# Determination of Methadone in Human Urine Using Salting-Out Effect and Dispersive Liquid-Liquid Microextraction Followed by HPLC-UV

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## Abstract

The salting-out effect combined with the dispersive liquid-liquid microextraction based on solidification of floating organic drop (DLLME-SFO) has been developed as a high preconcentration technique for the determination of drug in urine samples. Methadone was employed as model compound to assess the extraction procedure and were determined by high performance liquid chromatography-ultraviolet detection (HPLC-UV). In this method, initially, NaCl as a separation reagent is filled into a small column and a mixture of urine and acetonitrile is passed through the column. By passing the mixture, NaCl is dissolved and the fine droplets of acetonitrile are formed due to salting-out effect. The produced droplets go up through the remained mixture and collect as a separated layer. Then, the collected acetonitrile is removed with a syringe and mixed with 30.0 μL 1-undecanol (extraction solvent). In the second step, the 5.00 mL $K_2CO_3$ solution (2% w/v) is rapidly injected into the above mixture placed in a test tube for further DLLME-SFO. Under the optimum conditions, calibration curves are linear in the range of 2-2000 μg $L^{-1}$ and limit of detection (LOD) is 0.7 μg $L^{-1}$. The extraction recovery and enrichment factor were 83% and 140%, respectively. Repeatability (intra-day) and reproducibility (inter-day) of method based on seven replicate measurements of 100 μg $L^{-1}$ of methadone were 4.1% and 5.3%, respectively. The relative recoveries of urine samples spiked with methadone are 92%-106%.

## Introduction

Methadone (MET) is a synthetic analgesic drug, which has been widely used for the treatment of opioid dependence since the mid-1960s [1]. The treatment has been controversial as it replaces a short-acting opioid (heroin) with a long acting one [2]. The mechanism of action by which MET can alleviate opioid dependence and diminishing symptoms in affected individuals, have been discussed thoroughly [3]. Many analytical methods have been applied to the quantitation of MET in biological fluids. However, isolation and preconcentration of the drug have been the main challenges in the analysis of MET due to the complex matrix of biological samples and the low concentration of residual MET.

Among LLE methods, dispersive liquid-liquid microextraction (DLLME) [4] garnered considerable attention from researchers due to its fast, simple, and efficient extraction procedure, and direct injection of the extracted phase to various instruments. Up to now, DLLME has been successfully applied to the extraction of various families of organic and inorganic compounds from different matrices [5-10], and several reviews have been written on this issue [11-13]. Despite the many benefits of DLLME, the high density of the extraction solvents is its main drawback.

DLLME integrated with the solidification of a floating organic drop (DLLME-SFO) [14] has overcome the abovementioned drawbacks. It is based on an organic extractant that has lower density than water, but solidifies at near ambient temperature. After centrifugation, the test tube is cooled by inserting into an ice bath for 5 min. The enriched analyte in the floated solid phase is collected simply by a spatula and melted at room temperature; then, it is finally determined by chromatography or spectrometry methods.

The performance of DLLME-SFO was illustrated by extraction of

different organic and inorganic compounds [15-18]. In the previous research, we applied DLLME-SFO for extraction and preconcentration of methadone in urine samples [19]. Despite many benefits of the DLLME-SFO, the pretreatment and dilution of urine samples is its main drawback. Because of decrease in matrix effect, urine samples should be pretreated and diluted before DLLME-SFO.

The aim of this work is the combination of salting-out effect and DLLME-SFO, as a sample-preparation method for high performance liquid chromatography (HPLC). Methadone was chosen as model analyte to investigate the feasibility of the improved salting-out-DLLME-SFO technique. To the best of our knowledge, for the first time, the salting-out-DLLME-SFO is developed and applied to the analysis of methadone in human urine without pretreatment and dilution of the samples.

## Materials and Methods

### Reagents

Standards of MET (with a certified purity >98%) was obtained from Cerilliant (Round Rock, TX, USA) as 1 mg $mL^{-1}$ methanol solution. The methadone stock standard solution was prepared in methanol

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at the concentration levels of 1.00 mg L$^{-1}$ for MET. Afterwards, they were stored in a freezer at -20°C. Working standard solutions were prepared daily by diluting the stock solution with methanol. The ultra-pure water (six times distilled) was purchased from Shahid Ghazi Company (Tabriz, Iran). Methanol (for spectroscopy), acetone (Suprasolv for gas chromatography), acetonitrile (Hyper grade for liquid chromatography), acetic acid, sodium dihydrogen phosphate, sodium dodecyl sulfate, sodium chloride, 1-undecanol, n-hexadecane, 2-dodecanol and 1-decanol were obtained from Merck (Darmstadt, Germany). Drug free urine sample (blank) collected from healthy volunteer in our lab was used for the study. Actual human urine samples taken from two young people who were suspicious to consumption of MET were stored at -20°C and analyzed within 48 h after collection without any previous treatment or filtration.

### Instrumentation

Quantitative analysis of the MET was performed on a Knauer HPLC system (Berlin, Germany) equipped with aSmartline-1000 binary pumps and Smartline-UV-2500 detector variable wavelength programmable, an on-line solvent vacuum degasser and manual sample injector fitted with a 20 µL injection loop (model 7725i, Rheodyne, Cotati, CA, USA). Chromatographic separation was achieved on an ODS-3 column (25 cm × 4.0 mm, with 5 µm particle size) from Waters (Milford, MA, USA). The mobile phase consisted of water/methanol (50:50, v/v) adjusted to pH 2.7 with phosphate buffer (0.1 M). A mobile phase flow-rate of 1.0 mL min$^{-1}$ was used in isocratic elution mode and the detection was performed at the wavelength of 210 nm. The Hettich Zentrifugen (EBA20, Tuttlingen, Germany) was used for centrifugations. Chromatographic data were recorded and analyzed using Chromgate software version 3.1.

### Extraction procedure

In the first step, a 10 mL glass syringe barrel was cleaned with pure water and then a frit was placed in the bottom of the barrel and a stopcock was installed. Afterwards, 5 g of NaCl was poured into the barrel and slightly compressed with the syringe plunger. A 5.0 mL of urine sample (spiked or not with MET) was mixed with 1.5 mL acetonitrile and passed through the barrel at a flow rate of 0.5 mL min$^{-1}$. By passing the above homogenous solution through the barrel, fine droplets of acetonitrile were formed at the interface of solid (NaCl) and solution due to dissolution of salt into solution (salting-out effect). The produced droplets moved through the remained solution to top of the barrel and floated on the surface of solution as a separated layer due to lower density of acetonitrile with respect to water. During this step, the analytes were extracted into the fine droplets of acetonitrile. After passing all aqueous solution, the stopcock was closed. The volume of the acetonitrile (separated phase) on the top of remained NaCl solid was about 0.70 ± 0.03 mL. Subsequently, the organic phase obtained from the first step was transferred into a 10 mL glass test tube and 30.0 µL 1-undecanol (extraction solvent) was added to the test tube. Then, $K_2CO_3$ solution (2% w/v, 5.00 mL) were rapidly injected into a test tube, using a 5.00 mL syringe (Gastight, Hamilton, Reno, NV, USA). A cloudy solution, resulting from the dispersion of the fine 1-undecanol droplets in the aqueous solution, was formed in the test tube and the mixtures were centrifuged for 5 min at 4000 g. Accordingly, the organic solvent droplet was floated on the surface of the aqueous solution due to its low density. The sample vial was there after put into an ice bath for 4 min; at this time, the floated solvent was solidified because of the low melting point (14°C). The solidified solvent was transferred into a conical glass sample cup where it was melted immediately. Finally, 25 µL of the extractant was collected with a syringe and injected onto the HPLC-UV.

## Results and Discussion

In this research, the salting-out and DLLME-SFO conjunction was designed and employed for extraction of different drugs from urine samples. To reach a high extraction recovery and enrichment factor with the employment of salting-out-DLLME-SFO, the salting-out and DLLME-SFO conditions must be examined and optimized. Since the DLLME-SFO conditions had been optimized in our previous research [19], those results were used in this research. Only the salting-out conditions together with some notable parameters in the salting-out-DLLME-SFO combination were studied. The enrichment factor (EF), the extraction recovery (%ER) and the relative recovery (%RR) were calculated according to the equations described in our previous research [20].

### Selection of extraction solvent in salting-out step

In salting-out-DLLME-SFO procedure, the extraction solvent in salting-out step should be able to play the role as a disperser solvent in the following DLLME-SFO step. For this purpose, acetone, acetonitrile, methanol, and tetrahydrofuran, displaying this ability, were selected. The obtained results showed that only acetonitrile formed a two-phase system, while other solvents could not be separated from the aqueous solution by passing through a syringe barrel filled with NaCl. Therefore, acetonitrile was selected as an extraction solvent for the further studies.

### Selection of extraction solvent volume in salting-out step

For obtaining optimized volume of extraction solvent in salting-out step, various experiments were performed by using different volumes of acetonitrile (i.e., 0.50, 0.75, 1.00, 1.50, 2.00, and 2.50 mL) and all experiments were performed in triplicates (n=3). The results are shown in Figure 1. According to Figure 1 and considering the experimental errors on the data points, the enrichment factor of MET was found to increase by increasing volume of acetonitrile up to 1.50 mL; while, further increase in volume of acetonitrile caused a small decrease in the enrichment factor. This observation could be attributed to the fact that at lower acetonitrile volumes, the cloudy suspension of the 1-undecanol droplets was not formed well (in DLLME-SFO step), resulting in a decrease in the enrichment factor. By using more than 1.50 mL acetonitrile, the solubility of MET in aqueous phase increases and it causes a small decrease in the enrichment factor. Also, no collected phase was obtained in the case of 0.50 mL acetonitrile. Thus, according to the results, 1.50 mL of acetonitrile was chosen as the optimum volume of extraction solvent in salting-out step.
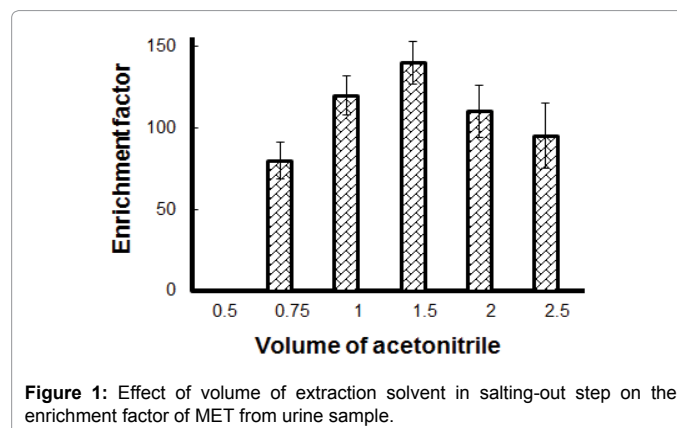


**Figure 1:** Effect of volume of extraction solvent in salting-out step on the enrichment factor of MET from urine sample.

## Effect of the flow rate of the sample solution

The flow rate of the sample solution through the solid (NaCl) is an important factor because it controls the time of analysis and extraction recovery. The flow rate of the sample solution must below enough to perform an effective salting-out. On the other hand, it must be high enough not to waste time. The effect of the flow rate of sample solution was examined from 0.5 to 5 mL $min^{-1}$. As it is illustrated in Figure 2, the flow rates up to 2 mL $min^{-1}$ have no effect on enrichment factor of MET while, at higher speeds, the enrichment factor decreased. This behavior can be explained because the amount of dissolved salt in aqueous phase is decreased at high flow rates and leads to a decrease in efficiency. Thus, a flowrate of 2 mL $min^{-1}$ was selected for further studies.

## Analytical performance of the method

The optimized salting-out-DLLME-SFO and HPLC-UV procedure was validated with respect to limit of detection (LOD), precision (intra-day and inter-day), linear range (LR), EF, and ER. Table 1 summarizes the analytical characteristics of the optimized method. The repeatability (intra-day) and reproducibility (inter-day) were studied by extracting the spiked urine samples (100 μg $L^{-1}$ for MET). The repeatability and reproducibility were calculated and were 4.1% and 5.3%, respectively. Determination coefficients ($r^2$) was 0.988. Good linearity 2-2000 μg $L^{-1}$ were obtained. The limits of detection, based on a signal-to-noise ratio (S/N) of 3, were 0.7 μg $L^{-1}$. Moreover, the EF and the ER of MET were 140 and 83%, respectively.

## Analysis of MET in real samples

The proposed method was firstly applied to determination of the concentration of MET in human urine samples, provided by one male volunteer in our lab, who was not exposed to any drugs or MET for at least 10 months. The results from urine samples showed that they were free of MET. These samples were spiked with MET standards at different concentration levels to assess matrix effects. The results of relative recovery of urine samples are shown in Table 2. As seen, the relative recoveries for MET in spiked urine samples are between 92% and 106%. Four actual urine samples taken from male and female young persons who were suspicious to consumption of MET were also subjected to the proposed procedure. MET was detected in all of the actual urine samples in the range of 38.5-74.6 μg $L^{-1}$. The concentration of MET in different actual urine samples are listed in Table 2. The presences of MET in these samples were confirmed by spiking MET at the different concentration levels. The results of relative recoveries and concentrations obtained by analysis of spiked actual urine samples are also included in Table 2. Figure 3 shows the obtained chromatograms of blank urine sample (A), blank urine sample spiked with 0.7 μg $L^{-1}$ of MET (B), actual urine sample taken from a 22-year-old male (C) and corresponding spiked ones at concentration level of 100.0 μg $L^{-1}$ for MET (D). These results demonstrate that the matrices of the analyzed actual urine samples have little effect on salting-out-DLLME-SFO followed by HPLC-UV for determination of MET.

## Conclusion

In this study, the salting-out effect was combined with the DLLME-SFO technique for the determination of MET in urine samples prior to analysis by HPLC-UV. This combination not only resulted in a high enrichment factor, but also it could be used in complex matrices (such as urine, fruit juice and highly saline solution) without any pretreatment or dilution. As compared with the other sample preparation methods, the analytical procedure offered numerous advantages such as simplicity, ease of operation, high preconcentration factor, low detection limit
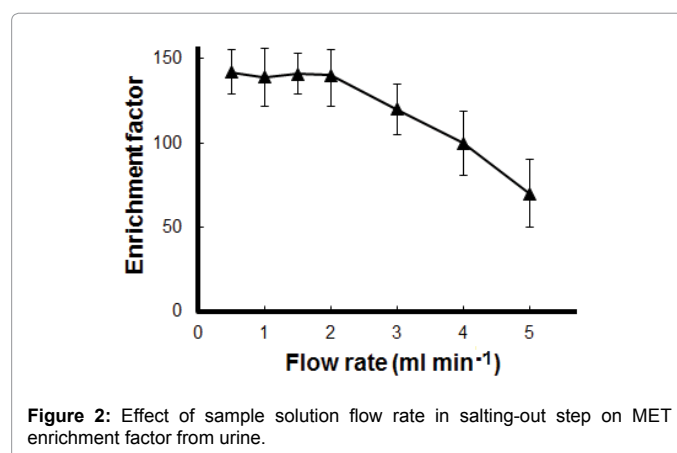


**Figure 2:** Effect of sample solution flow rate in salting-out step on MET enrichment factor from urine.
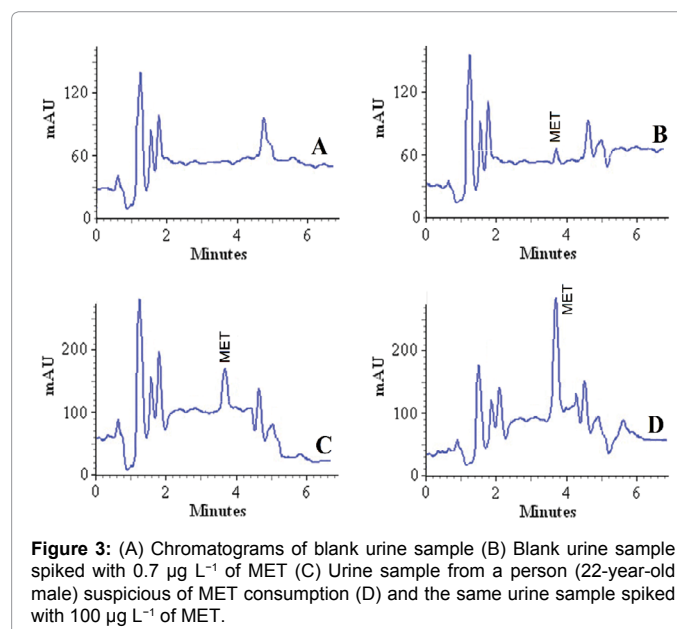


**Figure 3:** (A) Chromatograms of blank urine sample (B) Blank urine sample spiked with 0.7 μg $L^{-1}$ of MET (C) Urine sample from a person (22-year-old male) suspicious of MET consumption (D) and the same urine sample spiked with 100 μg $L^{-1}$ of MET.

| Analyte | RSD[a]% (Intra-day, n=7) | RSD% (Inter-day, n=7) | EF[b] | ER[c]% | LR[d] (μg $L^{-1}$) | $r^{2e}$ | LOD[f] (μg $L^{-1}$) |
|---|---|---|---|---|---|---|---|
| MET | 4.1 | 5.3 | 140 | 83 | 2–2000 | 0.988 | 0.7 |

[a]RSD% at a concentration of 100 μg $L^{-1}$ for MET; [b]EF: Enrichment factor; [c]ER: Extraction recovery; [d]LR: Linear range; [e]$r^2$: Correlation coefficient; [f]LOD: Limit of detection for a S/N=3

**Table 1:** Figures of merit of salting-out DLLME-SFO in a MET-free urine sample.

| Urine samples | Added (μg $L^{-1}$) | Found mean ± SD[a] (μg $L^{-1}$) | Relative recovery (%) |
|---|---|---|---|
| Taken from healthy volunteer (blank) | 0 | ND[b] | - |
|  | 100 | 97.5 ± 6.3 | 97.5 |
|  | 200 | 198.4 ± 14.7 | 99.2 |
| Taken from a 22-year-old male | 0 | 38.5 ± 2.6 | - |
|  | 100 | 135.0 ± 7.4 | 96.5 |
|  | 200 | 247.8 ± 12.2 | 104.6 |
| Taken from a 24-year-old female | 0 | 74.6 ± 5.3 | - |
|  | 50 | 120.6 ± 8.4 | 92.0 |
|  | 100 | 180.6 ± 137.0 | 106.0 |

[a]SD: Standard deviation (n=3); [b]ND: Not detected

**Table 2:** Analysis of blank and actual urine samples for determination of MET.

and relatively short analysis time. Although the obtained results in this work are related to determination of MET, the system could be readily applied for the determination of other drugs from complex biological and pharmaceutical matrices, using different analytical instruments.

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