MAGNETIC SOLID-PHASE EXTRACTION OF TARGET ANALYTES FROM LARGE VOLUMES OF URINE

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INTRODUCTION: Analysis of urine is often used to detect the presence or determine the concentration of various biologically active compounds or xenobiotics. Urine has become the preferred specimen since many compounds can be detected for longer time periods in urine than in blood. Furthermore, urine collection does not require phlebotomy, and urine is typically an ample and stable sample. In most cases the concentration of target analytes is low and thus the analytes have to be preconcentrated before the application of an appropriate analytical procedure.

There is a wide range of preconcentration procedures available that can be used individually or sequentially according to the complexity of the samples, the nature of the matrix, the analytes, and the instrumental techniques available. The use of an extraction technique is common in the pretreatment of most types of sample. Both liquid-liquid and solid-phase extraction procedures are widely used for the urine analytes analysis.

A great increase in the use of solid-phase extraction (SPE) as a preconcentration step has occurred recently. Solid-phase extraction can effectively handle small samples using only small volumes of organic solvents and very simple equipment (usually a small column and a syringe). In some cases, however, it is needed to extract very low amounts of the target analytes from larger volumes of samples. In this case standard SPE requires additional equipment such as solid-phase extraction vacuum manifolds.

Recently a new extraction procedure, called magnetic solid-phase extraction (MSPE), has also been developed [1]. This procedure based on the adsorption of the target analyte on relatively small amount of magnetic specific adsorbent enables to handle liter volumes of samples. Using MSPE with subsequent elution very low concentrations in ppb (µg/L) range of some compounds can be detected. There are two main advantages of this procedure when compared with standard column SPE. At first, it is the possibility to perform the extraction steps in a very simple way, without the need of expensive equipment, even using larger amount of sample (up to 1000 mL of liquid can be manipulated without problems). At second, MSPE can be performed not only in solutions, but also in suspensions. This is a general advantage of magnetic separation techniques due to the fact that majority of accompanying impurities are diamagnetic and do not interfere with magnetic particles during the magnetic separation step.

The use of MSPE in urine analysis was tested using silanized magnetite particles with immobilized reactive copper phthalocyanine dye (C.I. Reactive Blue 21; see Fig. 1) as an affinity ligand. This ligand interacts specifically with polyaromatic hydrocarbons having three or more conjugated rings [2] and with diamino- and triaminotriphenylmethane dyes [3]. This adsorbent can thus be used for the preconcentration of various compounds with proven or suspected carcinogenic properties [2].

![Fig. 1: Possible structure of the C.I. Reactive Blue 21.](image)

For experiments crystal violet (Basic Violet 3, CI 42555; also known as gentian violet or hexamethylpararosaniline chloride; chemical structure is shown in Fig. 2) was used as a model analyte. This dye belongs into the letter group of compounds interacting with copper phthalocyanine derivative. Because of its potential cancerous
property FDA put this dye on the Food and Drug Administration’s (FDA’s) priority list for drugs that need analytical methods development.

Fig. 2: Chemical structure of crystal violet

Concern about the health risks and carcinogenicity associated with the use and contact with various dyes and other xenobiotics requires a routine laboratory method to be developed to monitor the presence of target dyes and dye metabolites in body fluids. In this paper we demonstrate the use of MSPE for the preconcentration of a model dye (crystal violet) from human urine. Due to the fact that this dye exhibits high extinction coefficient (112,000 M⁻¹ cm⁻¹ at 590 nm) subsequent spectrophotometric detection can be easily be performed.

METHODS: In model experiments fine magnetite particles with immobilized reactive copper phthalocyanine dye (blue magnetite) were used as an affinity adsorbent. Blue magnetite was prepared in a similar way as described recently [3]. Iron(II,III) oxide (10 g; Aldrich, USA) was suspended in 5 % nitric acid and boiled in a closed vessel at 100 °C for 60 minutes. After thorough washing with distilled water, 40 mL of 10 % water solution (pH 4.0) of 3-aminopropyl-triethoxysilane (Sigma, USA) were added to the sedimanted magnetite. The suspension was mixed in a water bath at 80 °C for 4 h. Then the silanized magnetite was thoroughly washed with water, suspended in 200 mL of water and mixed with 4 g of Ostazin turquoise V-G (C.I. Reactive Blue 21; produced by Spolek pro chemickou a hutni vyrobu, Usti nad Labem, Czech Republic) and 12 g of sodium chloride. The suspension was warmed to 70 °C and 15 min later 10 g of anhydrous sodium carbonate were added. The suspension was stirred at 70 °C for 4 h and then the mixture was left overnight at ambient temperature without mixing. The blue magnetite particles were thoroughly washed with water and the remaining free dye was removed using an extraction with methanol in a Soxhlet apparatus. The extracted particles were then repeatedly washed with methanol – 2 % acetic acid (50:1, v/v). The washed blue magnetite particles were stored in water at 4 °C. The dry weight of 1 mL of the settled blue magnetite was 322 mg. The copper phthalocyanine content of the blue magnetite was ca 80 μmol per g of the dry adsorbent (determined from elemental analysis for copper using a PU 7450 ICP spectrometer (Pye-Unicam, England) after mineralization of blue magnetite with concentrated nitric acid).

Various volumes of human urine (usually 100 mL or more; obtained from healthy male donors) were spiked with various amounts of crystal violet and subsequently 400 μL of water suspension of magnetic adsorbent (corresponding to 100 μL of sedimented adsorbent) was added. The suspension was stirred for 3 – 4 hours at room temperature. After that magnetic particles were captured to the bottom of the beaker or flask using an appropriate magnet or flat magnetic separator and urine was poured off taking care not to lose the adsorbent. The adsorbent was washed several times with water and transferred into a test tube (test tube magnetic separators MPC-1 or MPC-6 from Dynal, Norway were used for magnetic separation). After washing and removing all water 1.5 mL of methanol – 2 % acetic acid (50:1, v/v) solution was added to the adsorbent. The elution proceeded for ca 20 minutes. The eluate was used for the spectrophotometric measurement when the position of the crystal violet peak was verified using a crystal violet standard.

RESULTS: Crystal violet was used as a model dye in order to test a new preconcentration procedure MSPE (magnetic solid-phase extraction) for the detection and determination of low concentration of target analytes in urine. The procedure was developed for standard spectrophotometers having the possibility to record spectra in visible region of light and for use of standard semi-microcuvettes (working volume ca 1.5 ml).

In previous experiments magnetic carrier with immobilized copper phthalocyanine dye (blue magnetite) efficiently adsorbed chromatic form of crystal violet from water solutions [3]. Nevertheless this dye can also be separated from other liquid samples, such as urine. It was shown in preliminary experiments that majority of the tested dye (added
in amounts 0.25 - 1 µg into 100 mL of urine) was adsorbed on blue magnetite in approximately 120 min; further prolongation of the incubation time did not improve the dye adsorption and subsequent recovery (data not shown). This incubation period (ca 2 hours) was used in all further experiments.

Fig. 3 shows an example of eluates spectra obtained after MSPE of crystal violet from male urine. It can be seen that very low amounts of the dye can be detected spectrophotometrically. After MSPE clearly visible peak of crystal violet can be found at the concentration 0.5 µg/100 mL urine (i.e., 5 ppb) and even at the half concentration (2.5 ppb). It corresponds to the concentration of crystal violet 6 - 12 nmol/L. The samples showing a peak corresponding to that one of crystal violet (with the maximum at ca 585 - 590 nm) can be considered as presumptive positive and the presence of crystal violet can be verified e.g. using a HPLC procedure.

The recovery of crystal violet was ca 60 – 75 % depending mainly on the batch of magnetic affinity adsorbent used for the MSPE. The reproducibility of the technique was determined from five replicate measurements for one concentration (1.0 µg per 100 mL of urine) of crystal violet. The typical relative standard deviation of the adsorption and elution efficiencies (measured as the absorbance of the eluate at the peak maximum) was 9.3 %.

**DISCUSSION & CONCLUSIONS:** Isolation and preconcentration of compounds present in solutions or suspensions in very low concentrations belongs to the basic operations in analytical, bioanalytical and environmental chemistry. The aim of this work was to develop a simple and ready to use procedure for the preconcentration and subsequent detection or determination of low concentrations of target analytes in urine. Crystal violet was chosen as a model compound because it belongs (together with e.g. malachite green) to a group of compounds that are linked to an increased risk of cancer [4]. This dye is commonly used for many purposes (e.g. such as an antimicrobial or antifungal agent) nevertheless its use has already been controlled in some individual member states within the European Community.

The results confirmed the applicability of MSPE procedure for a treatment of biological samples, specifically urine. Using MSPE low concentrations (5 ppb) of the native dye (in a chromatic form) can be detected spectrophotometrically. Of course, different situation can be expected if the dyes (or other xenobiotic compounds) enter the human body and their metabolic modification takes place. Rapid metabolic conversion of crystal violet to the colorless leuco form can be expected followed by its slow release into urine. The leuco form cannot be simply separated using the described procedure. An appropriate oxidizing process may lead to the conversion of leuco form into the chromatic form of crystal violet, which could then be separated and subsequently detected using the described procedure. The optimized oxidation / preconcentration procedure for the detection of metabolically modified crystal violet will be studied soon.

It can be supposed that MSPE can also be used for the preconcentration of other xenobiotics from urine provided that suitable affinity ligands (e.g., antibodies) will be available. MSPE could thus be efficiently used as a simple screening and preconcentration procedure that can be followed by a more precise chromatography analysis in case suspected urine samples are found. Currently we test other types of magnetic adsorbents (both specific and non-specific ones) which could be used for magnetic solid-phase extraction of various xenobiotics from human urine.
It can also be expected that MSPE could be used for the isolation and preconcentration of important minority biologically active compounds directly from urine and other body fluids such as blood and milk.

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ACKNOWLEDGEMENTS: The research is a part of ILE Research Intention No. AV0Z6087904. The experimental work was supported by the NATO Collaborative Linkage Grant No. LST.CLG.977500, Ministry of Education of the Czech Republic (Project No. OC 523.80) and Grant Agency of the Czech Academy of Sciences (Project No. S6087204).