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Two Phase Solvent Extraction Method for Analysis of Methadon in Immature Human Milk, A Breast Feeding Recommendation in Early Postpartum Period

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In this work we introduced a new two phase freezing (TPF) method coupled with gas chromatography for the extraction, clean up and determination of methadone (MT) in human milk samples. TPF procedure was optimized for extraction of MT from immature milk sample. The extraction of MT was performed from 1.0 ml of milk that contain 0.2 ml of Briton Robinson buffer (pH=2.5) and 0.3 ml of acetonitrile. For separation of acetonitrile from aqueous solution, the solution was placed in refrigerator at −40 °C. The MT was analyzed by gas chromatography. The results demonstrated that the amount of MT that transferred to milk is significantly different from other published reports. The immature milks of six women who were used MT (dose of 90 mg/day) in duration of 1, 2, 3, and 5 h after consumption were analyzed. Our data demonstrated that, before one hour and also 5 h after MT consumption the breastfeeding is safe and between 2-4 h after consumption dose not safe neither.

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Introduction

The full breastfeeding for the first 6 months of infant's life is very important, because it obviate all of essential vitamins, lipids, proteins, liquids, nutrition, immuno oligosaccharides and minerals for infants. Several reports demonstrated that the presence of environmental chemicals [1], pesticides [2], toxins [3] and abuse drugs [4] in human milk may have the potential infant exposures, and therefore, increase the risks of exposures to those compounds [5]. Unfortunately over the last decades, the misuse of opioids in women has noteworthy elevated and in duration of pregnancy it caused the birth of infants with serious health problems [6]. Additionally, due to exertion of small part of opioids into the milk, some parts of them transfer into the infant body and caused deleterious effects to infants.

Methadone (MT) is a synthetic opioid drug and is widely used in maintenance program of opioid-dependent especially in women. MT usually has a half-life around 27 h [7], metabolized at liver by *N*-demethylation ring cyclisation and conjugation [8] and has a very high protein binding affinity (98%) [9]. Also, MT is a weak base with pKa of 8.25 and n-octanol/water partition coefficient of 120 [10]. Atkinson and Begg demonstrated that the MT has a milk/plasma (M/P) ratio about 0.65 [10]. Several old reports existed for breastfeeding in duration of MT therapy and all of them demonstrated that in duration of treatment and at low dose of MT the breastfeeding may be safe [11-17]. In 1977 one infant death was reported which related to the mother MT therapy [18]. Although the previous studies had been clarify that the dose of passed drugs into milk were reduced and may have not produce a pharmacological effects in infants, but due to high frequency of breast feeding, small size of infants and the especial metabolism of infants, this transfer may be harmful for them. Wojnar-Horton et al. have quantified the distribution and excretion of MT into the human milk and investigated the exposure of breastfeed infants to the MT.

They obtained a mean milk/plasma ratio of 0.44. They also studied on 8 infants that gave 0.15 l kg^{-1} day^{-1} milk from their mothers who were taking MT in daily doses ranging from 20–80 mg. They reported that the MT concentration in seven infants were below the limit of detection of their HPLC procedure, while one infant had a plasma MT concentration of 6.5 mg l^{-1}. Therefore they concluded that due to minim exposure of infants to MT, the infants should not be discouraged from breastfeeding [19]. In 2007 Jansson et al., was obtained the concentration of MT in human milk in the range of 21 to 314 ng/ml, and recommended to continue of breastfeeding for methadone-maintained women [20]. In addition several other reports existed that advised to continue the breastfeeding [21, 22].

Choo and coworkers introduced a validated liquid chromatography-atmospheric pressure chemical ionization–tandem mass spectrometric method for the quantification of methadone, in human milk [23].

However in all of these studies a simple extraction method followed by HPLC was used for analyzing of MT in milk or plasma and based on they concluded that the breastfeeding is safe. However, the protein content of immature milk in two or three weeks of postpartum is more than the fat [9, 23] and therefore the extraction and purification of MT from milk is a critical point and remarkably affected the free MT concentration at eluent solution. In other words, due to high affinity binding of MT with milk's proteins and presence of systematic error in common extraction methods (such as liquid-liquid extraction and solid phase extraction (LLE, SPE)), some of MT may lost in extraction step and that provide a systematic error in determination of real amount of MT in milk. For overcome of this problem, the using of several miscible organic solvents such as methanol and/or acetonitrile is strongly recommend to produce a homogeneous solution for complete precipitation of proteins and completely break of MT-protein bonds. In continue the solution is centrifuge and supernatant directly injected into the analytical apparatus [24]. Due to noteworthy contact between organic phase and protein's milk, the MT may completely remove and extract into the liquid phase. Although, on first looks this manner seems good but as the supernatant is a combine of aqueous and non-aqueous solvents therefore, some of aqueous soluble proteins may transfer

into the supernatant and bonded to some of MT and resulted to decrease of free concentration of MT. So, the previous reported data may not valid. For overcome of this problem, recently we introduced a new simple, sensitive, and reproducible extraction procedure entitled two phase freezing (TPF) method [25] in which both of aqueous and non-aqueous solvents in supernatant solution is physically separate. In TPF method the organic phase is completely separate from the aqueous solution and a clear solution with lower interference and higher MT is obtained. Our studies demonstrated that the TPF method dissolved the protein binding problem of MT and provided a clear solution with higher recovery in one step that allows having a better judgment about breastfeeding of mother who consumed the MT. However, in this study in opposite of previous reports we shows that the amount of MT is different with previous reports and we are recommending the breastfeeding perform in special times after consumption of MT by mother.

Materials and Methods

Chemical reagents

Organic solvents such as: acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), propanol (Pro), n-butanol were purchased from Merck (Darmstadt, Germany). Other chemical compounds such as: orthoboric acid (H_3BO_3), phosphoric acid (H_3PO_4), hydrochloric acid (HCl), acetic acid (CH_3COOH), sodium hydroxide (NaOH), ammonia solution (NH_3), were purchased from Merck (Darmstadt, Germany). The Briton Robinson buffer solution (B-R buffer; 0.1 mol L^{-1}) was prepared in according to our previous work [26] as follows: 1.236 g of H_3BO_3, 1013 µl of H_3PO_4, and 857 µl of CH_3COOH were transferred into a 500 ml of volumetric flask and dissolved by double distilled water. The stock solution of MT (purchased from Sigma–Aldrich; St. Louis, MO, USA) was prepared by dissolving of 30.944 mg in 10 ml of ACN.

Apparatus

A Varian CP-3800 gas chromatograph equipped with an automatic split–split less injector and a flame ionization detector (FID) system was used for the analysis of MT. A fused silica capillary column (CP-Sil 5 CB) with 30 m × 0.25 mm I.D., and 0.25 µm film thickness, supplied by Varian (Chrom Pack capillary column No. CP8752) was used with helium as carrier gas at a constant pressure of 6.894×10^4 Pa. The determination of MT was performed according to our previous work [24] as follows: the temperature of injector and detector was, 280, and 290 °C, respectively. The temperature changes of oven were as follow: the oven temperature was set at 80 °C and after 1.0 min it changed to 290 °C by temperature rate of 40 °C min^{-1} and held for 5.0 min. The total analysis time was 11.25 min. Under these instrumental conditions the MT's chromatogram was appear at about 8.3 min. The chromatogram area of each concentration was plotted versus the concentration of MT in milk. A Metrohm pH meter model 827 was used for measuring of pH of solutions. For separating of ACN from the supernatant at -40 °C by TPF method a refrigerator model Ang-platilab 500 was used. A Hettich centrifuge model EBA20 was used for protein precipitation.

Human milk samples

For optimization of method the immature human milk samples were obtained from three healthy mothers living in Kermanshah, Iran. Samples were stored at −20 °C until used in our laboratory.
Additionally, six milk samples of mothers who were participated in MT maintenance were obtained from the Imam Reza hospital (Kermanshah, Iran) and stored at −20 °C. Six mothers take 90 mg/day of MT at 10:00 AM throughout the study period and human milks were obtained from them two days after delivery of baby. Specimens were collected at 1, 2, 3, and 5 hours after single daily methadone dose.

Ethic approval

The study protocol was approved by ethics committee of Immam Reza hospital, affiliated to Kermanshah university of Medical sciences, Kermanshah, Iran. All volunteers signed the informed consent form.

The spiking of milk samples

For optimization of TPF procedure exactly 1.0 ml of healthy milk sample was used. Into each sample exactly 20 µl of standard solution of MT was added to obtain a final concentration of 1.0 µg mL^{-1} for MT. For complete interaction of MT with the human milk, the samples were standing about 12 h at 4 °C in the refrigerator. Next, the milk samples were used for method validation purposes.

Extraction and clean-up of MT by two phase freezing method

Into 1.0 ml of milk sample exactly 0.2 ml of B-R buffer (pH=2.5, 0.01 mol L^{-1}), and 0.3 ml of ACN was added and vigorously shacked for 3 min. the solution was centrifuged for 3 min with 6000 rpm and the supernatant was carefully removed by a Pasteur pipette. After removing of supernatant solution, both volumes of supernatant and proteins were measured. Into the supernatant solution an appropriate of KCl solution (1.5 mol L^{-1}) was added in which the KCl reach to a final concentration of 1 %W/V, then the solution was placed in refrigerator at −40 °C for 3.0 min. The tube was centrifuged at −20 °C by a universal centrifuge (model PIT 320R) to separate the ACN from the frozen aqueous phase and the ACN was removed by Pasteur pipette from the aqueous frozen. The organic phase evaporated under N_2 gas at 50 °C and the residue was dissolved in 30 µl of ACN and 1.0 µl was injected into the GC apparatus.

Method validation

The linear range (L.R), limit of detection (LOD), limit of quantification (LOQ) intra-day precision, inter-day precision, and ruggedness of proposed method was studied. For evaluation of method validity we used one blank immature milk sample after one week of postpartum of a healthy volunteer who was 22 years old. The calibration curve of milk sample was constructed with 10 concentration levels in the range of 0.03-92 µg ml^{-1}. The extraction and clean-up was performed according to section 2.5. The 3.0 replicate analyses for each concentration level were performed. The trueness (absolute recovery) of method was evaluated on 1.0 ml of three milk samples of healthy volunteer that spiked with two different concentration level of MT (0.5 and 1 µg ml^{-1}) under optimum conditions. The ruggedness of TPF method was evaluated by comparison of RSD results of intraday assay of two different analysts. The experiments were performed in the same laboratory on same milk samples [27-30].

Results and Discussions

It is well known that the FID is a powerful and sensitive detector in GC but the presence of lipids, proteins and other matrix interferences can deteriorate the sensitivity of the detector and also harmful for capillary column of GC. Therefore, for determination of drugs in plasma and milk samples an efficient clean-up step is necessary to overcome this problem. The clean-up method must be completely break the drug-protein matrix bonds and efficiently extract the drugs. In this regards, the parameters such as: pH of solution, type and volume of miscible organic solvent, the ratio volume of organic to aqueous solvent, extraction cycles, and salting out were optimized. The percent extraction yield (%EY) was considered as a response for each optimization parameter.

Extraction factor (EF) is defined as the ratio of the MT concentration in the ACN phase (C_{ACN}) to the initial concentration of MT (C_0) within the aqueous solution:

—— (1)

The C_{ACN} was obtained from the calibration graph that was obtained by a series of MT solution in MeOH against the corresponding concentration.

Percent extraction yield (%EY) is defined as the percentage of the ratio of MT amount extracted into the ACN phase (n_{ACN}) and the total MT amount (n_0) in aqueous sample.

$$\%EY = \frac{}{} \frac{}{} 100 \quad (2)$$

Where V_{ACN} and V_{aq} are the volumes of ACN phase and aqueous solution, respectively.

Effect of pH of aqueous solution

The MT is a weak base with pKa=8.5 and has an amine of type III and a carbonyl moieties, that caused the MT has different charges in various pHs (see Fig. 1). Therefore, the effect of pH on extraction of MT into the ACN was evaluated. To this regard, the efficiency of TFP on the extraction of MT in various pHs of 1.5 to 11.0 was studied at specific concentration of the MT (1 µg ml^{-1}). The results were shown that in the pH range of 2.5 to 3.8 the peak area of MT was relatively constant, but by increasing of pH the peak areas were noteworthy decreased (Fig. 2). At higher pHs (pH>4) due to the increase role of donor ability of MT and hydrogen-bonding between both moieties of MT and H_2O molecules the extraction decreased. In other words, at relative acidic conditions (pH=2.5-3.8) the type III amine of MT is protonated and therefore, the hydrogen binding between MT and H_2O molecules is disrupted, so the strength interaction of MT with H_2O molecules is weaken [31]. We previously reported that the ACN has basic properties [32] therefore, in the pH range 2.5-3.8 the protonated MT has acidic properties and an acid-base interaction was occurred between MT and ACN molecules. In continue the pH=2.5 were selected for next studies.

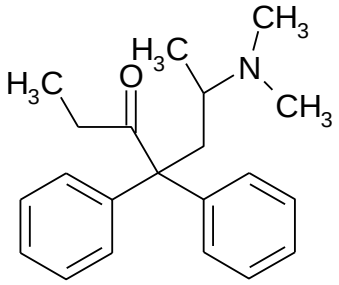

Fig. 1. Structure of Methadone

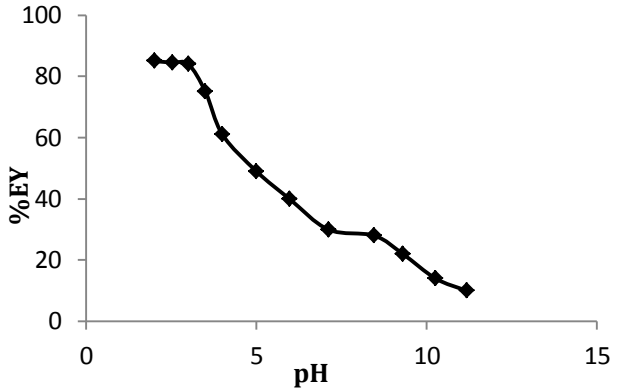

Fig. 2. Extraction yield (%EY) of MT in the organic phase of ACN versus pH of aqueous phase, the conditions were as follows: V_{milk}=1.0 ml, V_{ACN}= 0.3 ml, V_{buffre}= 0.2 ml, the amount of MT=1.0 µg ml^{-1}, number of extraction cycle=1, no salt was added.

Selection of organic solvent

Due to basic and lipophilic properties of MT, the transfer of MT into the organic solvent may affect by several factors including: type of organic solvent, acceptor and donor ability of organic solvent, and solvent-solute hydrogen-bonding properties. In TPF extraction, the miscible organic solvent should be separate the MT from proteins and removed the H_2O molecules in first solvation shell of MT and replaced with its molecules. The effect of various organic solvents such as MeOH, EtOH, ACN, and propanol on extraction efficiency of MT (1 µg ml^{-1}) was studied in the pH=2.5. As these organic solvents are well miscible with milk therefore, the milk's proteins denatured and the MT is completely remove, so, a well contact will be obtained between organic solvents and MT in solution and caused higher extraction efficiency obtain. Fig. 3 show that ACN provided the

maximum extraction efficiency in pH=2.5. We well know that the water molecules have high solvating (Donor Number (DN=33)) and hydrogen-bonding ability, while the ACN is an aprotic and protophobic dipolar solvent and rarely interacts with MT by hydrogen bonding (DN=14.1) in neutral and or alkaline pHs [33]. Therefore, in pH=2.5 due to hydrophobic and acid-base interactions between ACN and protonated MT, the highest extraction efficiency were obtained by ACN in comparison with other organic solvents.

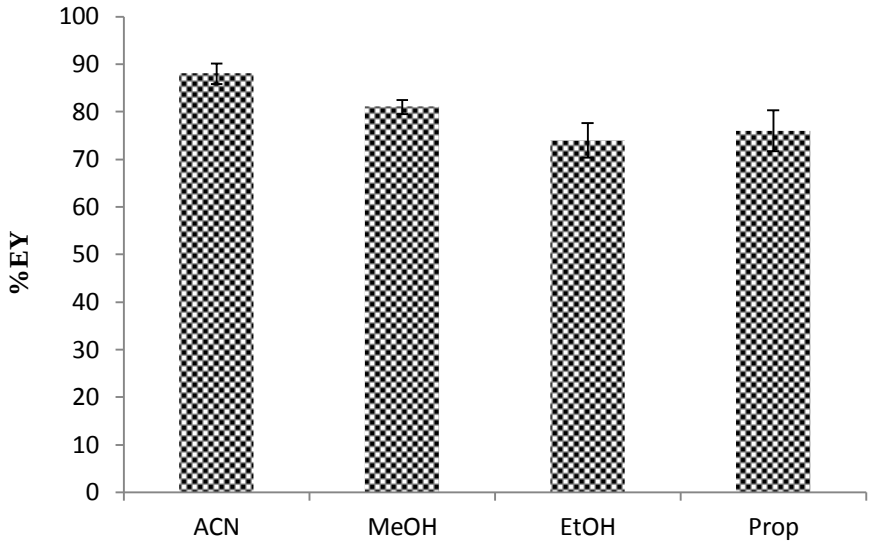

Fig. 3. The effect of various organic phases on extraction yield (%EY) of MT, the conditions were as follows: V_{milk}=1.0 ml, V_{ACN}= 0.3 ml, V_{buffre}= 0.2 ml, the amount of MT=1.0 µg ml^{-1}, number of extraction cycle=1, no salt was added, pH aqueous phase=2.5. All measurements were three times repeated (n=3).

Salting out effect and cycle of extraction

In extraction step of a drug from a biological fluid, the addition of a salt noteworthy helps to decrease of solubility of drug in the aqueous sample and increased it's partitioning into the sorbent or organic phase. Therefore, the change of ionic strength of binary homogenous was investigated by using KCl in the range of 0.0 to 4.0 %W/V at pH=2.5. As it is shown in Fig. 4, at above of 0.0 %W/V of KCl, a remarkable enhancement in extraction yield of MT in ACN phase was obtained and reached constant at 1.0 %W/V, therefore, the 1.0 %W/V of KCl was selected for next studies. The addition of KCl may changes the physicochemical properties of the aqueous solvation shell in MT and thus increasing the diffusion rates of the MT molecules into the ACN phase. We also, studied the effect of number of extracting cycle on %EY and founded that, this factor does not have an important role on extraction efficiency of TPF. Also, the best solvent ratio (milk solution/ACN) of 3 with one extraction cycle gives the highest recovery (more than 90%) and reproducibility (RSD<4%).

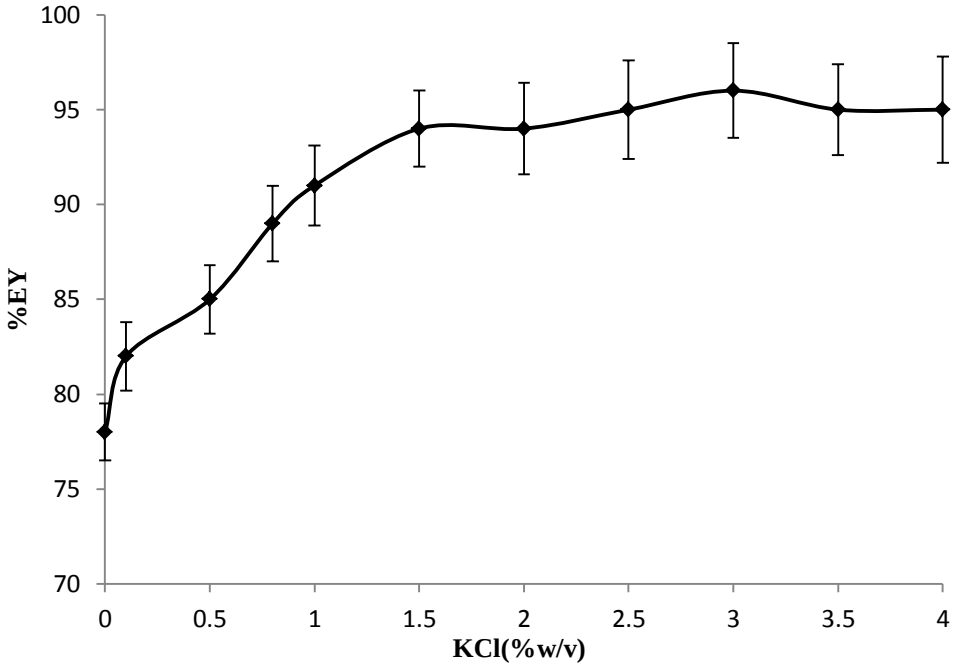

Fig. 4. The effect of salting out on extraction efficiency, the conditions were as follows: V_{milk}=1.0 ml, V_{ACN}= 0.3 ml, V_{buffre}= 0.2 ml, the amount of MT=1.0 µg ml^{-1}, number of extraction cycle=1, pH aqueous phase=2.5. All measurements were three times repeated (n=3).

Validation of the method

For plotting of milk-matched calibration curve by the TPF method, one immature milk sample of a healthy volunteer (22 years old) was used. To 1.0 ml of sample milk various amounts of MT in the concentration range of 0.01-100 µg ml^{-1} was spiked and at each concentration the analysis was repeated three times (n=3). Each sample treatment and extraction was carried out according to section 2.5 and the mean peak area was used for next evaluations. The limit of detection (LOD) and limit of quantification (LOQ) was calculated according to our previous work [34] as: 3×S/N and 10×S/N, respectively and the data were placed in Table1.

Table 1. The analytical merits of TPF method for determination of MT in immature human milk.

Compound	L. R[a] (µg ml^{-1})	L. E[b]	LOD (µg ml^{-1})	LOQ (µg ml^{-1})	%RSD(n=3) intraday	%RSD(n=3) interday	R^2	%A. R[c] (n=3)
MT	0.03-92	A= 497.61C_{MT} + 3769	0.01	0.03	2.2	3.5	0.993	95.4±2.8

[a] **Linear range;** [b] **Linear Equation;** [c]**Abslute Recovery**

The intraday and interday repeatability on milk samples that spiked with 1.0 µg ml^{-1} of MT was also studied. The intraday repeatability studies were carried out on one milk sample that spiked with 1.0 µg ml^{-1} of MT (n=3) on the same day and the interday was studied in three consecutive days (n=3; total analysis=3×3). The data were shown as %RSD of the %EY, in Table 1. The ruggedness of proposed method was evaluated by comparison of RSDs that obtained by two analysts in the same laboratory and the results demonstrated that none of them gave the RSD more than 4.0 %.

Real sample application of the method

Several studies have shown that the sub therapeutic level concentration (STLC) of MT is less than 5 ng ml^{-1} in milk and therefore the breastfeeding is safe and has not any neonatal abstinence syndrome side effect [21]. For example Wojnar-Horton and et al., demonstrated that the level of 6.5 ng ml^{-1} of MT in blood of infants is insufficient to development of a neonatal abstinence syndrome [19].

The enrichment factor (EF) of proposed method is 3 and the LOQ of method is equal to STLC. However for achieve to higher EF and monitor of MT in below of STLC in real samples the extracting ACN sample is manually evaporated and the residue was resolved in 30 µl of ACN and reanalyzed for presence of MT. In this manner the EF increased to 10. Six milk samples of mothers who were taken MT in four different times (1, 2, 3 and 5 h after MT consumed) were analyzed and the results were shown in Table 2.

Table 2. Maternal demographics, absolute maternal doses, average milk concentration in various times.

Subject NO.	Age (years)	Weigh (Kg)	Daily maternal Dose (mg Kg^{-1})	Immature milk MT concentration (µg ml^{-1})			
				1h [a]	2h	3h	5h
1	28	65	1.38	ND [b]	0.056	0.095	0.045
2	29	63	1.25	ND	0.117	0.120	0.048
3	25	75	1.43	ND	0.031	0.010	0.040
4	22	65	1.25	ND	0.041	0.087	0.035
5	24	62	1.41	0.02	0.142	0.159	0.077
6	26	64	1.35	ND	0.078	0.099	0.049

[a] after MT taken
[b] not detected

The advantages of proposed method

In previous studies the extraction of MT from human milk were performed by various methods such as: combination of protein precipitation with ACN and then solid phase extraction [9, 34, 35]; liquid-liquid extraction [19]; combination of protein precipitation by centrifuge and solid phase extraction [21] and liquid-liquid back extraction [22]. The most methods used the ACN for precipitation of proteins and solid phase extraction for separation of MT from the binary mixture of water-ACN solution. As the ACN is freely mixed with water therefore the some proteins significantly soluble in binary mixture of ACN-water, and these proteins may adsorbed the free MT in solution and prevent of complete extraction of MT from solution by SPE. In addition, the presence of ACN in loading solution caused the polarity of solution changed and the efficiency of SPE remarkably reduced. In other words, the ACN washed the retained MT in SPE cartridges and the concentration of MT in final elution step reduced. Therefore, the reported data of MT in milk may not very valid. For overcome of this problem the physical phase separation seems give higher recoveries and more clear solution [25]. Our results demonstrated that the recovery and reproducibility of extraction of MT from immature milk matrix is very better than the reported methods [9, 14, 20, 21]. Table 2 shows the amount of MT in immature milk of six mothers who were taken the maternal dose of 90 mg/day of MT at 4 different times. As it is observed the MT appeared into the immature milk on 2h after taken the MT by mothers and increased in 3 h after taken and reduced 5h after that. Milk is a mixture suspension of fats and proteins in a carbohydrate-based solution and the opioids transferred into human milk by mechanisms which are similar to those mechanisms governing passage into any other organ systems and or body fluids [1]. Opioids that enter into human milk via passive diffusion may reach to concentration equilibrium with the concentration in the blood and this completely dependence to degree of protein binding and lipid

solubility of opioids. It is demonstrated that the drugs with high lipid solubility have higher concentration penetrate into milk; while the drugs by higher protein binding almost have lesser penetrate into milk. Although no correlations between the methadone dose level and milk level were reported but an insight correlation between human milk level and fat content of the milk and also sampling time are reported [20]. McCarthy and Posey [21] were shown that the concentration of MT in the milk samples for maternal methadone dose between 25 to 180 mg/day were ranged from 27 to 260 ng/ml.

The Academy of Pediatrics' committee in accordance works of Dr. Blinick and coworkers which dedicated on the transfer of MT into human milk [12, 36], in years of 1983 [37], 1989 [38] and 1994 [17] recommended on breastfeeding of mother who consumed the MT. These grope reported for 20 mg/day dose of MT the concentration that appears in milk was ranged from 50 to 570 ng/ml [12, 36]. Also, Kreek and coworkers reported that for maternal dose of 50 mg/day of MT, it is appears in milk in the range 20 to 120 ng/ml [14]. Also, they estimated, if the infant daily intake about 475 ml/day of milk that contain 120 ng/ml of MT, the baby might ingest 0.05 mg/day of MT and advised the breastfeeding. The same results and recommendation for breastfeeding were reported [13, 11].

Also Wojnar-Horton et al., measured the level concentration of MT in milk and plasma samples of 12 women who were taken 20 to 80 mg/day of MT, in the range of 39 to 232 ng/ml in milk and 121 to 603 ng/ml in plasma. They didn't found the MT in plasma of 7 infants and only fined in one infant with level concentration of 6.5 [19]. While Dr. Blinick et al. [12] reported the cord levels of plasma in birth were from 30 to 250 ng/ml.

However due to high inconsistency in literature on MT levels in human milk, McCarthy state that "minimal transmission of methadone into human milk occurs regard less of the mother's methadone dose" [21]. However, by introduce of new TPF method for extraction of MT from immature milk that contain higher proteins and lower lipid and sugar, the results (see Table 2) remarkably depends on the time of MT use. However, one hour after MT consumption the breastfeeding is safe while, it is better to ovoid to breastfeeding in duration of 2-4 h after MT consumption, and after 5 h of consumption, the MT level concentration is lower the reported data of Dr. Blinick et al., [12, 36]. So, according to our data we advise to reconsideration of the American Academy of Pediatrics' recommendations and revised the criteria that used as a guide decisions about breastfeeding in women who are use the MT.

As the breastfeeding has well-documented benefits and provide special substance-exposed to babies, therefore, our scientific evidence may does not strongly support a policy of breastfeeding that limits to time frames in breastfeeding women specially in the early postpartum period and need more studies.

Conclusion

In this study, an efficient extraction and clean up method based on two phase separating solvent was developed for simultaneous extraction of MT and remove of proteins from immature human milk. The parameters of two phase solvent method were optimized to achieve the maximum efficiency. MT was separated by careful optimization of GC conditions. Our data is reasonable with comparison to other reported data (See Table 3).

Table 3. The comparison of work with other works.

Work	method	Linear range(µg ml^{-1})	Absolut recovery	LOD (µg ml^{-1})	R^2
[23]	LC-MS	0.01-0.5	66%	0.005	0.992
[35]	EI-GC-MS	2.0-1000	85%	0.5	0995
Our work	GC-FID	0.03-92	95.4±2.8	0.01	0.993

We have applied the method in the evaluation of six real immature milks samples that collected from six women who were used MT (dose of 90 mg/day) in duration of 1, 2, 3, and 5 h after consumption. Our data demonstrated that, before one hour and also 5 h after MT consumption the breastfeeding is safe and between 2-4 h after consumption dose not safe neither.

Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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