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Scientific paper

Thin-Layer Chromatography: an Efficient Technique for the Optimization of Dispersive Liquid-Liquid Microextraction

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Abstract

Thin-layer chromatography (TLC) is an often omitted analytical technique due to its lower sensitivity and separation capacity. Even in the era of high-performance liquid chromatography (HPLC), thin-layer chromatography still offers many advantages, such as simplicity, rapidity, and cost-effectiveness, which predict TLC to be the first-choice method for the laborious optimization process requiring analysis of numerous samples. In this work, a thin-layer chromatography method with chemical and densitometric detection was used to optimize a dispersive liquid-liquid microextraction (DLLME) process for the extraction and preconcentration of estradiol in human urine. The chromatographic system consisted of silica gel plates as the stationary phase and toluene-ethanol (9:1; *v/v*) mixture as the developing solvent. The plates were dyed with 10% phosphomolybdic acid reagent and sequentially evaluated densitometrically at $\lambda = 430$ nm. In the context of DLLME optimization, parameters including the type and volume of extraction and dispersive solvents, centrifugation, salt addition and extraction time, were studied. The proposed DLLME-TLC method was successfully applied to the determination of estradiol in real human urine samples.

Keywords: Thin-layer chromatography; post-chromatographic detection; dispersive liquid-liquid microextraction; estradiol; human urine

1. Introduction

Estrogens are human hormones primarily responsible for the development and function of female gonadal system, regulation of menstrual cycle, and maintenance of pregnancy[1] which also take part in various biochemical processes within the organism in both males and females.[2] Estrogens originate in steroidogenesis with estradiol (E2) being the primary product and the most potent human estrogen. Urinary estradiol levels are essential for the monitoring of regular pregnancy progress as well as for the diagnosis of reproductive and hormonal diseases.[3] Estradiol concentrations in urine are typically low and fluctuate during pregnancy. While mean daily excretion of estradiol in menstruating women is 3.5 μg, it usually elevates up to 259–330 μg *per* day during pregnancy.[4] Besides clinical applications, development of new analytical methods for the monitoring of estrogens in environmental and food samples is required due to their adverse effects on living organisms and environment.[5,6] Chromatographic methods, primarily high-performance liquid chromatography, are the most commonly used for the quantification of estrogens owing to their high sensitivity.[7,8] Still, sample pretreatment remains a crucial step in the analysis of estrogens because of their low concentrations and complex character of the samples.[9]

Dispersive liquid-liquid microextraction (DLLME) was introduced in 2006[10] as a fast and straightforward sample preparation technique offering high preconcentration factors and recoveries. DLLME is based on a ternary solvent system in which a dispersive solvent causes dispersion of an extraction solvent within the aqueous sample. A mixture of extraction and dispersive solvents is rapidly injected into the aqueous sample with a syringe resulting in the formation of tiny droplets of extraction solvent in the solution thus allowing fast transfer of the desired analyte into the extraction solvent. This state, called cloudy solution, disappears during centrifugation when the aqueous and organic phases are separated. The organic drop sedimented at the bottom of the tube is removed with a mi-

crosyringe and analyzed by the compatible analytical system.[11] Although DLLME was initially introduced as an extraction technique for organic compounds from predominantly water samples, it has evolved to be suitable even for the extraction of analytes from more complex matrices, including biological samples.[12] Few DLLME applications for estrogen extraction have been described[13–17] so far; however, most of them were limited to water samples, except for two DLLME modifications used for the extraction of estrogens from milk[18] and urine.[19]

This work describes optimization of a dispersive liquid-liquid microextraction for the extraction and preconcentration of estradiol in human urine samples using thin-layer chromatography (TLC) with chemical and densitometric detection which has, to the best of our knowledge, not been described yet. The main advantage of open planar arrangement over column chromatography lies in the possibility for simultaneous analysis of numerous samples at once, while column arrangement allows analysis of only one sample at a time. This factor significantly decreases the time needed for the analysis itself, making TLC the first-choice method for laborious optimization process, despite its lower sensitivity. Moreover, new aspects regarding the DLLME application to biological sample are presented, emphasizing the effects of centrifugation and salt addition on extraction recovery.

2. Experimental

2. 1. Chemicals and Reagents

Standard of 17β-estradiol (≥98%) was purchased from Cayman Chemical (USA). Acetone, methanol, toluene, ethanol, tetrachloromethane, tetrachloroethane, chloroform and phosphomolybdic acid were obtained from Lambda Life (Slovakia), sodium chloride, sodium carbonate and potassium carbonate were from Mikrochem (Slovakia). All reagents were of analytical grade. Distilled water was used throughout all experiments.

2. 2 Instrumentation

End-capped plastic 5-mL test tubes, 100-μL microsyringe (Hamilton, Switzerland), centrifuge MPW-310 (Poland), thermal chamber (Laboratorni pristroje Praha, Czech Republic), 5-μL microsyringe (Hamilton, Switzerland), alumina-backed TLC plates with silica gel coating ALUGRAM Sil G/ UV 254 (Macherey-Nagel, Germany) and vertical chromatographic chamber (Lublin, Poland) were used throughout the experiments.

2. 3 Thin-Layer Chromatography

Chromatographic separation was carried out on alumina-backed silica gel 60 TLC plates (10 × 10 cm, 0.20 mm) with fluorescent indicator UV_{254}. The samples were applied manually with a 5 μL microsyringe to the starting line 1 cm from the bottom edge. The TLC plates were placed into the chromatographic chamber saturated with vapors of developing solvent, toluene-ethanol (9:1; *v/v*) mixture, at laboratory temperature. The developing distance was 8 cm and chromatographic separation in this system took 20 min. Developed TLC plates were dried in the stream of air at laboratory temperature for approximately 5 min and subjected to post-chromatographic detection. TLC plates were shortly (1–2 s) immersed in the detection reagent (10% phosphomolybdic acid in methanol[20]) and then heated in a thermal chamber at 110 °C for approximately 10 min, until dark-blue estradiol spots appeared on a yellow-greenish background. For quantification, the spots were evaluated densitometrically at λ = 430 nm. The volume of sample applied to the plates was 0.3 μL.

2. 4. Standard Solutions and Calibration Curve

Estradiol stock solution at a concentration of 5 mg mL^{-1} was prepared in methanol. Working standard solutions were prepared by further dilution of stock solution with methanol. The calibration curve in the range of 0.10 – 5.00 mg mL^{-1} consisted of the following calibration points (n = 3): 0.10, 0.25, 0.50, 0.75, 1.00, 2.50 and 5.00 mg mL^{-1}.

2. 5. Urine Samples

Urine samples obtained from healthy individuals were checked with diagnostic strips for the presence of pathological constituents such as blood, sugar, or proteins. The urine samples were frozen and stored at –10 °C. On the day of analysis, the urine samples were thawed in lukewarm water, centrifuged at 10 000 rpm for 5 min and only the supernatants were used for further study.

Urine of a 3-year old boy, spiked with estradiol at concentration of 0.1 mg mL^{-1}, was used as a model urine sample throughout the optimization process. The real urine sample was prepared by blending pregnancy urines obtained from five healthy women in the second half of pregnancy as follows: 5 mL from each urine sample was combined together and diluted to 50 mL with distilled water.

2. 6. Dispersive Liquid-Liquid Microextraction

A mixture of tetrachloromethane as an extraction solvent and methanol as a dispersive solvent (500 μL; 1:9, *v/v*) was rapidly injected into 2 mL urine sample with 1.5 mol L^{-1} NaCl. After gentle agitation for 30 s, the sample was centrifuged at 10 000 rpm for 5 min. Sedimented organic drop (75 μL) was removed by a microsyringe and subjected to TLC analysis.

3. Results and Discussion

3. 1. Thin-Layer Chromatography

Silica gel aluminum-backed TLC plates were after the application of samples developed in vertical chromatographic chamber previously saturated with the vapors of developing solvent, a mixture of toluene and ethanol (9:1; *v/v*). After development, estradiol on TLC plate was visualized with 10% phosphomolybdic acid detection reagent as dark-blue spots on greenish background[20] and it was quantified densitometrically at wavelength of λ = 430 nm. TLC chromatogram and densitogram of estradiol standard analyzed in the described chromatographic system are demonstrated in Figure 1.

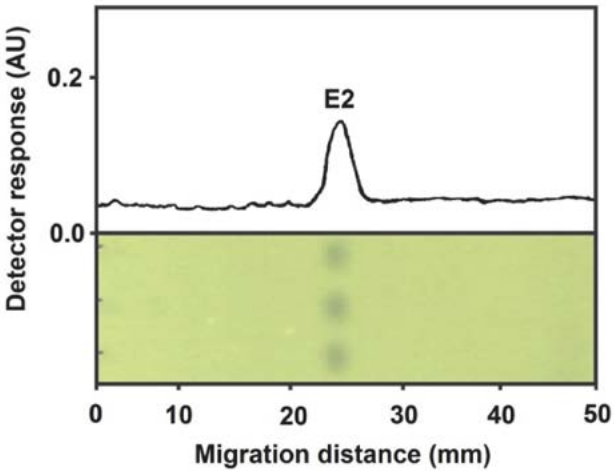

Figure 1. TLC chromatogram and densitogram of estradiol (E2) standard (0.25 mg mL^{-1}; n = 3); stationary phase: silica gel, developing solvent: toluene-ethanol (9:1; *v/v*), detection reagent: 10% phosphomolybdic acid in methanol, λ = 430 nm, sample volume: 0.3 μL

Qualitative chromatographic parameter – R_f value for estradiol in this system was 0.28 (n = 3, RSD = 0.23%). Optimized TLC method was linear within the concentration range of 0.1 – 5.0 mg mL^{-1} (r = 0.9979) with LOD = 0.03 mg mL^{-1} and LOQ = 0.1 mg mL^{-1}. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the standard deviation of response (σ) and slope of calibration curve (b): LOD = 3 σ/b and LOQ = 10 σ/b.

3. 2. Optimization of Dispersive Liquid-Liquid Microextraction

The efficiency of DLLME procedure is significantly influenced by several factors which were studied in the following manner: (1) type and volume of extraction and dispersive solvents, (2) centrifugation conditions, (3) salt addition, and (4) extraction time. A model urine sample containing 0.1 mg mL^{-1} estradiol was used throughout the optimization process so the influence of real matrix on the extraction efficiency could be assessed.

The selection of dispersive and extraction solvents is crucial in order to attain efficient extraction of the analyte. The requirement for extraction solvent is higher density than water, good extraction capacity for the target analyte and low miscibility in water. On the other hand, high miscibility with dispersive solvent is mandatory. According to these requirements, three halogenated solvents were selected as extraction solvents: chloroform (ρ = 1.5 g cm^{-3}), tetrachloromethane (ρ = 1.6 g cm^{-3}) and tetrachloroethane (ρ = 1.6 g cm^{-3}). The only demand for the dispersive solvent is high miscibility in both extraction solvent and the aqueous phase. Two dispersing solvents were tested in combination with selected extraction solvents: methanol and acetone. Dispersive solvent (500 μL) was mixed with 100 μL extraction solvent before injection into the model urine sample (2 mL). As a result of the rapid injection, a cloudy solution of a different character was formed, depending mainly on the type of the dispersive solvent: while acetone induced poor dispersion, methanol resulted in fine droplets of extraction solvent scattered within the sample. Consequently, the type of dispersive solvent greatly affected the quality of sedimented organic phase after centrifugation. The sedimented organic phase emerged as a compact drop and was well separated from the solution containing methanol. On the other hand, the sedimented organic phase obtained with acetone was inconsistent and exceeded the initial volume of extraction solvent which suggests that the phase also contained some undesirable portion of the sample or dispersive solvent besides the extraction solvent. According to the obtained results, methanol was selected as a suitable dispersive solvent. The extraction solvents were compared for extraction recovery. Chloroform provided the least extraction potential for estradiol in combination with both methanol and acetone, reaching extraction recoveries of 41% and 40%, respectively. Extraction recoveries obtained with tetrachloroethane and tetrachloromethane in both dispersive solvents ranged from 68% to 74%, reaching their highest level with tetrachloromethane in combination with methanol. Since the requirements for the optimal progress of DLLME procedure were fulfilled with methanol and tetrachloromethane, they were tested in various ratios for adequate development of dispersion, volume of sedimented organic phase and extraction recovery. These demands were met with 500 μL tetrachloromethane-methanol (1:9; *v/v*) mixture which was used throughout the following experiments.

After the selection of extraction and dispersive solvents, the centrifugation conditions were investigated. Although most of the literature published about DLLME so far claims that 4 000 rpm is sufficient for extraction of estrogens in environmental water samples,[13–17] this study indicated that urine sample requires higher centrifugation speed for adequate separation of phases. Centrifugation causes the separation of phases and is essential for the formation of compact organic drop sedimented on the bot-

tom of the tube. This parameter is of critical importance to the following removal of extraction solvent which should be free of dispersive solvent or sample components. Centrifugation reached optimal conditions for the separation of phases at 10 000 rpm.

The complex character of urine sample resulted in the forming of precipitate which, during centrifugation, covered the sedimented organic drop and hindered the removal for the following analysis. Three types of salts, including potassium carbonate, sodium carbonate, and sodium chloride, were tested in order to prevent the development of this undesired state. Potassium carbonate and sodium carbonate had no influence on the presence of the precipitate. However, the addition of NaCl significantly reduced the amount of precipitation and, moreover, resulted in an unforeseen increase in extraction recovery. This led to further study of the NaCl effect on the extraction efficiency with series of additions ranging from NaCl concentration of 0.5 mol L^{-1} to 2.0 mol L^{-1} which is demonstrated in Figure 2. The highest extraction recovery was reached with the NaCl concentration of 1.5 mol L^{-1} in the sample.

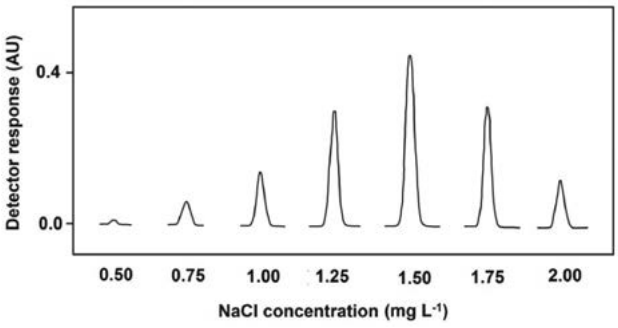

Figure 2. Effect of NaCl concentration on extraction efficiency. Experimental conditions: 2 mL model urine sample containing estradiol (0.1 mg mL^{-1}) with NaCl addition; 500 µL tetrachloromethane – methanol (1:9; *v/v*), centrifugation: 5 min, 10 000 rpm

In the final step, the extraction time (time from injection of extraction and dispersive solvents mixture into the sample until the start of centrifugation) was optimized. The initial extraction time in previous experiments was 60 s while the sample was gently agitated to enhance the extraction process. However, the study of the extraction time ranging from 0 to 120 s showed that the extraction efficiency rapidly increases during the first 30 s and does not noticeably change afterwards.

3. 3. Evaluation of Extraction Process

Optimal conditions and characteristics of DLLME procedure for extraction and preconcentration of estradiol in human urine are summarized in Table 1 and Table 2.

Figure 3 presents the preconcentration obtained with optimized microextraction technique as the difference in estradiol concentration detected in model urine sample before and after DLLME.

Table 1. Conditions for optimized DLLME method

Studied parameters	Optimal conditions
Extraction solvent (ES)	Tetrachloromethane
Dispersive solvent (DS)	Methanol
Volume of ES and DS mixture	500 µL (1 : 9, *v/v*)
Salt addition	1.5 mol L^{-1} NaCl
Extraction time	30 s
Centrifugation conditions	5 min, 10 000 rpm

Table 2. Evaluation of optimized DLLME method with model urine sample spiked with estradiol (0.1 mg mL^{-1})

Evaluation of DLLME	
Volume of sample	2.0 mL
Volume of organic phase	75 µL (n=3, RSD = 2.67%)
Preconcentration factor	25 (n = 3, RSD = 6.81%)
Extraction recovery	93.75% (n = 3, RSD = 6.81%)

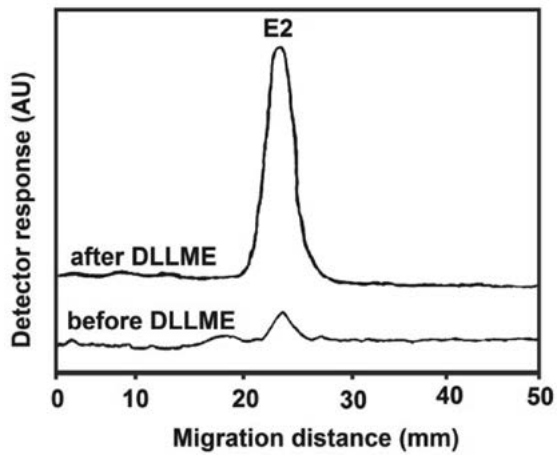

Figure 3. Chromatogram of model urine sample spiked at 0.1 mg mL^{-1} with estradiol (E2) before and after DLLME. Experimental conditions: 2 mL model urine sample with NaCl addition (1.5 mol L^{-1}); 500 µL tetrachloromethane – methanol (1:9; *v/v*) mixture, extraction time: 30 s; centrifugation: 5 min, 10 000 rpm

3. 4. Application to Real Samples

An optimized DLLME-TLC method was applied to the analysis of real urine prepared from five urine samples obtained from women in the second half of pregnancy. The real urine sample was spiked with estradiol at two concentration levels. Table 3 shows found estradiol concentrations in both spiked and non-spiked real urine samples analyzed by the DLLME-TLC method.

Table 3. Extraction recovery (ER) obtained from the determination of estradiol in real urine samples (n = 3)

Spiked (mg mL^{-1})	Found (mg mL^{-1})	ER (%)	RSD (%)
non-spiked	*ND*	–	–
0.20	0.18	89%	1.12%
0.10	0.09	91%	1.10%

ER: Extraction recovery; RSD: Relative standard deviation, ND: Not detected

4. Conclusion

Thin-layer chromatography with chemical and densitometric detection was used to optimize a DLLME procedure for the extraction and preconcentration of estradiol in human urine. TLC method enabled fast chromatographic separation of 19 samples within 20 min and, therefore, allowed fast optimization of extraction technique which provided preliminary results for further experiments carried out with HPLC. Optimum conditions for DLLME were reached after rapid injection of 500 μL tetrachloromethane-methanol (1:9; *v/v*) mixture into 2 mL urine sample containing NaCl (1.5 mol L^{-1}). The samples were after 30 s of gentle agitation centrifuged at 10 000 rpm for 5 min. This study proved TLC to be an efficient method for the laborious optimization process.

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Povzetek

Tankoplastna kromatografija (TLC) je zaradi svoje slabše občutljivosti in ločljivosti pogosto spregledana analizna tehnika. Vendar celo v eri visokozmogljive tekočinske kromatografije (HPLC) tankoplastna kromatografija še vedno nudi mnoge prednosti, kot na primer: preprostost, hitrost, cenovno ugodnost; to pa postavlja TLC na prvo mesto pri izbiri metode za delovno intenziven proces optimizacije, ki zahteva analizo številnih vzorcev. V tej raziskavi smo uporabili tankoplastno kromatografijo s kemijsko in denzitometrično detekcijo za optimizacijo disperzivne mikroekstrakcije tekoče-tekoče (DLLME) za ekstrakcijo in predkoncentracijo estradiola iz človeškega urina. Kromatografski sistem je bil sestavljen iz silikagelskih plošč kot stacionarne faze in mešanice toluen-etanol (9:1; *v/v*) kot topila za razvijanje plošče. Plošče smo orosili z reagentom 10% fosfomolibdensko kislino in nadalje denzitometrično ovrednotili pri λ = 430 nm. V kontekstu optimizacije DLLME smo preučevali naslednje parametre: tip in volumen ekstrakcijskega in disperzijskega topila, centrifugiranje, dodatek soli ter čas ekstrakcije. Predlagano DLLME-TLC metodo smo uspešno uporabili za določitev estradiola v realnih vzorcih človeškega urina.