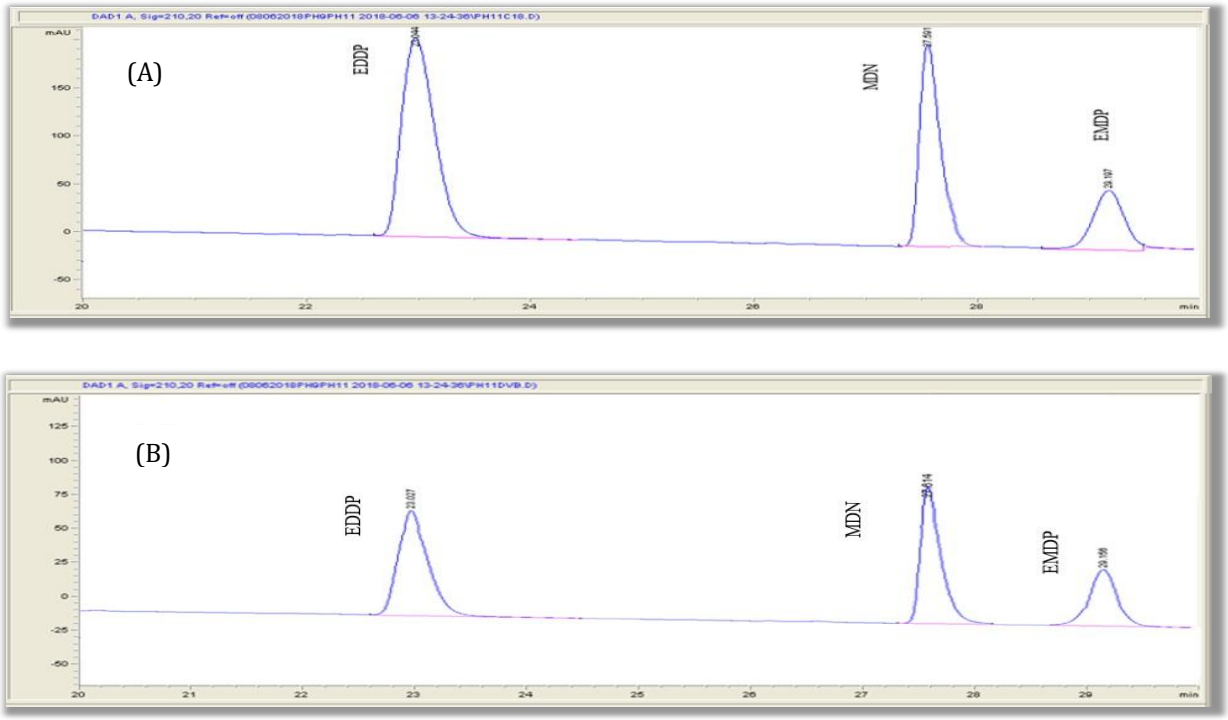

Figure 6. Chromatograms of 1µg/mL extracted sample shows comparison of two coating performance at PH11 (A) C18 and (B) PDMS/DVB.

This causes a decrease in the solubility of the targeted analytes and an increase in the amount of analyte extracted by the fibre coating. As a result, the sensitivity can be significantly increased for these polar compounds.

3.5. Effect of desorption solvent type

To study the effect of desorption solvent, owing to high MDN polarity, several polar solvents such as methanol, isopropanol, acetonitrile, acetone, ethanol and ethyl acetate were studied. Based on the obtained results (Figure 7), ethyl acetate had comparatively higher extractability than acetonitrile, isopropanol, acetone, ethanol and methanol.

Ethyl acetate extracts both methadone and its metabolites in high levels, and the remaining five have a variety of extraction results for methadone and

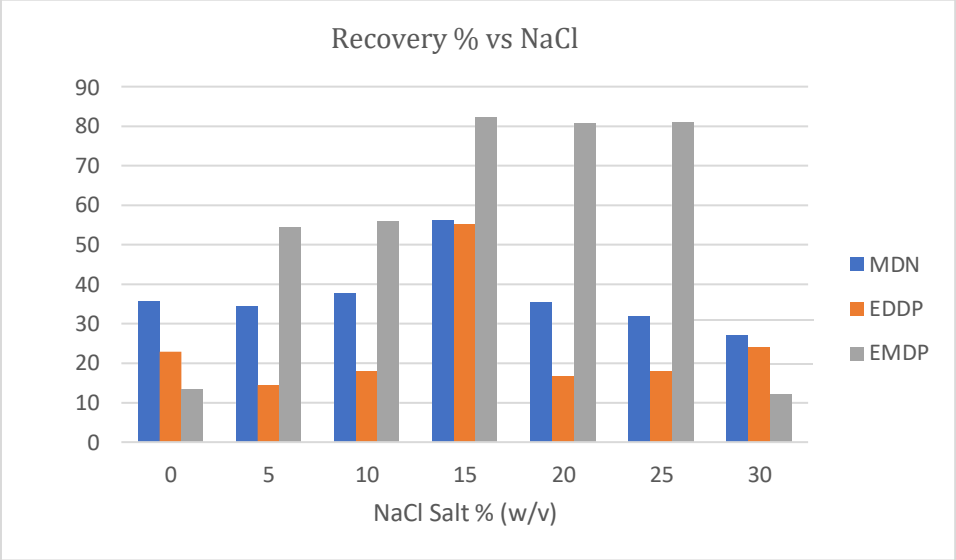

Figure 7: Bar chart of the effect of NaCl on the recovery of MDN, EDDP and EMDP ranging 0–30 %.

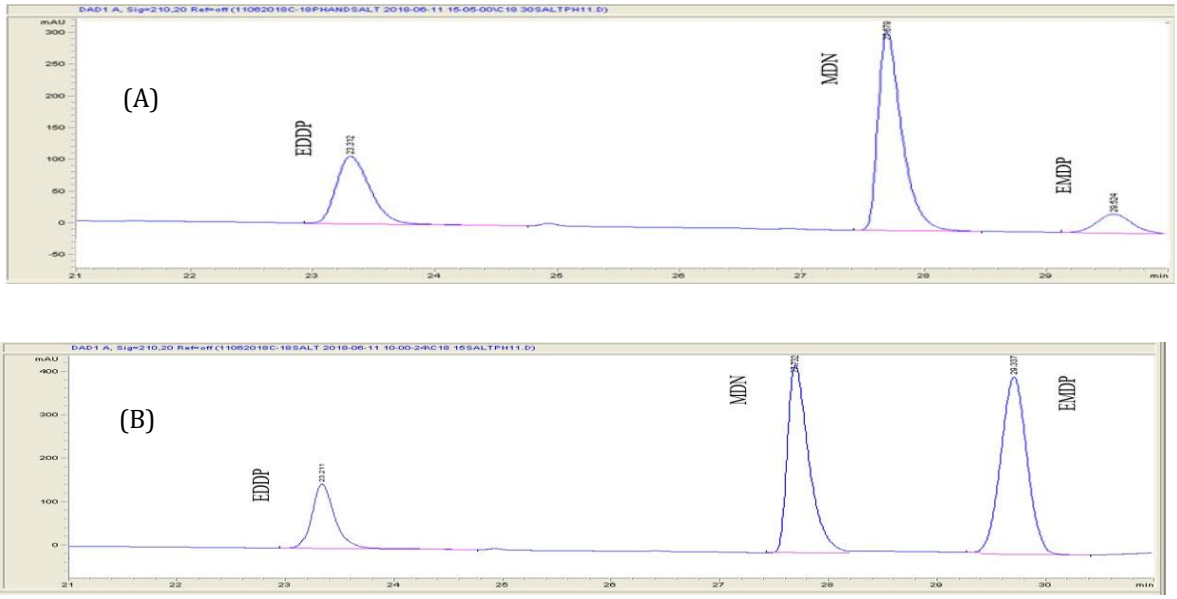

Figure 8: Chromatograms of 1 µg/mL extracted sample with salt addition. (A) NaCl 30% and (B) NaCl15%.

its metabolites.

To illustrate these results, there are various parameters contributing to the strength of the solvent that can be manipulated to achieve better desorption of analytes. These parameters are the nature of the functional groups, solubility of solvent in water, dipole moment, hydrogen bonding ability and other parameters which describe the physiochemical nature of a solvent [20]. Ethyl acetate achieved the best recovery, with MDN 90.8%, EDDP 89.3% and EMDP 86.5%. These analytes were most influenced by the desorption solvent type, showing a significant improvement in recovery percentages when the desorption solvent was changed from methanol to ethyl acetate. Ethyl acetate is capable of extracting these polar compounds resulting from an H acceptor bond [21], and it is easily dissolved in water [22]. However, acetonitrile, isopropanol, acetone, ethanol and methanol are miscible in water, even thoughthey have a hydrogen band [22]. They may cause a change in the chemical properties of these compounds in the urine sample, so diffusion into the organic phase was reduced, and the recoveries of these two metabolites were still unacceptably low. Given these results, ethyl

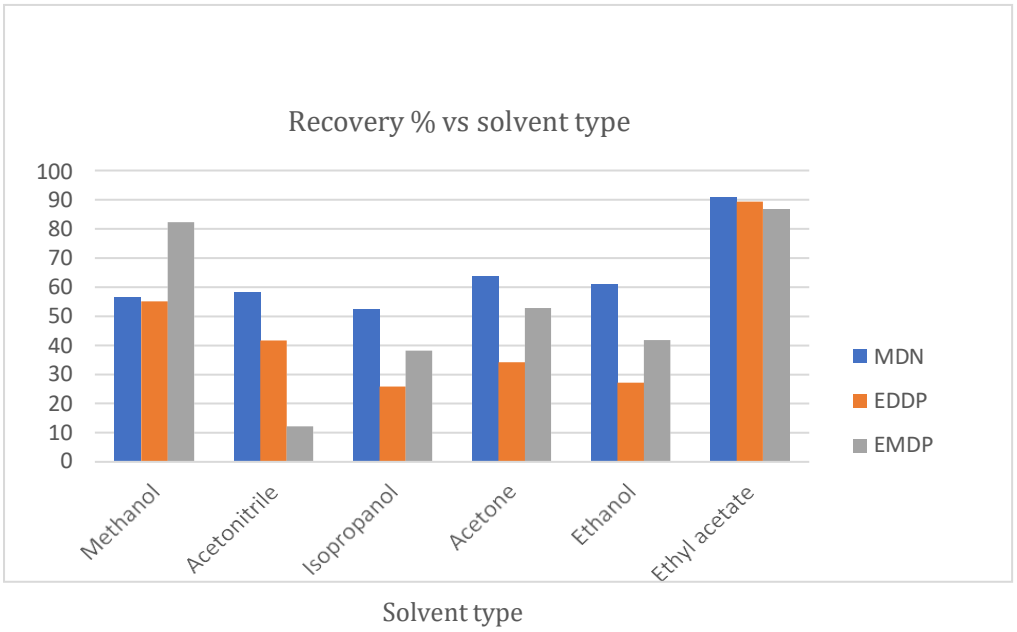

Figure 9: Bar chart of the effect of different desorption solvent types (methanol, acetonitrile, isopropanol, acetone, ethanol and ethyl acetate) on the recovery of MDN, EDDP and EMDP in urine samples, which shows the best desorption solvent is ethyl acetate.

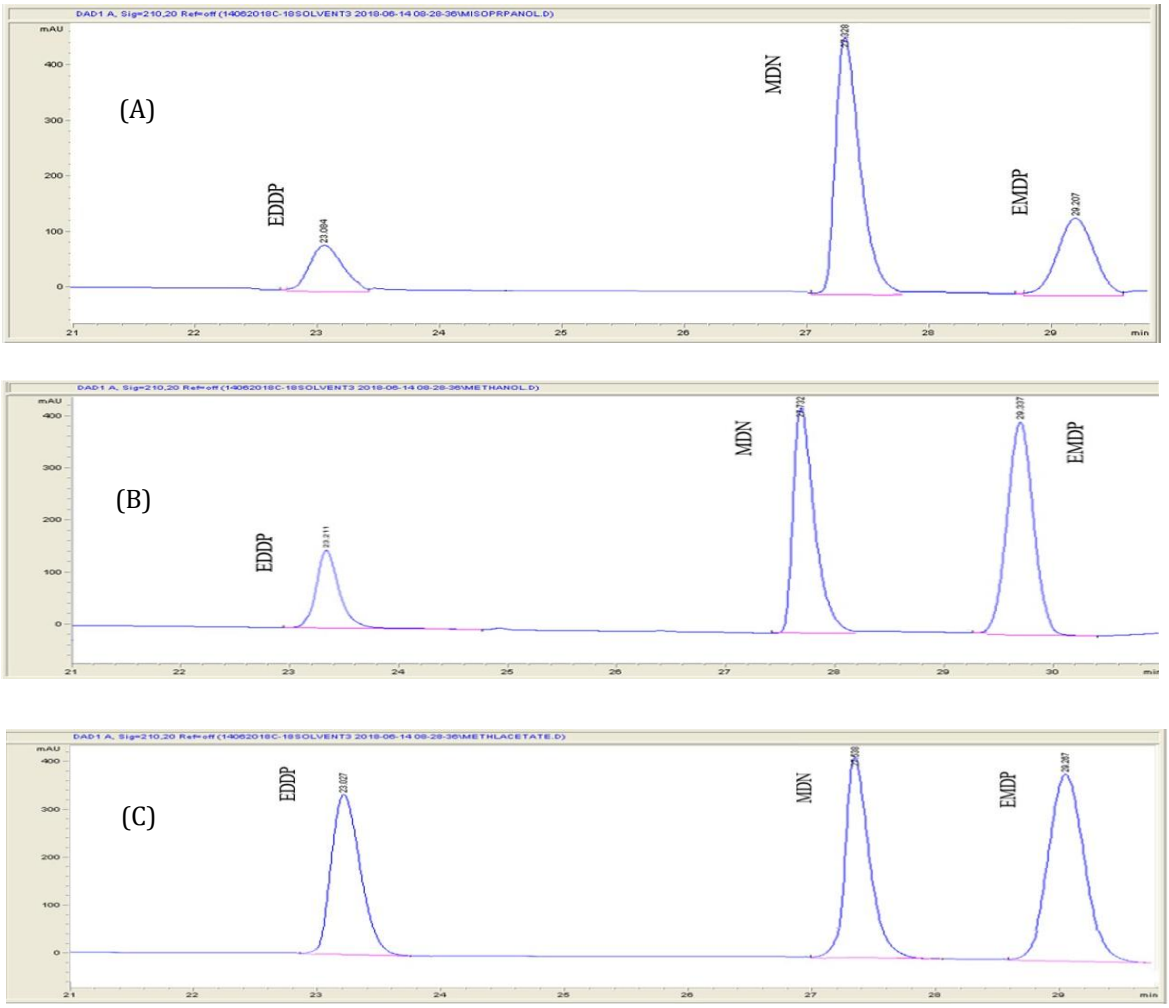

Figure 10: Chromatograms of the effect of desorption solvent types on extraction efficiency for MDN, EDDPand EMDP; (A) isopropanol, (B) methanol, and (C) ethyl acetate.

Table 2. Summary of the optimum conditions of the method development parameters formethadone and its metabolites and their recoveries under these conditions.

Parameters	**Optimum Condition**
pH sample	11
Coating fibre	C18
NaCl	15%
Desorption solvent type	Ethyl acetate
Recovery of methadone and its metabolites under these conditions	
Analytes	**Recovery %**
MDN	90.8
EDDP	89.3
EMDP	86.5

acetate was applied as the desorption solvent for methadone and its metabolites in SPME- C18 fibre at a pH of 11 and NaCl 15.

The difference between isopropanol and methanol in Figure 8 (A) and (B) is that isopropanol has a lower recovery for EDDP and EMDP; whereas methanol has a lower recovery for EDDP only. These two metabolites were most influenced by the desorption solvent type, showing a significant improvement in response when the desorption type changed to ethyl acetate (Figure 8(C)). A very clean chromatogram was obtained with a clear peak, narrow shape, Gaussian distribution, smooth baseline and good separation for all analytes. Ethyl acetate, for example, was employed because it exhibited more powerful desorption abilities for these analytes. Salami Asl et al [23]. pointed out that methadone can be extracted using ethyl acetate as a desorption solvent, because it is similar to the chemical nature of this compound, which may provide it a specific attachment to the targetcoating. It is an H acceptor and has a hydrogen bond to help extract these analytes and enhance their partitioning into the acceptor phase. In contrast, isopropanol and methanol are miscible in water, resulting in reducibility of diffusion of these analytes into the organic phase, and the recoveries were still unacceptably low. Moreover, ethyl acetate is a polar aprotic solvent with a dipole moment of 1.78 and dielectric constant of 6.02. Higher desorption efficiency of this solvent was attributed to the highest polarity and dipole moment (1.7 and 1.68 for methanol and isopropanol, respectively) [21,24]. Consequently, the lower extraction of these analytes is due to the dipole moment of isopropanol being the lowest of the solvents and resulting in a decrease in the abilities of the hydrogen bond acceptor, thus providing the lowest extraction efficiency for these metabolites. However, the highest recovery of these analytes when using ethyl acetate could be due to these compounds having similar skeletons as this solvent, causing these compounds to contain a hydrogen bond acceptor (C=O). According to the physical properties of flammable solvents, flash point is one of the main indicators for evaluating a fire hazard. Flashpoint isdefined as the lowest temperature at which a material can form an ignitable mixture with air and produce a flame when a source of ignition is present [25]. Ethyl acetate has a fruity smell [26] and its flash point is -4˚ C, whereas both isopropanol and methanol have a flash point of 12˚ C, acetone is -20˚ C, acetonitrile is 6˚ C and ethanol is 13˚ C with a bad odor [22]. Shen et al. reported that using ethyl acetate for HPLC gave better chromatographic results with higher resolution and was less toxic than methanol [27-28]. Therefore, comparison between these solvents indicates that ethyl acetate achieves better chromatographic results than others and is safer.

Based on the experiments mentioned above, the optimum SPME conditions are summarized in Table 2.

4. Conclusions

In this project, direct immersion SPME followed by HPLC-UV detection of methadone and its

metabolites was developed. Moreover, the separation of this drug from its metabolites on a Gemini C18 (octadecyl carbon chain) analytical column (150 x 2.0 mm, 5 µm) was achieved and detected by an ultraviolet (UV) detector at 210 nm. Moreover, the factors influencing the SPME procedure, such as sample pH, fibre coating type, salt addition and desorption solvent type were optimised. The best conditions were obtained with a C18 coating at a pH of 11, with NaCl 15 %, and ethyl acetate as the desorption solvent, with recoveries of MDN, EDDP and EMDP at 90.8%, 89.3% and 86.5%, respectively. The calibration curves for urine samples showed good linearity under optimum conditions (R^2 range 0.9983–0.9988) in the concentration range of 0.025–4 µg/mL for the analytes, using hydrocodone as the IS. All samples were analysed using validated method. Moreover, the selectivity of this method was evaluated and provided clean chromatograms with no interference in the analysis. Finally, the novel SPME fibre tips have relatively high extraction efficiency for methadone and its metabolites using HPLC-UV.

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