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Sensitive determination of methotrexate in plasma of children with acute leukemia using double-solvent supramolecular systemas a novel extractant for dispersive liquid-liquid microextraction

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ABSTRACT

Methotrexate, as a folate antagonist, is one of the first anti-neoplasm drugs offered and is still used as an effective drug in the treatment of various malignancies. Methotrexate has a narrow treatment index and is associated with numerous side effects.In thisresearch, for the first time a double-solvent supramolecular system (DSS) was developed as an extractant without disperser solvent for dispersive liquid–liquid microextraction (DLLME). DSS – DLLME was applied to the extraction of methotrexate in plasma of children with acute leukemiaprior to its-determination by high-performance liquid chromatography–ultraviolet detection (HPLC – UV). In the present method, two long normal chain alcohols are mixed in a particular ratio, and then it is injected into the sample solution, which is on the magnetic stirrer. In this case, the mixture of the two alcohol changes to new supramolecular aggregate. This new supermolecule is used as an extractant, which has a higher extraction power than any of its components alone. Under the optimum conditions, the calibration graph was linear in the rage of 0.1–150 μg L^{-1} with detection limit of 0.03 μg L^{-1}. Relative standard deviations (RSDs) including intra–day and inter–day of method based on7 replicate determinations of 100.0 μg $L^{-1}$of methotrexate were 2.6% and 4.8%, respectively. The results proved that DSS – DLLME is a sensitive, very simple, inexpensive, environmental friendly, rapid and efficient method for the preconcentration of trace amount of drugs in biological samples.

1. Introduction

The accuracy of prescribing drugs, especially drugs with a narrow treatment index (NTI), is one of the most critical health system issues. Despite the problems that arise in the field of irrational use of drugs, it is less systematically and scientifically addressed [1]. One of the most important goals of drug use evaluation (DUE) studies can be mentioned to increase the quality of health care through the accurate prescription of drugs, improving the quality of life of patients and training physicians in health, timely prevention of side effects and drug interactions and proper use of resources and facilities [2]. Methotrexate, as a folate antagonist, is one of the first anti-neoplasm drugs offered and is still used as an effective drug in the treatment of various malignancies [3]. Methotrexate, in addition to its anti-tumor activity, has anti-inflammatory and immunomodulatory effects and is used in various doses for various therapeutic indications, including psoriasis, rheumatoid arthritis, and malignancies [4]. Methotrexate has an NTI and is associated with numerous side effects. In some cases, it is life-threatening if the recommended dosage is not followed or if monitoring is not observed during treatment [5]. Gastrointestinal side effects commonly occur with methotrexate, and renal toxicity due to drug deposition in the renal tubules and glomeruli may occur. Methotrexate also causes renal failure by contracting afferent arteries or mesangial cells [6]. Hematologic side effects, hepatotoxicity, and pulmonary toxicity are other side effects of methotrexate [7]. Therefore, accurate measurement of methotrexate concentrations in patients' plasma is necessary to generate an optimized dose and minimize toxicity. The therapeutic dose varies in different diseases and different phases of treatment and is between 10 mg and 12 g/m^2. In leukemia in the consolidation phase, it is 2 to 5 g/m^2. Serum methotrexate levels depend

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on the dose prescribed. For example, in the administration of 2 g/m^2, after 24 h, the serum level of methotrexate is on average about 4.54 mg/L [8].In general, the expected concentration of methotrexate in clinical samples of children from previous studies is greater than 3.5 mg/L [3,5,7].

Various analytical instrumentals such as high performance liquid chromatography (HPLC) [9–12],liquid chromatography − mass spectrometry/mass spectrometry (LC − MS/MS) [13–15], capillary electrophoresis (CE) [16], fluorimetry [17] and electrochemical techniques [3,7] have been used for the determination of methotrexate in different matrices.LC-MS and especially LC-MS/MS are usually employed for determination of drugs in biological samples because of high sensitivity, but due to the high cost, the use of these techniquesis limited. On the one hand, HPLC − UV has less sensitivity and specificity than LC-MS and LC-MS/MS and can not detect trace amounts of drugs in biological samples. On the other hand, the HPLC − UV is known to be simple, inexpensive, and found in most laboratories. However, due to the extraction and preconcentration of the samples by the microextraction methods, acceptable results were obtained by HPLC-UV. Isolation and extraction of methotrexate is an important stage for its determination in biological fluids. These samples are very complex due to the large number of different compounds and the selectivity of the method may have a problem. However, spite the use of a suitable analytical instrument, an extraction procedure is required before the methotrexate analysis.

The sample preparation step is the most critical part of the analysis process, often associated with spending much time, high consumption of organic solvents, and inaccuracy. To overcome these problems, miniature extraction techniques based on liquid–liquid extraction (LLE) have been developed today. One of the best of these techniques is dispersive liquid–liquid microextraction (DLLME), the conventional mode of which was first introduced by Assadi and co-workers [18]. The advantages and disadvantages of this technique are mentioned in the articles [19–23]. Selecting the extraction solvent type and high consumption of disperser solvent (in milliliters) are problems of DLLME technique [24,25].To fix these disadvantages, innovations have been made on DLLME, such as DLLME based on solidification of floating organic droplet (SFO)[26–28] and DLLME based on the deep eutectic solvent (DES) [29–32]. These innovations are aimed at further reducing the consumption of organic solvents and using organic extraction solvents lighter than water with less toxicity, cheaper, and more environmentally friendly.

In recent years, attention has been paid to the use of alternative solvents, such as supramolecular solvents in order to higher extraction of target analytes and mitigate pollution of the environment by organic solvents [32,33]. Supramolecular solvents are green water-immiscible solvents that are composed of amphiphile aggregates [34]. Some review articles have been published already on supramolecular solvents that the history and stages of their formation are mentioned in these review articles [35-37].

In the present work, a double-solvent supramolecular system (DSS) was developed as an extractant without disperser solvent for DLLME. The DSS − DLLME was evaluated to determine the level of methotrexate in plasma by HPLC − UV. High extraction recovery, lower organic solvent consumption, ease of operation, environmentally friendly and low cost are some of the advantages of this method. Compared to theconventional DLLME, DSS − DLLME does not require a disperser solvent and centrifugation.

2. Experimental

2.1. Reagents and solutions

Methotrexate (purity ≈ 99%) and 8-chlorotheophylline (purity ≥ 98%) as an internal standard (IS) were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). The methotrexate stock standard solution was prepared in distilled water and methanol (50:50, v/v) at the concentration level of 1000 mg L^{-1} and was stored at −20 °C. More diluted working solution were obtained by diluting the stock standard solution. Stock solution of internal standard was prepared by dissolving an accurately weighed amount of 8-chlorotheophylline in 0.2 M phosphate buffer with pH 6.0, to obtain a final concentration of 1000 mg L^{-1}, then stored at 4 °C. The ultra-pure water was purchased from Shahid Ghazi Pharmaceutical Co. (Tabriz, Iran). Methanol, acetonitrile, phosphate salt (analytical grade), 1-undecanol, 1-dodecanol, 1-decanol, sodium dodecyl sulfate (SDS), Na_2HPO_4 and NaCl were obtained from Merck (Darmstadt, Germany).

2.2. Instrumentation

The analysis of methotrexate was achieved on a HPLC Knauer equipped with a quaternary pump, online degasser, detector Smartline-UV-2500 variable wavelength programmable (Berlin, Germany) and a 20 μL injection loop injector (model 7725i, Rheodyne, Cotati, CA, USA). Separation wascarried out with H5-ODS C_{18} column (15 cm × 4.6 mm, with 5 μm particle size) from Anachem (Luton, UK), preceded by a Security Guard Cartridge C_{18}(Anachem, Luton, UK).The mobile phase consisted of 85% buffer containing 10.0 mM Na_2HPO_4 and 0.70 mM SDS with pH 6.5 and 15% acetonitrile. A mobile phase flow-rate of 0.8 mL min^{-1} was used in isocratic elution mode and the analytes were detected at 305 nm. The Metrohm pHmeter Model 692 (Herisau, Switzerland) was used for the pH values measurement.

2.3. Sampling and preparation of sample

Blank plasma samples (drug-free) was provided by healthy volunteers, not exposed to any drug for at least 10 months for method development and validation. Real plasma samples were taken from patients with acute leukemiawho were admitted and treated in the Dr. MohammadKermanshahi Hospital from Kermanshah, Iran. In this way2girls and 2boys (aged 8 to 13 years) were randomly chosen and 1.0 mL blood of each of them was taken and transferred to advanced research laboratory. This research is approved by the ethics committee of Kermanshah University of Medical Sciences. (Approved code of ethics: IR.KUMS.REC.1398.531). For preparation and clean up of samples, 400 μL of whole blood was placed in EDTA-contained glass test tube and one mLmixture of acetonitrile and $ZnSO_4$(15%, w/v)(2:3) was added to a test tube and vertex for 12 min. For better separation of colloidal particles in centrifugation stepthe test tubewas kept at 4°Cfor a few minutes andit was centrifuged at 5000 rpm for 4 min. The obtained supernatant were transferred to another clean tube andwas reached to a volume of 5 mL by distilled water to reduce the effects of matrices. The resulting solution was then subjected to the presented procedure.

2.4. Extraction procedure

An aliquot of 5.0 mL of a pretreated and diluted plasma sample (spiked or not with methotrexate) containing 10.0 μg L^{-1}of 8-chlorotheophylline (IS) was placed in a 10-mLsamplevial. The sample solutions pH were adjusted to areasonable amount 5 by using phosphate bufferand vial fixed on a magnetic stirrer. Forty-fivemicroliter of double extractant (1-undecanol/1-dodecanol; 1:2 v/v) was injectedinto the diluted plasma sample and the magnetic stirrer was turned on at 1200 rpm for 30 min. The double extractant diffused in tiny droplets with a very high contact surface (without the need for a disperser solvent) in the sample solution. In this case, the extraction of analytes with different polarities is done through intermolecular hydrogen bonding or high-efficiency hydrophobic interaction. When the stirring stops, the supermolecules, which also contain analytes slowly accumulate on the surface of the sample solution and float in a droplet. The sample vial was thereafter put into afreezer for a few minutes; at this time, the floated double extractant was solidified because of the low melting point. The solidified double extractant was transferred into a conical glass test tube where it was melted at room temperature. Finally, 30 μL of the solution

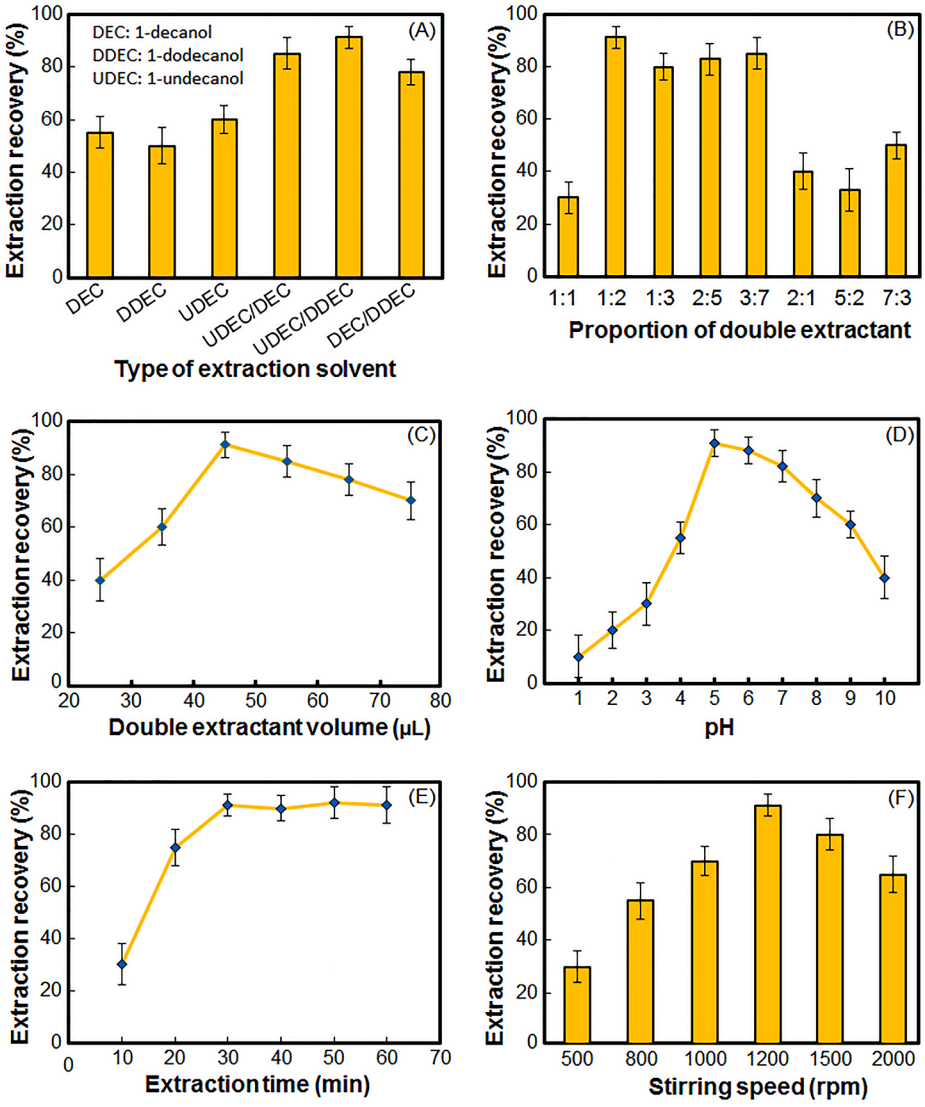

Fig. 1. Effect of the different types of extractant (A), proportion of double extractant (B), double extractant volume (C), sample solution pH (D), extraction time (E) and stirring speed (F) on the extraction recovery of the methotrexate. Extractionconditions: types of extractant, 1-undecanol/1-dodecanol; proportion of 1-undecanol/1-dodecanol, 1:2; volume of the samplesolution, 5 mL; sample solution pH, 5; volume of the extraction solvent, 45 µL; stirring-speed, 1200 rpm; extraction time, 30 min; room temperature.

was used for HPLC analysis.

3. Results and discussion

3.1. Type of double extractant

Selecting anappropriate double extractant is crucial in the present method. The double extractant must have melting point close to RT, high extraction efficiency, less toxic and low solubility in the aqueous phase. For this purpose, 1-decanol, 1-dodecanol and 1-undcanol solvents were selected. At first, each of the solvents was used as an extraction solvent, and then their double mixture with a specific ratio was used. Average extraction recoveries (three riplicate measurements) and standard deviations (SD) for different extractants alone and double extractants are shown in Fig. 1A. The results showedthat the ER% of methotrexatewith each solvent alone is not more than 60%, but when two of the solvents are mixed in a particular ratio, the ER% increases. Although the extraction recoveries are comparable in all double extraction systems, in the 1-undcanol/1-dodecanol double extraction system, the extraction recoveries are slightly better, and the standard deviation is low. As a result, 1-undcanol/1-dodecanol double system was selected as the optimal double extraction system.

3.2. Proportion of double extractant

In the presentstudy, the most suitable proportion of double extractant was obtained to achieve bestER%. To this end, the double extractants wereobtained by using 1-undecanol and 1-dodecanol with different ratios of 1:1, 1:2, 1:3, 2:5, 3:7, 2:1, 5:2 and 7:3. Experiments in Fig. 1B show that 1-undecanol and 1-dodecanol at a 1:1, 2:1, 5:2 and 7:3 M ratios could not form DSS. The mixture of 1-undecanol and 1-dodecanol in other ratios has a positive effect on the extraction of the methotrexate. However, double extractant obtained from a mixture of 1-undecanol and 1-dodecanol in a 1:2 ratio, has higherER% and lower SD. So, the 1:2 ratio was chosen as the bestratio of 1-undecanol and 1-dodecanol.

3.3. Type of double extractant volume

Section 3.2 showed that 1-undcanol and 1-dodecanol with a 1:2 vol ratio were the best double-solvent supramolecular system. The double extractant volume plays a important role in the extraction recovery of methotrexate. To study the effect of double extractant volume on the extraction recovery of methotrexate, different volumes of double extractant (25, 35, 45, 55, 65 and 75 µL) were tested to select the optimum volume of double extractant to be applied in subsequent tests. According to the results in Fig. 1C, At volumes less than 45 µL, the

contact surface is not high, and the extraction recovery is low. Also, not enough volume is obtained for injection into the HPLC, and the repeatability is significantly reduced. At volumes more than 45 μL, the dual extractant cannot quickly accumulate on the surface of the sample solution, and the ER% decreases due to the dilution effect. Thus, in order to have a high ER% and good repeatability, 45 μL of double extractant was chosen as the optimum volume.

3.4. Effectof sample solution pH

The pH of sample solution will affect the existence form of the methotrexate in the sample solution, so the pH will play an significant role in the ER%. To evaluate the effect of sample solution pH, several experiments were carried out by different pH of sample solution (1–10). Other experimental conditions were kept constant. Fig. 1Dshows that the methotrexate can hydrolyze in strong acidic and alkaline environment while itis stable at pH = 5. Thus the pH of sample solution was fixed at 5by a phosphate buffer.

3.5. Salt effect

For evaluating the salt effect on the efficiency of DSS–DLLME, varioustests were carried out by different concentrations of salt (0–10% NaCl). The results indicated that with increasing NaCl from 0 to 5%, the extraction recovery of methotrexate remain nearly constant, because on the one hand, the salting-out effect increases the ER%. On the other hand, the solubility of the DSS in the sample solution decreases and the volume of the extraction phase increases. As a result, due to the dilution effect, the ER% decreases. At concentrations higher than 5%, the dilution effect prevails on salting-out effect and the ER% decreases. Therefore, the experiments were carried out in the absence of any salt.

3.6. Effect of extraction time

The time of extraction is an important parameter that may affect the analytes' ER% from sample solution into the extractant phase (DSS) [30]. In the present method, extraction time is defined as the time between injecting the double extractant and turn off the magnetic stirrer. The extraction time must be high enough to achieve an effective recovery of the target analyte. On the other hand, it must be low enough not to waste time. Thus, the effect of extraction time on the ER% of methotrexate was examined in the range of 10–60 min with constant experimental conditions. When the extraction time increased from 10to 30 min, the extraction recovery of methotrexatewas increased due to the mass transfer of analyte from cellular material to double extractant by diffusion and osmosis. However, the extraction recovery is kept constant upon further increase in extraction time (Fig. 1E). Therefore, the extraction timeof 30 min was chosen as the optimum extraction time.

3.7. Effect of stirring speed

Magnetic stirring can accelerate mass transfer and promote the dispersion of the double extractant into the sample solution; thus, stirring improves the ER% of the analyte in the double extractant [28]. As the stirring speed increases, the extraction recovery of methotrexate improves. However, if the stirring speed is too high, the solution will spatter and organic droplets will be destroyed. Different stirring speeds (500, 800, 1000, 1200, 1500 and 2000 rpm) were investigated. The stirring speed of 1200 rpm obtained the highest extraction recovery (Fig. 1F). Thus, 1200 rpm was chosen as the stirring speed.

3.8. Quantitative analysis and method validation

The DSS–DLLME procedure was validated with respect to selectivity, linearity (LR), limit of quantification (LOQ), limit of detection (LOD), accuracy, precision including repeatability (intra–day) and reproducibility (inter–day), extraction recovery (ER), enrichment factor (EF), carry-over and stability. Method validation was carried out according to the US Food and Drug Administration (FDA) bioanalytical validation guidelines [38]. The characteristics of the calibration curve summarized in Table 1.

Table 1
Analytical characteristics of DSS − DLLME − HPLC − UV for determination of methotrexate.

Parameter	Analytical feature
Linear range (μg L^{-1})	0.1–150
RSD% (Intra-day, $n = 7$)	2.6
RSD% (Inter-day, $n = 7$)	4.8
Accuracy% ((Intra-day, $n = 7$)	92–104
Accuracy% (Inter-day, n = 7)	91.6–107.5
r^2	0.9991
Limit of detection (μg L^{-1}) (S/N = 3, $n = 7$)	0.03
Limit of quantification (μg L^{-1}) (S/N = 10, $n = 7$)	0.1
Extraction recovery (%)	91.2
Enrichment factor	152
Stability of plasma after 12 h at room temperature (Mean accuracy ± RSD, %)[a]	98 ± 8.5
Stability of plasma after 12 h at 4 °C (Mean accuracy ± RSD, %)	104 ± 7.6
Stability of freeze–thaw plasma (Mean accuracy ± RSD, %)	101 ± 9.2
Stability of post-preparative plasma after 24 h at 10 °C (Mean accuracy ± RSD, %)	96 ± 5.1

[a] The concentrations of plasma samples for stability were 10 and 100 μg L^{-1}.

Six drug-free human plasma samples from different sources were selected randomly to investigate the selectivity of the method developed. Interferences were assessed by comparing chromatograms of the blank plasma and the plasma samples spiked with the methotrexate and internal standard. The acceptance criteria for the experiment were achieved. The retention times of methotrexate and internal standard, were 9.12 and 13.63 min, respectively. No other peaks corresponding to these retention times were observed in the chromatograms of the blank plasma samples. The method is selective as it was able to differentiate and quantify the methotrexate in the matrix.

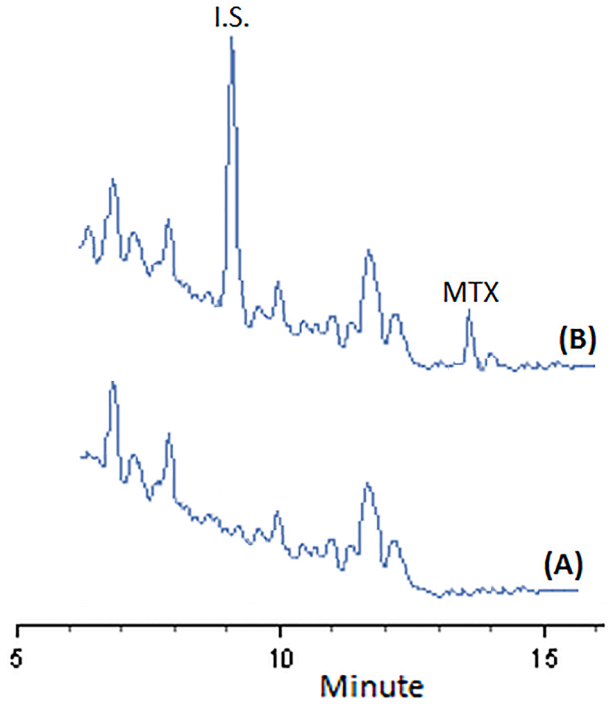

Fig. 2. Chromatograms of blank plasma sample (A) and plasma at LLOQ (B) obtained by using DSS − DLLME combined HPLC − UV. Peak identification: (MTX) methotrexate, (I.S.) internal standard.

Table 2
Determination of methotrexatein plasma samples and relative recovery of spiked methotrexatein these samples.[a]

Plasma samples	Added (mg L^{-1})	Found, mean ± SD[b] (n = 3) (mg L^{-1})	Relative recovery (%)
Taken from a 12-year-old boy (diluted 100 times)	0	8.6 ± 0.4	–
	5	13.8 ± 1.1	104
Taken from a 7-year-old girl (diluted 200 times)	0	11.3 ± 2.8	–
	10	21.1 ± 1.5	98
Taken from a 9-year-old boy (diluted 300 times)	0	5.5 ± 0.3	–
	15	20.8 ± 1.6	102
Taken from a 13-year-old girl (diluted 500 times)	0	17.2 ± 1.2	–
	20	35.6 ± 2.4	92

[a] These data are based on the diluted volumes of blood samples and dilution effect was considered for calculation of them.
[b] Standard deviation.

The linearity of methotrexate was obtained in the blank plasma spiked with different amounts of methotrexate. The concentration of internal standard was maintained at 10 μg/L. The samples were analyzed in triplicate, and calibration curve was constructed by plotting concentration against peak area ratio of analyte to internal standard. Linear range was 0.1 to 150 μg L^{-1} for methotrexate with coefficient of determination (r^2) of 0.9991.

The method was evaluated for accuracy and precision by analysis of quality control (QC) sample at four concentration levels(including 10, 20, 50 and 100 μg L^{-1}) within the calibration range in plasma. The prepared samples were analyzed in seven replicates on the same day for intra-day, and the same samples were analyzed on three consecutive days, for inter-day. For this purpose, specific quantity of methotrexate was added to the known concentration of plasma samples. Then the methotrexate was extracted using above mentioned procedure and the samples were analyzed by optimized HPLC procedure. The quantity recovered from plasma was estimated using respective regression equations. The accuracy was expressed as percent recovery and precision was depicted as percent relative standard deviation.Relative standard deviations (RSDs) including intra–day and inter–day of method based on 7 replicate determinations of methotrexate were 2.6% and 4.8%, respectively. The inter-day and intra-day accuracy ranged from 92.0 − 104.0% and 91.6–107.5, respectively.

The LOD (S/N = 3) and LOQ (S/N = 10) were 0.03 μg L^{-1} and 0.1 μg L^{-1}, respectively. The LLOQ was defined as the lowest concentration in the calibration curve that can be measured with acceptable accuracy and precision (≤20%). The LLOQ for methotrexate was 0.1 μg L^{-1} with accuracy 94.6%. Fig. 2 shows the chromatograms of blank plasma sample (A) and plasma at LLOQ (B).

The enrichment factor (EF) was defined as the ratio between the analyte concentration in the floated phase (C_{flo}) and initial concentration of analyte (C_0) within the sample.

$$EF = \frac{C_{flo}}{C_0}$$

The extraction recovery (%ER) was defined as the ratio between the amount of the analyte in the floating phase (n_{flo}) and the initial amount of the analyte (n_0) within the sample.

$$ER\% = \frac{n_{flo}}{n_0} \times 100 = \frac{C_{flo}.V_{flo}}{C_0.V_{sample}} \times 100$$

where V_{flo} and V_{sample} are the volumes of the floating phase and sample, respectively. The EF and ER% of the method were 152 and 91.2%, respectively, at the concentration level of 100 μg L^{-1} of methotrexate.

Sample carry-over was evaluated by running a blank plasma sample after the highest calibrator on calibration curves during the validation period. The average carry-over was < 10% of the calculated response of the LLOQ for methotrexate. LLOQ was determined to be 0.1 μg L^{-1} for methotrexate.

The stability of the analyte in plasma samples was assessed under the following three conditions: (1) after storing the plasma for 12 h at room

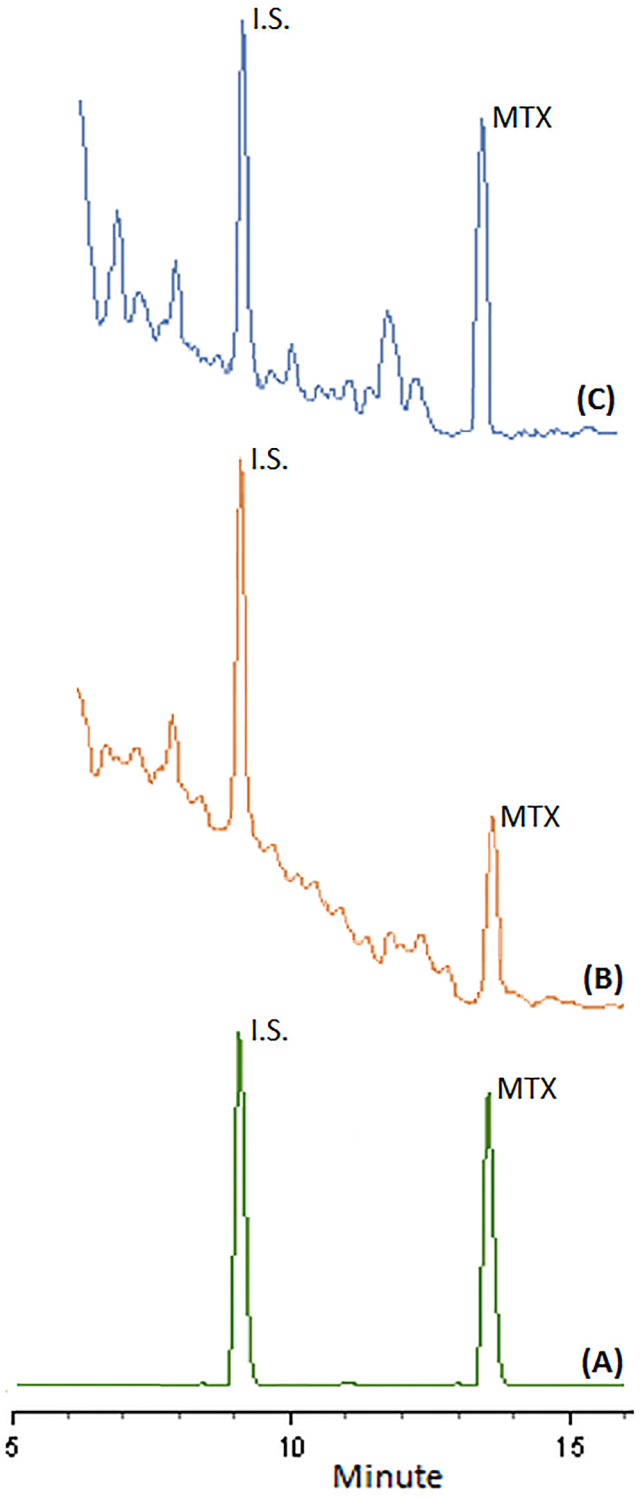

Fig. 3. Chromatograms of direct injection of methotrexateand 8-chlorotheophylline standards at concentration level of 1.00 mg L^{-1} (A), plasma sample taken from 7-year-old girl (B) and the corresponding spiked ones at concentration level of 10.0 μg L^{-1} for methotrexate (C) obtained by using DSS − DLLME combined HPLC − UV. Peak identification: (MTX) methotrexate, (I.S.) internal standard.

Table 3
Comparison of DSS–DLLME–HPLC–UV with other extraction methods for determination of different drugs in biological samples.

Methods	Aalyte	LOD[a] (μg L^{-1})	LR[b] (μg L^{-1})	RSD[c] %	EF[d]	Extraction time (min)	Extractant volume (μL)	Reference
rGO – IL – GCE[e]	Methotrexate	0.01	0.05–7.5	4.5	–	˃30	–	[7]
SPE–LC–MS/MS[f]	Methotrexate	1.0	2–2000	0.43–2.8	–	20	1000	[14]
LLE–LC–MS/MS[g]	Methotrexate	0.05	0.1–250	<15	–	14	500	[39]
HILIC–ESI–MS[h]	Methotrexate	61	200–120000	1.8	–	˃15	2000	[40]
DSS – DLLME–HPLC–UV	Methotrexate	0.03	0.1–150	2.6	152	˃20	50	This work

[a] LOD, limit of detection.
[b] LR, linear range.
[c] RSD, relative standard deviation.
[d] Enrichment factor
[e] Reduced graphene oxide-ionic liquid-modified glassy carbon electrode.
[f] Solid-phase extraction-liquid-chromatography-tandem mass spectrometry.
[g] Liquid-liquid extraction–liquid-chromatography-tandem mass spectrometry.
[h] Hydrophilic interaction liquid chromatography/positive ion electrospray mass spectrometry.

temperature and 4 °C (short-term), (2) after three repeated freeze and thaw cycles, (3) after storing the post-preparative plasma for 24 h at 10 °C. All of the stability studies were conducted at two concentrations (10 and 100 μg L^{-1}) with three determinations each. The stability experiment confirmed that plasma sample was stable through three repeated freeze and thaw cycles. Short-term stability was also confirmed for 12 h at 4 °C and room temperature.Post-preparative plasmastability was stable 24 h at 10 °C.

3.9. Analysis of blood samples

The efficiency of the DSS–DLLME procedure with HPLC–UVinstrument was validated with the monitoring of the methotrexate in plasma of patients with acute leukemia. Blood samples were taken from patients with acute leukemia who were admitted to and treated at Dr. Mohamad Kermanshahi Hospital. The results in Table 2 show that all blood samples contained methotrexate with different concentration levels in the range of 5.5 ± 0.3 to 17.2 ± 1.2 mg L^{-1}. It should be noted that the concentration of methotrexate in the blood samples was out of the calibration range. Therefore, before DSS–DLLME procedure, the blood samples were diluted for 100 to 500 times. Fig. 3 shows the chromatograms of direct injection of methotrexate and 8-chlorotheophylline standards at concentration of 1.00 mg L^{-1} (A), plasma sampletaken from 7-year-old girl (B) and the corresponding spiked ones at concentration of 10.0 μg L^{-1} for methotrexate (C).To evaluate the matrix effects and validation of the method, blood samples were spiked with different concentration of standard solution of methotrexate. Relative recovery of methotrexate in spiked samples at different concentrations is shown in Table 2, ranging from 92 to 104%.These results demonstrate that the plasma matrices, in our present context, have no significant effecton DSS – DLLME–HPLC–UV for determination of methotrexate.

3.10. Comparison of DSS – DLLMEwith other methods

The DSS – DLLMEcombined with HPLC–UV is compared with other procedures for determination of drugs in biological samples and the results are summarized in Table 3.According to Table 3, the detection limit of DSS – DLLME combined with HPLC–UV is lower than other techiques andconsumption of toxic organic phase is greatly reduced. The RSD and linearity of the DSS – DLLME are superior to those reported before. Compared to other methods, the extraction time is relatively short except for the DLLME method. However unlike the DLLME method, in this method the disperser solvent are not required and no centrifuge is required for the separation of phases. All these results indicate thatDSS – DLLME is a simple, inexpensive and reproducible technique that can be used for the extraction and preconcentration of methotrexatein plasmay samples.

4. Conclusions

In this study for the first time, a double-solvent supramolecular system as a novel extractant for dispersive liquid–liquid microextraction (DSS – DLLME) combined with HPLC – UV has been proposed for the determination of methotrexatein plasma of children with acute leukemia. The advantages of this method include a simple operational procedures, inexpensive, environmental friendly, dispersive-solvent-free and low organic solvent consumption. We expected this method will be a breakthrough in separation science for the extraction of variousdrugsinblood samples. In this method, the first two long normal chain alcohols are mixed in a particular ratio, and then it is injected into the sample solution, which is on the magnetic stirrer. In this case, the mixture of the two alcohol changes to new supramolecular aggregate. This new supermolecule is used as an extractant, which has a higher extraction power than any of its components alone.

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.

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Authors' disclosures of potential conflicts of interest.
No authors declared any potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122628.

References

[1] M.S. Phillips, J.E. Gayman, M.W. Todd, ASHP guidelines on medication-use evaluation, Am. J. Health Syst. Pharm. 53 (1996) 1953–1955.
[2] A. Addis, F. Rocchi, Drug evaluation: New requirements and perspectives, Recenti Prog. Med. 97 (2006) 618–625.
[3] S. Phal, B. Lindholm-Sethson, P. Geladi, A. Shchukarev, S. Tesfalidet, Determination of methotrexate in spiked human blood serum using multi-frequency electrochemical immittance spectroscopy and multivariate data analysis, Anal. Chim. Acta 987 (2017) 15–24.
[4] L. Janíková-Bandžuchová, R. Šelešovská, Determination of methotrexate at a silver solid amalgam electrode by differential pulse voltammetry, Anal. Lett. 49 (2016) 122–134.

[5] E. Asadian, S. Shahrokhian, A. Iraji Zad, F. Ghorbani-Bidkorbeh, Glassy carbon electrode modified with 3d graphene-carbon nanotube network for sensitive electrochemical determination of methotrexate, Sens. Actuat. B Chem. 239 (2017) 617–627.
[6] B.C. Widemann, P.C. Adamson, Understanding and managing methotrexate nephrotoxicity, Oncologist 11 (2006) 694–703.
[7] A.A. Ensafi, F. Rezaloo, B. Rezaei, $CoFe_2O_4$/reduced graphene oxide/ionic liquid modified glassy carbon electrode, a selective and sensitive electrochemical sensor for determination of methotrexate, J. Taiwan Inst. Chem. Eng. 78 (2017) 45–50.
[8] R. Zangooei, A.A. Hedayati Asl, P. Vossough, A. Mehrvar, M.R. Golpayegani, F. Yazdi, F. Azizzade, M. Alebouyeh, M. Faranoush, Medium and high-dose methotrexate-induced toxicity in children with acute lymphoblastic leukemia, Iranian J. Blood Cancer (IJBC) 5 (2012) 19–23.
[9] S. Sadray, S. Rezaee, S. Rezakhah, N on-linear heteroscedastic regression model for determination of methotrexate in human plasma by high-performance liquid chromatography, J. Chromatogr. B 787 (2003) 293–302.
[10] K. Fotoohi, T. Skarby, S. Soderhall, C. Peterson, F. Albertioni, Interference of 7-hydroxymethotrexate with the determination of methotrexate in plasma samples from children with acute lymphoblastic leukemia employing routine clinical assays, J. Chromatogr. B 817 (2005) 139–144.
[11] Y.D. Li, Y. Li, N.S. Liang, F. Yang, Z.P. Kuang, A reversed-phase high performance liquid chromatography method for quantification of methotrexate in cancer patients serum, J. Chromatogr. B Anal. Technol. Biomed, Life Sci. 1002 (2015) 107–112.
[12] D. Abd El-Hady, N. Abo El-Maali, R. Gotti, C. Bertucci, F. Mancini, V. Andrisanoa, Methotrexate determination in pharmaceuticals by enantioselective HPLC, J. Pharm. Biomed. Anal. 37 (2005) 919–925.
[13] M.A. Al-Ghobashy, S.A. Hassan, D.H. Abdelaziz, N.M. Elhosseiny, N.A. Sabry, A. S. Attia, M.H. El-Sayed, Development and validation of LC–MS/MS assay for the simultaneous determination of methotrexate, 6-mercaptopurine and its active metabolite 6-thioguanine in plasma of children with acute lymphoblastic leukemia: Correlation with genetic polymorphism, J. Chromatogr. B 1038 (2016) 88–94.
[14] I. Rodin, A. Braun, A. Stavrianidi, O. Shpigun, A validated LC–MS/MS method for rapid determination of methotrexate in human saliva and its application to an excretion evaluation study, J. Chromatogr. B 937 (2013) 1–6.
[15] A. Barbieri, L. Sabatini, P. Indiveri, R. Bonfiglioli, V. Lodi, F.S. Violante, Simultaneous determination of low levels of methotrexate and cyclophosphamide in human urine by micro liquid chromatography/electrospray ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 1889–1893.
[16] Z. Szakacs, B. Noszal, Determination of dissociation constants of folic acid, methotrexate, and other photolabile pteridines by pressure-assisted capillary electrophoresis, Electrophoresis 27 (2006) 3399–3409.
[17] S. Chen, Z. Zhang, D. He, Y. Hu, H. Zheng, C. He, Flow-injection-electrochemical oxidation fluorimetry for determination of methotrexate, Luminescence 22 (2007) 338–342.
[18] M. Rezaee, Y. Assadi, M.R. Milani Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, Determination of organic compounds in water using dispersive liquid–liquid microextraction, J. Chromatogr. A 1116 (2006) 1–9.
[19] M. Shamsipur, N. Fattahi, Extraction and determination of opium alkaloids in urine samples using dispersive liquid–liquid microextraction followed by high-performance liquid chromatography, J. Chromatogr. B 879 (2011) 2978–2983.
[20] M. Pirsaheb, N. Fattahi, M. Shamsipur, T. Khodadadi, Application of dispersive liquid–liquid microextraction based on solidification of floating organic drop for simultaneous determination of alachlor and atrazine in aqueous samples, J. Sep. Sci. 36 (2013) 684–689.
[21] J.-W. Wu, H.-C. Chen, W.-H. Ding, Ultrasound-assisted dispersive liquid–liquid microextraction plussimultaneous silylation for rapid determination of salicylate andbenzophenone-type ultraviolet filters in aqueous samples, J. Chromatogr. A 1302 (2013) 20–27.
[22] A.P. Birjandi, A. Bidari, F. Rezaei, M.R. Milani Hosseini, Y. Assadi, Speciation of butyl and phenyltin compounds using dispersive liquid–liquid microextraction and gas chromatography-flame photometric detection, J. Chromatogr. A 1193 (2008) 19–25.
[23] M. Sadeghi, Z. Nematifar, M. Irandoust, N. Fattahi, P. Hamzei, A. Barati, M. Ramezani, M. Shamsipur, Efficient and selective extraction and determination of ultra trace amounts of Hg^{2+} using solid phase extraction combined with ion pair based surfactant-assisted dispersive liquid–liquid microextraction, RSC Adv. 5 (2015) 100511–100521.
[24] M. Pirsaheb, N. Fattahi, M. Shamsipur, Determination of organophosphorous pesticides in summer crops using ultrasound-assisted solvent extraction followed by dispersive liquid-liquid microextraction based on the solidification of floating organic drop, Food Control 34 (2013) 378–385.
[25] X. Jing, J. He, W. Zhao, X. Huang, X. Wang, Effervescent tablet-assisted switchable hydrophilicity solvent-based microextraction with solidification of floating organic droplets for HPLC determination of phenolic endocrine disrupting chemicals in bottled beverages, Microchem. J. 155 (2020), https://doi.org/10.1016/j.microc.2020.104680 (in press).
[26] T. Ahmadi-Jouibari, N. Fattahi, M. Shamsipur, M. Pirsaheb, Dispersive liquid–liquid microextraction followed by high-performance liquid chromatography–ultraviolet detection to determination of opium alkaloids in human plasma, J. Pharm. Biomed. Anal. 85 (2013) 14–20.
[27] T. Ahmadi-Jouibari, N. Fattahi, M. Shamsipur, Rapid extraction and determination of amphetamines in human urine samples using dispersive liquid–liquid microextraction and solidification of floating organic drop followed by high performance liquid chromatography, J. Pharm. Biomed. Anal. 94 (2014) 145–151.
[28] R. Akramipour, M.R. Golpayegani, S. Gheini, N. Fattahi, Speciation of organic/inorganic mercury and total mercury in blood samples using vortex assisted dispersive liquid-liquid microextraction based on the freezing of deep eutectic solvent followed by GFAAS, Talanta 186 (2018) 17–23.
[29] S.C. Cunha, J. Fernandes, Extraction techniques with deep eutectic solvents, TrAC, Trends Anal. Chem. 105 (2018) 225–239.
[30] M. Pirsaheb, N. Fattahi, Development of a liquid-phase microextraction based on the freezing of a deep eutectic solvent followed by HPLC-UV for sensitive determination of common pesticides in environmental water samples, RSC Adv. 8 (2018) 11412–11418.
[31] Z. Meng, J. Zhao, H. Duan, Y. Guan, L. Zhao, Green and efficient extraction of four bioactive flavonoids from Pollen Typhae by ultrasound-assisted deep eutectic solvents extraction, J. Pharm. Biomed. Anal. 161 (2018) 246–253.
[32] N. Caballero Casero, S. Garcia Fonseca, S. Rubio, Restricted access supramolecular solvents for the simultaneous extraction and cleanup of ochratoxin A in spicessubjected to EU regulation, Food Contr. 88 (2018) 33–39.
[33] F. Rezaei, Y. Yamini, H. Asiabi, S. Seidi, M. Rezazadeh, Supercritical fluid extraction followed by nanostructured supramolecular solvent extraction for extraction of levonorgestrel and megestrol from whole blood samples, J. Supercrit. Fluids 107 (2016) 392–399.
[34] H. Musarurwa, N.T. Tavengwa, Supramolecular solvent-based microextraction of pesticides in food and environmental samples, Talanta 223 (2021), 121515.
[35] A. Ballesteros-Ǵomez, L. Lunar, M.D. Sicilia, S. Rubio, Hyphenating supramolecular solvents and liquid chromatography: tips for efficient extraction and reliable determination of organics, Chromatographia 82 (2019) 111–124.
[36] A. Ballesteros-Ǵomez, M.D. Sicilia, S. Rubio, Supramolecular solvents in the extraction of organic compounds: a review, Anal. Chim. Acta 677 (2010) 108–130.
[37] S. Rubio, Twenty years of supramolecular solvents in sample preparation for chromatography: achievements and challenges ahead, Anal. Bioanal. Chem. 412 (2020) 6037–6058.
[38] Food and Drug Administration, USFDA, Bioanalytical Method Validation, Guidance for Industry, 2001.
[39] S. Steinborner, J. Henion, Liquid-liquid extraction in the 96-well plate format with SRM LC/MS quantitative determination of methotrexate and its major metabolite in human plasma, Anal. Chem. 71 (1999) 2340–2345.
[40] H. Pligoropoulou, A. Vonaparti, I. Panderi, Hydrophilic interaction liquid chromatography/positive ion electrospray mass spectrometry for the quantification of deferasirox, an oral iron chelator, in human plasma, J. Chromatogr. B 893–894 (2012) 114–120.