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DEPARTMENT OF ANALYTICAL CHEMISTRY

MASTER THESIS

DETERMINATION OF CYCLOPHOSPHAMIDE IN
HUMAN URINE BY HPLC COUPLED TO TANDEM
MASS SPECTROMETRY

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LIST OF ABBREVIATIONS

CP	Cyclophosphamide
CUR	Curtain gas
CE	Collision energy
CEPM	Carboxyethylphosphoramidate
CarboxyCP	Carboxycyclophosphamide
DP	Declustering potential
DCM	Dichloromethane
DCCP	Dechloroethylcyclophosphamide
ESI	Electrospray ionization
HPLC	High performance liquid chromatography
i.d.	Internal diameter
IF	Ifosfamide
ISV	Ion spray voltages
KH ₂ PO ₄	Potassium dihydrogen phosphate
K ₂ HPO ₄	Potassium hydrogen phosphate
4-Keto CP	4- Ketocyclophosphamide
LC	Liquid chromatography
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MS/MS	Tandem mass spectrometry
MRM	Multiple reaction monitoring
<i>m/z</i>	Mass/Charge
MeOH	Methanol
N ₂	Nitrogen
4-OHCP	4- Hydroxycyclophosphamide
pKa	Dissociation constant
PBS	Phosphate buffer solution
RP-18	Reverse phase group C ₁₈
SPE	Solid phase extraction

UNITS

Unit	English term
Å	angstrom
°C	Celsius degree
cm	centimeter
h	hour
L/h	Liter per hour
μg	microgram
μg/L	microgram per liter
μg/mL	microgram per milliliter
μm	micrometer
mg	milligram
mg/L	milligram per liter
mg/mL	milligram per microliter
min.	minute
mL	milliliter
mL/min	milliliter per minute
mm	millimeter
msec	millisecond
ng/L	nanogram per liter
psi	Pound per square inch
s	second
V	Voltage

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1. INTRODUCTION

Antineoplastic drugs are commonly used to treat cancers which are generally referred to as chemotherapy. The majority of antineoplastic drugs are non-selective in their action which exhibits their effects in both cancerous and non-cancerous cells in most organs and body tissues. Chemotherapy agents are known to be mutagens and carcinogens in animals, humans and in patients treated with therapeutic dose [1]. Cyclophosphamide (CP) is known to be human carcinogenic according to International agency for research on cancer (IARC Group I).

Medical professionals were unaware of the risk from exposure to antineoplastic agents during performance of their daily routine. The routes of exposure are typically inhalation, dermal or oral. Workers may be exposed by inhalation via droplets, particulates and vapors when they create aerosols, generate dust by crushing tablets and clean up spills. Dermal exposure may occur when workers touch contaminated surfaces during preparation, administration or disposal of hazardous drugs and oral exposure may occur from hand to mouth contact. In most paper air sampling for antineoplastic drugs have little to no air borne contamination. Drugs particulates can become airborne after the drying of contaminated areas. Vaporization of antineoplastic agents has also been reported with various drugs such as carmustine, ifosfamide, thiotepa and CP [2, 3]. When food or beverages are prepared, stored or consumed in work areas, they may easily become contaminated with airborne particles of antineoplastic drugs. A potential source of exposure is direct skin contact when a spill or leak occurs and large volume of drug is released to the environment. Antineoplastic drugs can have chronic effects on reproduction, causing abortion, stillbirth, infertility and congenital abnormalities and other minor health effect include hair loss, headache, acute irritation and hypersensitive [4, 5].

Few methods have been described for the determination of CP in urine till now. The method used for the determination of CP is gas chromatography separation with nitrogen phosphorous detection or mass spectrometric detection. Other method used for analysis of CP is HPLC [6]. UV detection is not however, selective and sensitive enough to enable low exposure levels to be quantified because CP has weak chromophores [7, 8]. Polarographic and colorimetric methods have been described for the determination of CP and ifosfamide [9].

2. AIMS AND OBJECTIVE OF THE THESIS

The main objective of this study was

- a) To optimize SPE method for isolation of organic compound such as CP from urine sample. Optimization is achieved by using different sorbent, matrix, washing solvent and elution solvent. Biological samples such as plasma and urine are much more complex due to presence of proteins, salts, acids, bases and various organic compounds with similar chemistry to analytes of interest. As a result, the extraction methods for biological samples have been complicated. If unsuitable sample preparation methods have been employed before injection, the entire analytical process can be wasted. The main task is to remove maximum interfering substance from urine matrix and conversion of analytes into a more suitable form for injection.
- b) To optimize the separation conditions of the HPLC-MS/MS analysis to achieve the optimal (short retention time, no interferences) cyclophosphamide separation. Optimization is achieved by changing different parameter such as
 - Mobile phase and its composition
 - Flow rate
 - Scan type
- c) After developing suitable analytical method, this SPE-LC-MS/MS method is applied for the analysis of urine samples of health workers from Medical University of Gdansk, who were exposed with CP during their work in pharmacy. Hospital workers are exposed to antineoplastic drugs via dermal, inhalation route during preparing and handling.

3. THEORITICAL PART

CP general information

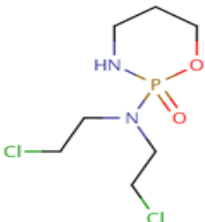
3.1. Cyclophosphamide

CP (2-[bis(2-chloroethyl)amino] tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide) is an alkylating agent that is frequently used as an antineoplastic drug. CP is a prodrug that requires activation by the cytochrome P450 enzyme system to form its pharmacologically active metabolite 4- hydroxycyclophosphamide [10]. The metabolites are genotoxic due to their ability to cross-link DNA and thereby cause DNA damages. CP is classified as carcinogenic to humans [11]. It has very narrow therapeutic index [12]. CP is rapidly converted to number of mutagenic and carcinogenic products, some of which have been isolated from urine [13].

3.1.1. Physical and chemical properties of CP

Basic physical and chemical properties of CP [11, 14-17] are summarized below in Table 1.

Table 1 Physical and chemical properties of CP

Chemical structure	
	
Molecular formula	C ₇ H ₁₅ Cl ₂ N ₂ O ₂ P
Molecular weight	261.09
Physical properties	Odorless, fine white crystalline powder
Melting point	49.5 – 53 ° C
Boiling Point	336° C
Density	1.479 g/cm ³
Solubility	Soluble in chloroform, dioxane and glycols; slightly soluble in benzene, carbon tetrachloride, very slightly soluble in ether and acetone.
Partition coefficient	0.63
pKa	4.5-6.5
Stability	Hydrolysis occurs at temperatures above 30 °C, with removal of chlorine atoms. Sensitive to oxidation, moisture and light.

3.1.2. Metabolism of CP

CP is a prodrug which metabolized to both active and inactive metabolites, as shown in figure 1. CP is activated by hepatic microsomal mixed function oxidases cytochrome P450 to form 4-hydroxycyclophosphamide (4-OHCP), which exists in equilibrium with its tautomer aldophosphamide (AldoCP). 4-OHCP is very unstable, readily diffuses in to cells and spontaneously decomposes into phosphoramidate mustard (PM) by β elimination of acrolein [18]. PM is an active alkylating species which is responsible for alkylating effect of CP [19]. Acrolein is an unwanted by product which may enhance CP-induced cell damage, possibly by depletion of cellular glutathione by conjugation [20]. The aldehyde dehydrogenase enzymes are involved in formation of carboxyphosphamide (CarboxyCP) from aldophosphamide [21]. 4- Ketocyclophosphamide (4-KetoCP) are formed due to oxidation of 4-OHCP by alcohol dehydrogenase. Other inactive metabolite includes 2-dechloroethylcyclophosphamide (DCCP) produced by separate oxidative N-dealkylation reaction [22].

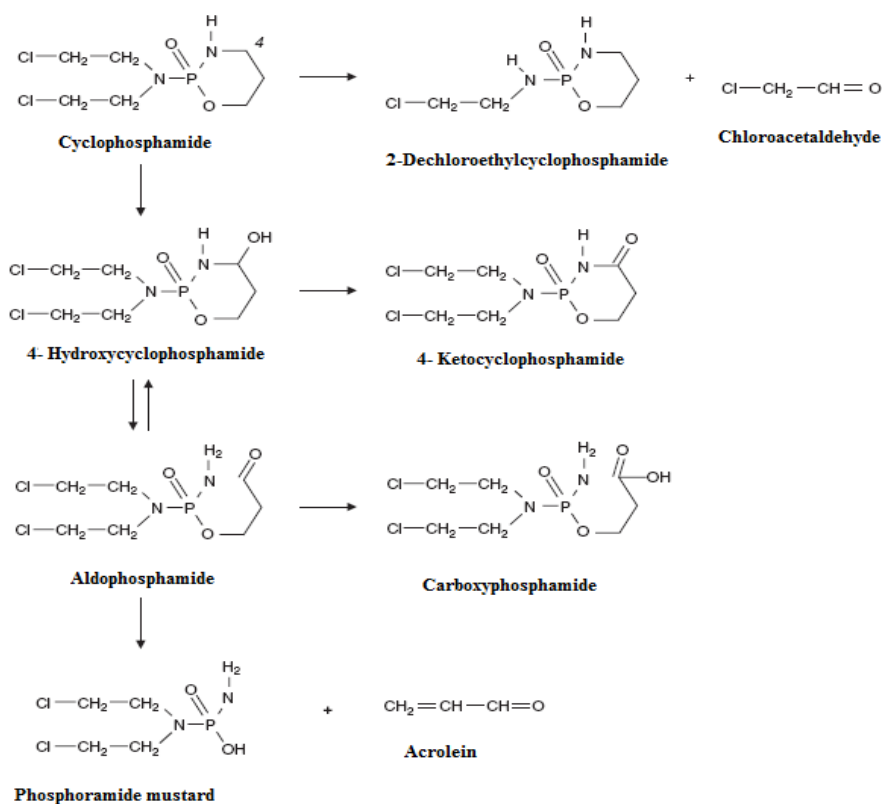


Figure 1. Metabolism of cyclophosphamide.

3.1.3. Absorption, distribution and elimination of CP

CP is well absorbed orally, with peak concentrations occurring after 1-3 hours and has bioavailability of 85-100% [23]. The drug is rapidly absorbed from the blood after IV injection [11].

CP is distributed with a volume of distribution of 30 -50 L, which approximates to the total body water [10]. Penetration of CP and its metabolites into body fluids is limited. Metabolites like CarboxyCP and DCCP were not found in cerebrospinal fluid whereas PM and CP could be detected [24, 25].

CP and its metabolites are eliminated by urine in 24 hours after the start of treatment. The major function of CP in the body is eliminated by hepatic metabolism, but small fraction is eliminated by renal excretion of unchanged drug in urine. Less than 20 % of the administered dose is eliminated unchanged in the urine. Between 30-60% of the total CP dose is eliminated renally as CP or metabolites. CarboxyCP is major metabolites found appearing in urine [26, 27]. Small fraction of CP dose is eliminated via feces and expired air. In children and young adults plasma half life is shorter than adults. The elimination half life of CP ranges between 5-9 hours over a large concentration range. Total systemic clearance of CP ranges from 4-5 L/h of which the greater part is non-renal clearance. The plasma half life of CP is approximately 5 h in human during the first day after the dose [29].

3.1.4. Mechanism of action of CP

First CP is converted by the liver into two chemicals acrolein and phosphoramidate. Acrolein and phosphoramidates are the active compounds that prevent cell division by cross-linking DNA strands and decreasing DNA synthesis. These drugs can't distinguish between normal and cancerous cells. CP also possesses potent immunosuppressive activity [30].

3.1.5. Cyclophosphamide use:

CP has been widely in different disease treatments:

- Breast cancer [31-33]
- Ovarian cancer [34]
- Prostate cancer [35-38]
- Lung cancer [39,40]
- Lupus erythematosus [41,42]
- Rheumatoid arthritis [43,44]
- Multiple Sclerosis [45,46]
- Idiopathic pulmonary fibrosis [47-48]
- Wegener granulomatosis [49-51]
- Hodgkin's Lymphoma [52-54]
- Thrombocytopenic purpura [55-58]
- Polyarteritis nodosa [59]
- Anemia [60,61]

3.2. Solid Phase Extraction (SPE):

SPE is one of the techniques available to an analyst to bridge the gap that exists between the sample collection and analysis step. In SPE, extraction is performed by absorbing the analyte from the matrix onto a solid sorbent. The solid phase is then separated from the solution and other solvents added. The first solvent is usually a wash to remove possible adsorbed matrix components; eventually an eluting solvent is brought into contact with sorbent to selectively desorb the analyte.

Today SPE is the most popular sample preparation method. SPE is choice of method when handling biological samples for liquid chromatography (LC), a sample is mandatory to limit column clogging, presence of co-eluting substances as well as matrix effects frequently encounter with mass spectrometric detection. SPE allow good sample clean up.

It is a very active area in the field of separation science [62-65]. Liquid-liquid extraction (LLE) has remained the preferred technique for the preparation of the liquid samples for several years especially in the environmental field. LLE is labor intensive, difficult to automate, and is frequently plagued by practical problems, such as emulsion formation. In addition, it consumes relatively large volumes of high-purity solvents with expensive disposal requirements [66]. The increased development of SPE has occurred during the past five or six years; with many improvements in formats, automation and introduction of new phases. One reason was the pressure to decrease organic solvent usage in laboratories which has encouraged the requirement for solvent free procedures and has greatly contributed to the growth of SPE at the expense of LLE procedure [67]. Other reasons for the growing interest in SPE techniques are the large choice of sorbents with the capability for new ones of trapping analytes, shorter processing time and simpler procedure [68].

The general procedure of SPE is to load a solution onto the SPE phase, wash away undesired components and then wash off the desired analytes with another solvent into a collection tube.

3.3. Literature review

Literature review concerning CP extraction and analyses has been summarized in table 2.

Table 2 Literature review presenting different methods of extraction and analysis of CP.

Drugs	Matrix	Sample Preparation	HPLC Parameter	Mobile phase	MS	Reference
CP	Urine	Liquid-Liquid	Hypersil BDS C ₈ 150 × 4.6 mm, 5 μm	Solvent A: Methanol Solvent B: 0.02 M ammonium acetate, pH 4.5	Triple quadrupole mass spectrometer	[69]
CP and metabolites (DCL-CP, 4KetoCP, CarboxyCP)		-	Beta Basic C ₈ 100 × 3 mm, 5 μm	Solvent A: Water with 0.1% formic acid Solvent B: Methanol	MS/MS	[70]
CP and other drugs (IF, Doxorubicin, Epirubicin Daunorubicin)		SPE	Hypersil BDS C ₈ 150 × 4.6 mm, 5 μm	Solvent A: Water + 0.1 % formic acid Solvent B: Acetonitrile +0.1 % formic acid	Triple quadrupole mass spectrometer	[12]
Metotrexate CP		SPE	Max RP Capillary column 0.5 × 50 mm, 4 μm	Solvent A: 20 mM ammonium acetate, pH 4 with acetic acid Solvent B: Methanol	Triple quadrupole mass spectrometer	[71]
CP		LLE	GC- Methyl siloxen capillary column 30 m × 0.25 mm, film thickness 0.25 μm	-	GC-MS	[72]
CP	Urine Plasma	Centrifuged the sample for 5 min.	Genesis C18 50 × 2.1mm, 4 μm	Solvent A: 0.5 % acetic acid Solvent B: Methanol with 0.5 % acetic acid	Triple quadrupole mass spectrometer	[73]

CP and other drugs (Doxorubicin, Doxorubicinol)	Plasma	SPE	Symmetry C ₁₈ 30 × 2.1 mm	Solvent A: 5 mM acetate buffer, pH 3.5 Solvent B: Methanol	Triple quadrupole mass spectrometer	[74]
CP and metabolites (4-OHCP)		Centrifused for 15 min.	Zorbax Extend C18 analytical column 150 × 2.1 mm, 5 μm	Solvent A: 1 mM ammonium hydroxide in water , pH 10 Solvent B: Acetonitrile	Triple quadrupole mass spectrometer	[75]
CP and metabolites (4- OHCP, CEPM)	Plasma	Centrifused for 5 min.	Zorbax Extend 50 × 2.1 mm, 5 μm	Solvent A: Water: methanol Solvent B: 10 mM ammonium acetate pH 8.5	Single quadrupole	[86]
CP and metabolites (4KetoCP CarboxyCP 3- Dechloroethylifosfamide)		SPE	Ultrasep ES 100 Pharm RP18 (125 × 2 mm, 5 μm	Solvent A: Methanol Solvent B: 20 mM ammonium acetate with 1 % acetic acid.	Single quadrupole	[77]
CP and metabolites (4KetoCP CEPM 4-OHCP DCCP)		Centrifused	Zorbax SB-C8 15cm × 4.6 mm	Solvent A: Methanol Solvent B: 10 mM ammonium acetate ,pH 4	Micromass Quattro II tandem quadrupole mass spectrometer	[78]
CP and other drug IF		SPE LLE	Supelcosil LC-18 150 × 4.6 mm, 5 μm	Solvent A 10 mM phosphate buffer pH6 Solvent B Acetonitrile (75:25)	-	[79]

CP and other drug IF	Urine	Liquid- Liquid extraction	Hypersil C8 BDS 15 cm × 4.6 mm, 5 μm	Solvent A: Methanol Solvent B: 0.02 M acetate buffer pH 4.0	Triple quadrupole mass spectrometer	[83]
CP		-	GC	-	GC-MS/MS	[81]
CP	Wipe samples	-	GC	-	GC-MS/MS	[82]
CP and other drugs Methotrexate 5-Fluorouracil	Wipe sample	-	Synergi 4u Max RP capillary column 0.5 × 50 mm, 5μm	Solvent A: 20 mM ammonium acetate pH 4 Solvent B: Methanol	Triple quadrupole mass spectrometer	[76]
CP and other drugs Taxol Gemcitabine		SPE			Solvent A: 0.1 % HCOOH in distilled water/ acetonitrile (80:20v/v) Solvent B: 0.1 % HCOOH in distilled water/ACN (30:70 v/v)	Triple quadrupole mass spectrometer
CP and metabolite (OH-CP)	Mouse plasma and tissue	Centrifused for 5 min.	YMC C18 basic column 50 × 2 mm, 5μm	Solvent A: ACN Solvent B: 0.1 % formic acid	LC-MS/MS	[85]
CP	Water	Lyophilization extraction	Nucleosil C ₁₈ 0.2 × 10 cm, 3 μm	Solvent A: Ammonium formate buffer pH 5.7 Solvent B Methanol	MS/MS	[14]

4. EXPERIMENTAL PART

4.1. Chemicals and reagents

- Ammonia (POCH, Poland)
- Cyclophosphamide (Baxter, Poland)
- Deionized water (0.22 μm , Hydro lab HLP 5, Poland)
- Dichloromethane (POCH, Poland)
- Ethyl acetate (POCH, Poland)
- Formic acid 80 % (POCH, Poland)
- Hydrochloric acid (POCH, Poland)
- Methanol (J.T. Baker, Holland) HPLC grade, LC-MS grade, HPLC gradient grade
- Nitrogen gas (Oxygen S.C., Poland)
- Potassium dihydrogen phosphate (KH_2PO_4) (POCH, Poland)
- Potassium hydrogen phosphate (K_2HPO_4) (POCH, Poland)
- Sodium hydroxide (POCH, Poland)

4.2. Apparatus and Laboratory equipment

- Disposable pipette tips (Eppendorf, Germany)
- Laboratory glassware (volumetric flask, measuring cylinder, beaker)
- Microliter Syringe 10 μL , 50 μL (Hamilton, USA)
- Magnetic stirrer
- pH meter Basic 20+ (Crison, Spain)
- Pipettes model (P10, P100, P2500) (Eppendorf, Germany)
- 4000 Q Trap mass spectrometer (Applied Biosystems MDS SCIEX)
- Refrigerator (Siemens, USA)
- Sonicator (Labart Sp. z o.o, Poland)
- Series 1200 HPLC (Agilent Technologies, Palo Alto, CA, USA)
- Syringe filter device
- Turbo Vap LV Evaporator (Caliper Life Science, USA)
- Ultra low temperature freezer (New Brunswick, USA)
- Visiprep SPE vacuum manifold (Sigma- aldrich, USA)

- Weighing balance (Mettler Toledo, USA)
- SPE Cartridges from different manufacturers are summarized in Table 3.

Table 3 Cartridges applied for SPE during CP isolation from urine sample

Sorbent	Manufacturer	Mass/ Volume (mg/mL)	Porosity (Å)	Particle diameter (μm)	End- capping	% Carbon
Chromabond easy	Machery–Nagel	200/6	50	80	n.a	n.a
Chromabond C ₁₈ ec	Machery–Nagel	1000/6	60	45	Yes	14 %
Lichrolut RP-select B	Merck	200/3	n.a	40-63	n.a	n.a
Octadecyl	J.T. Baker	500/3	47-60	n.a	Yes	17-18
Oasis HLB	Waters	150/6	80	30	n.a	n.a
Strata- X	Phenomene	200/3	85	33	n.a	n.a
Zorbax C18	Agilent	200/3	80	50	No	11

4.3. Analytical procedure

4.3.1. Chromatographic conditions

HPLC analysis was performed by injection volume 50 μL of the sample onto the Lichrospher® 100 RP-18 column (125 \times 4 mm) with particle size of 5 μm (Merck, Germany). The column outlet was coupled with to an ESI 4000 Q Trap mass spectrometer (Applied Biosystems MDS SCIEX) equipped with an electrospray ionization.

The flow rate was 1000 $\mu\text{L}/\text{min}$. The mobile phase, under gradient conditions, was as follows: mobile phase A, methanol; mobile phase B, 10 mM ammonia and formic acid buffer adjusted to pH 3; time program, 0, min 20 % A/80 % B; 7 min, 80% A/20% B; 9 min, 80% A/20% B; 10 min, 20% A80% B and this mobile phase composition was maintained for 4 minutes. Total run time was 15 min.

4.3.2. Mass spectrometry parameters

MS/MS analysis was performed on the 4000 Q Trap triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in positive ion mode. Analyst software (Applied Biosystems/ MDS Sciex, Analyst software version 1.5) was used for the instrument control and data collection. The instrument was operated in multiple reaction monitoring (MRM) mode and the following ion transition (Precursor \rightarrow product) were monitored: m/z 261.00 \rightarrow 140.00. The electrospray voltage

was 5000 V and dwell time was 200 msec. Curtain gas (CUR) 40 psi; collision energy (CE) 31 V; declustering potential (DP) 80 V; collision cell exist potentials (CEX) 22 V were used for the analysis of CP.

4.3.3. Preparation of stock and working standard solutions

Stock solution was prepared by dissolving 25.0 mg of pure CP drug in 25.0 mL of methanol to give concentration of 1.0 g/L. Stock solution was stored at refrigerator (4°C). Working standard solution was prepared weekly by diluting 10 μ L of stock solution in 10 ml of volumetric flask using mixture MeOH: Water (1:1) as a solvent, it was kept at 4°C. Final concentration of CP was 1 μ g/mL.

4.3.4. Urine collection:

Urine samples from fifteen health workers from Medical university of Gdansk who handle CP were collected in 100 mL of bottles. Two blank samples were collected from six year old boy and 26 year old girl. Table 4 shows the characteristics of health workers. Collected urine samples were labeled and stores at – 80°C until sample preparation. Sample preparation was done after bringing the urine sample in ambient temperature. No drug spillage or accidents were reported in connection with the urine sampling.

Table 4: Characteristics of health workers

Person	Sex	Person	Sex
1	F	8	F
2	F	9	F
3	F	10	F
4	F	11	F
5	F	12	F
6	F	13	F
7	F	14	F
		15	M

4.3.5 Comparison of LC-MS and LC-MS/MS in term of sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) were determined by analyzing blank urine spiked with working standard solution. LOQ was determined by comparing the measured signals of the samples with known low concentrations of analytes to those of blank samples, thereby establishing the minimum amount of compounds that could be reliably quantified with signal to noise ratio 10:1. LOD and LOQ were, respectively, defined as three and ten times the standard deviation of the LC-MS/MS and LC-MS peak areas detected at the retention times of the analytes of interest.

Table 5 Sensitivity comparison of LC-MS and LC-MS/MS

Linearity	LC-MS	LC-MS/MS
LOD	9.36 $\mu\text{g/mL}$	0.27 ng/mL
LOQ	93.6 $\mu\text{g/mL}$	0.54 ng/mL

For CP detection limits, 1000 times lower sensitivity with LC-MS operating in the SIM mode than with the triple quadrupole were achieved. In terms of sensitivity and selectivity LC-MS/MS is markedly superior.

4.3.6. SPE extraction

The most important parameters in SPE are the selection of the type and amount of the sorbent, the determination of the sample volume that can be applied without loss in recovery, the composition and volume of the washing solution that can be applied without loss of the analytes, and finally the composition and volume of the elution.

For optimizing SPE methods different parameters were changed such as the cartridge stationary adsorbent, pH, solvent used for cleaning, solvent used for elution and elution volume. SPE methods were carried out using different cartridge such as Octadecyl from J.T. Baker (500 mg/3mL), Chromabond C₁₈ ec from Machery- Nagel (1000 mg/6mL), Strata- X from phenomene (200 mg/3mL), Lichrolut RP- select B from Merck (200 mg/3mL), Oasis HLB from Waters (150 mg/3mL), Zorbax C₁₈ from Agilent (200 mg/mL),

Chromabond Easy from Machery- Nagel (200 mg/mL). Among them recovery values were higher with Oasis HLB and Strata- X. So further analysis were carried out using Strata-X.

Buffer PBS pH 7, buffer pH 3, urine adjusted to pH 3, normal urine were used to see the recoveries. Normal urine has high recovery so analysis was carried out with it.

For cleaning different solvents were used such as water, buffer PBS pH 7, buffer pH 3 and methanol: water (1:1). In order to remove interference from the analyte mixture of methanol: water (1:1) was used.

Whereas for elution dichloromethane, methanol were checked. To see the impact different elution volume (1ml, 2ml, 3ml, 4ml, 5ml, 6ml, 7ml, and 8ml) were used and it was found that 3ml was enough to elute the analyte.

SPE procedure used for CP determination in urine samples has been shown on figure 2.

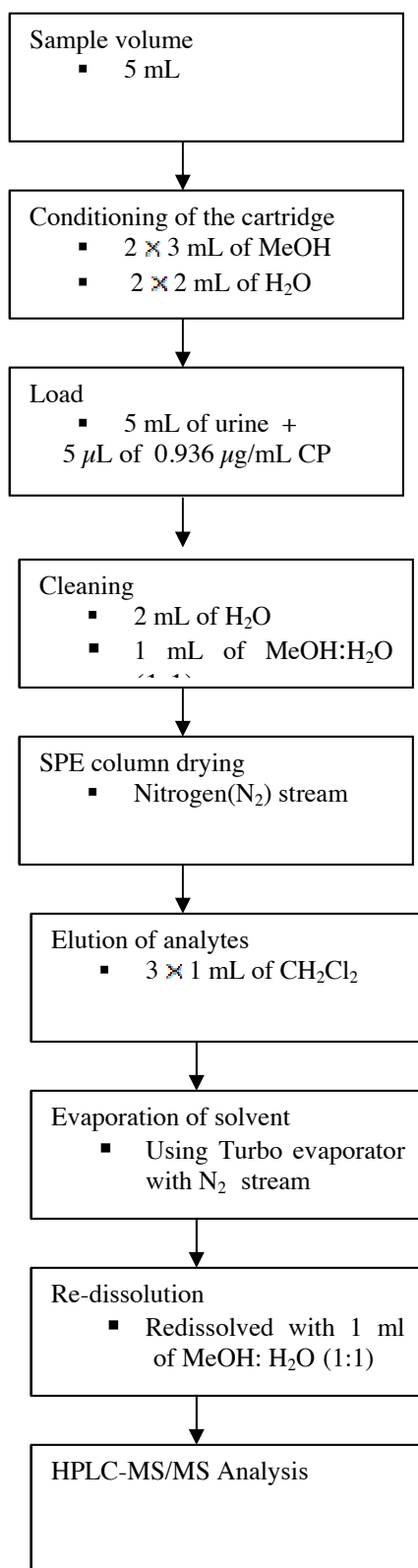


Figure 2 Schematically presentation of SPE procedure

4.3.7. Calibration

Calibration curves were prepared with known concentrations of CP:

- 1) diluted in methanol: water (1:1)
- 2) spiked in 5ml of blank urine

Five calibrations standards were made by adding working solution of CP to 1 mL methanol: water (1:1) and directly analyzed. The CP concentrations of the standard were 0.608, 1.17, 2.34, 4.68, 9.36 ng/mL.

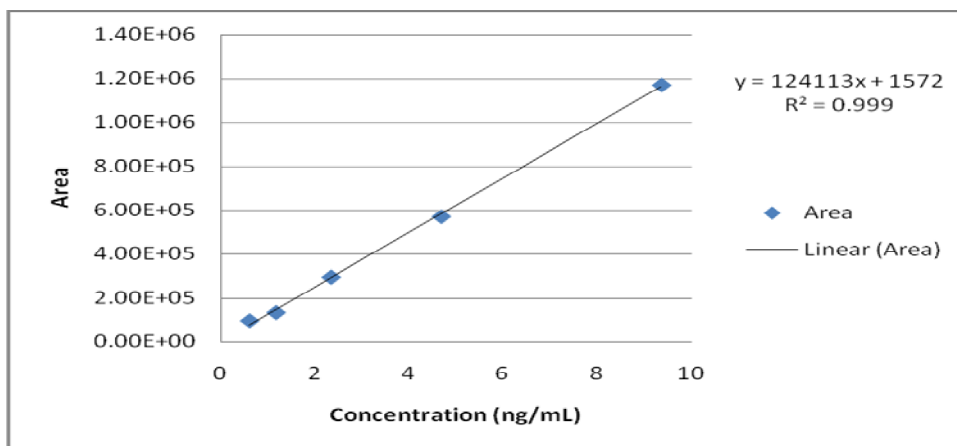


Figure 3 Calibration curve of CP diluted in methanol: water (1:1).

Calibrations urine samples were prepared by spiking human urine samples from unexposed subject (blank urine) with CP, in order to obtain five concentration (0.187, 0.374, 0.936, 1.4, 1.87 ng/mL). Calibration samples were extracted following the above described procedure and analyzed by HPLC-MS/MS.

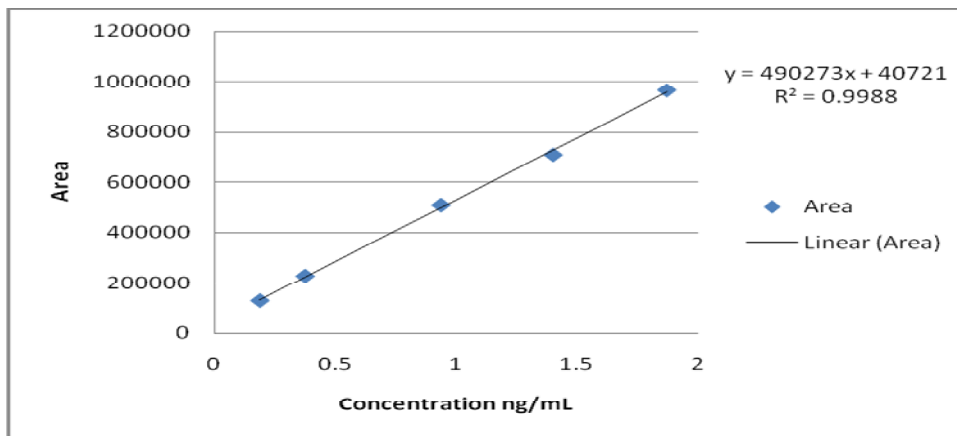


Figure 4 Calibration curve of urine sample spike with CP.

5. RESULTS AND DISCUSSION

5.1. Mass spectrometry

In ESI positive ion mode the compounds yielded abundant $[M+H]^+$ ions. This protonated molecule at m/z 261 for CP was selected as the precursors ions for the MS/MS fragmentation of the analyte. The most abundant fragment was detected at m/z 140 and proposed fragmentation is shown in figure 6.

Cleavage of the nitrogen-phosphorus bond gave fragment ion at m/z 140, resulting in the loss of di(2-chloroethyl) amine. The transition from m/z 261 to 140 was used in MRM analysis. The MRM parameter and precursor- product ion combinations used are summarized in table 6.

Table 6 MRM parameters

Compound	Retention time	Precursor ion	Product ion
CP	6.75 min	261.0	140

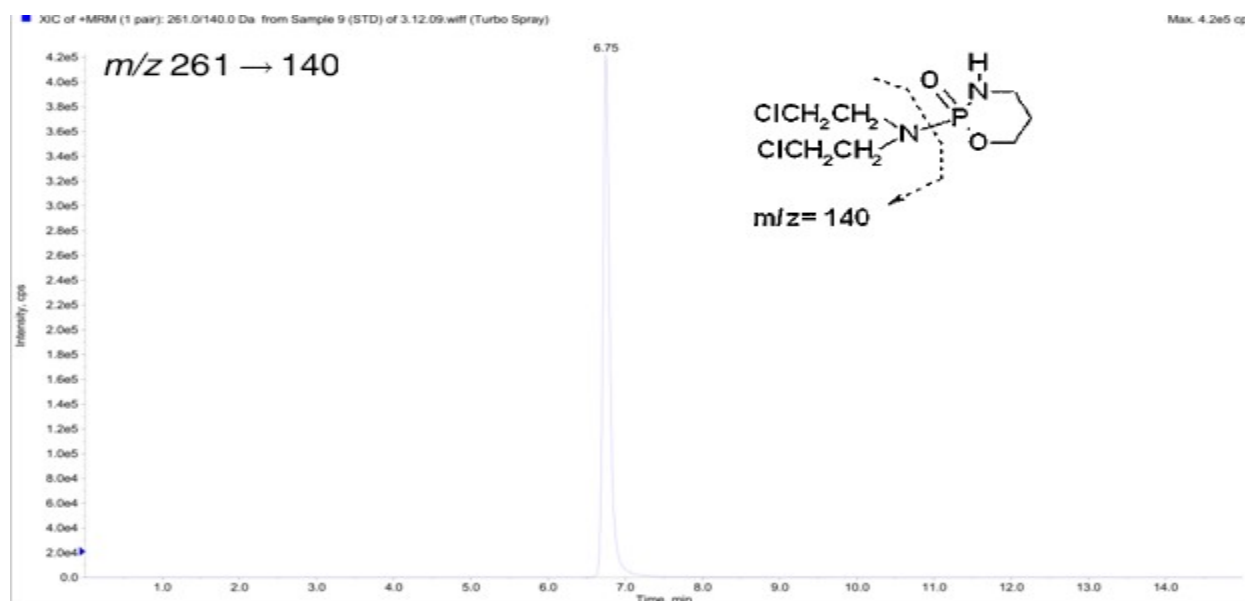
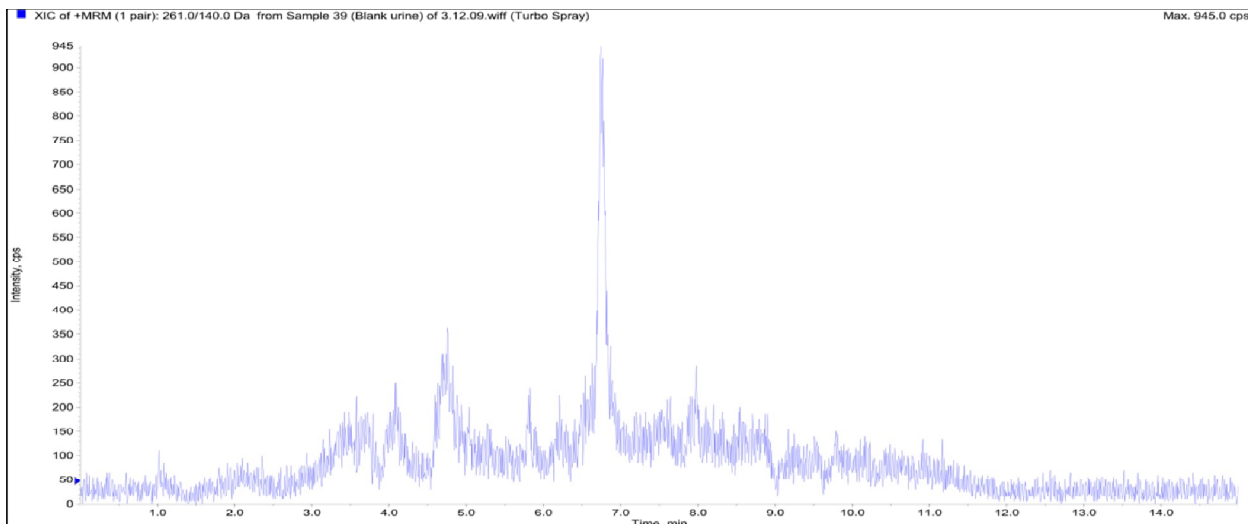
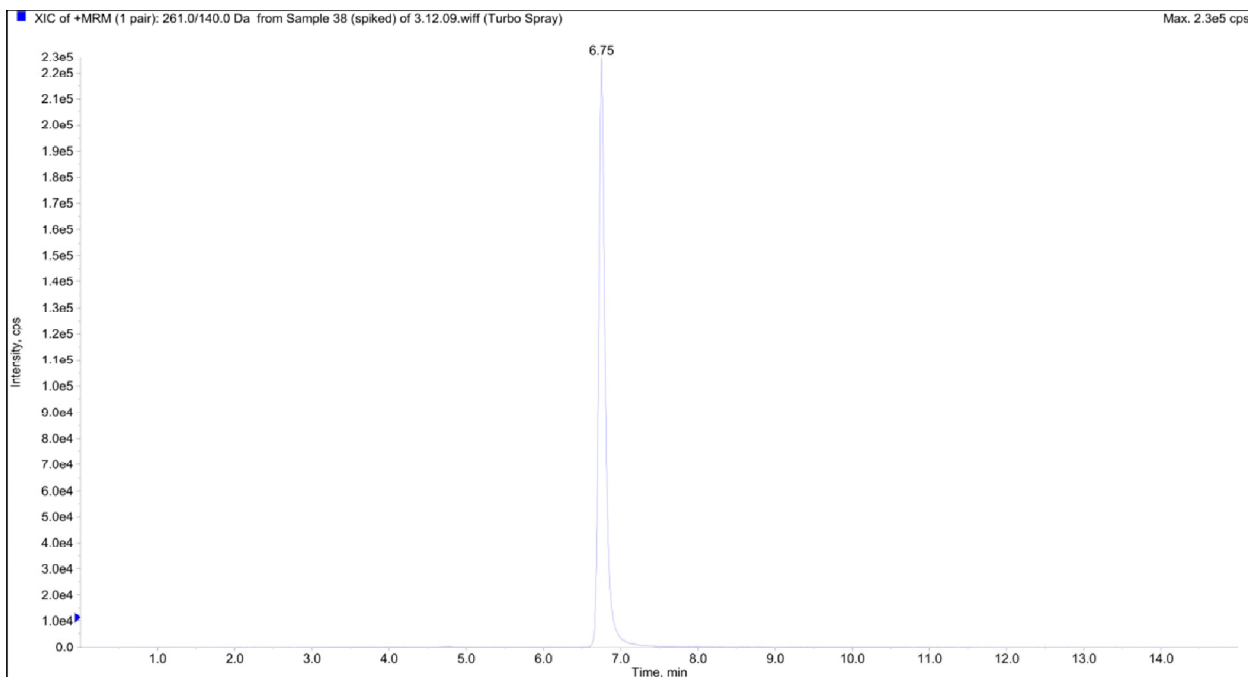


Figure 5 HPLC-MS/MS working in MRM mode obtained for CP standard solution. Proportion of CP fragmentation has been included.

Figure 6 (a) shows result for blank urine sample and (b) shows a typical MRM chromatogram corresponding to a blank urine sample spiked with 4.68 ng/mL of CP. No interfering peaks were presented at the retention time of CP.



a)



b)

Figure 6 The HPLC/MRM chromatograms a) for a blank urine sample and b) for a blank urine sample spiked with 0.936 $\mu\text{g/mL}$ of CP.

5.2. Liquid Chromatography:

The elution gradient and influence of mobile phase pH were studied in order to optimize the analytical performance. A short analytical column and elution gradient with 10 mM formic acid with ammonia buffer pH 3 and methanol were chosen as the best compromise between retention time and ionization of analyte. Under these chromatographic conditions, CP was separated in 15 min in a flow rate 1 mL/min.

5.3. Linearity

Calibration curves showed high linearity over the concentration range 0.187 -1.87 ng/mL with correlation coefficient greater than 0.998.

5.4. Sensitivity

The limits of detection (LOD) and LOQ were determined by analyzing blank urine samples spiked with the working standard CP. LOD and LOQ were found to be 0.27 ng/mL and 0.54 ng/mL. These values allowed the analysis of CP in human urine sample at trace level.

5.5 Carry over

To determine the carry over, blank samples were injected after a sample. No carry over from analytes were seen in blank sample.

5.6 Recovery

The recovery was determined using urine sample spiked with CP (5 μ L of 0.936 μ g/mL in 5 mL urine). Final concentration in urine was 4.68 μ g/mL. The obtained peak areas of the spiked urine standards at these levels after sample preparation were compared with the peak areas resulting from aqueous standard solutions at the same concentrations without sample preparation.

5.7. Influence of solvent used to prepare CP standard solution:

CP at concentration 4.68 ng/ml was prepared in five different solvent:

- a) Methanol
- b) Methanol: Water (1:1) mixture
- c) Urine: Water (pH=3) mixture (1:1)
- d) Urine: Buffer (pH=3) mixture (1:1)
- e) Buffer pH=3

Data obtained from CP diluted in MeOH: Water (1:1) mixture have been treated as 100 %, therefore CP diluted in all the other solutions gives different recoveries, as shown in figure 7.

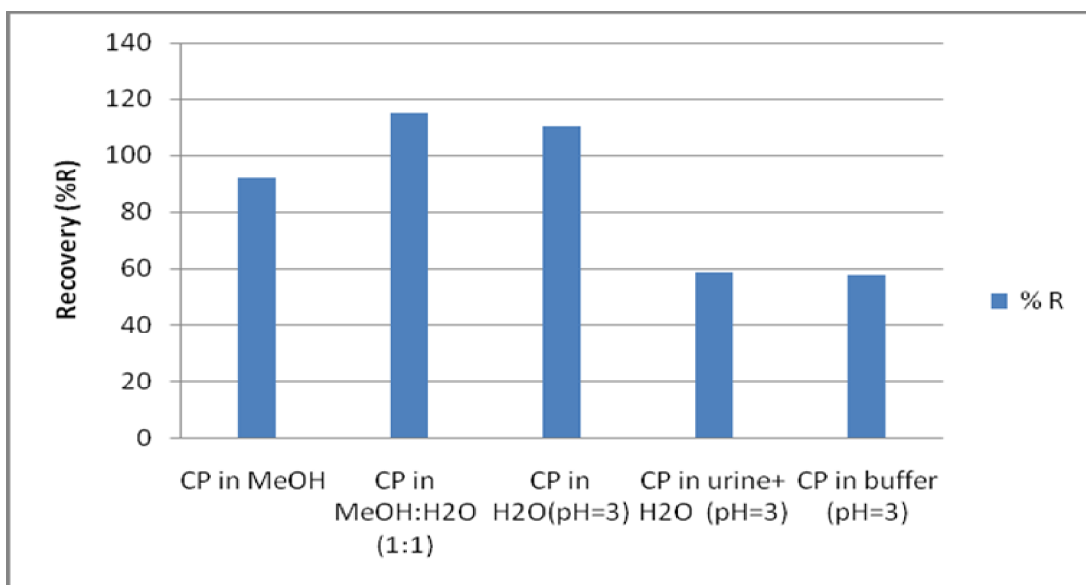


Figure 7 Influence of solvent used to prepare CP standard solution.

Analysis of these CP solutions gave different chromatograms, as it has been presented in figure 8. It was found that methanol: water (1:1) mixture give highest peak area whereas those dilutions of CP with urine sample show very low recovery. Also baseline and peak shape of CP are not good probably due to presence of interferences.

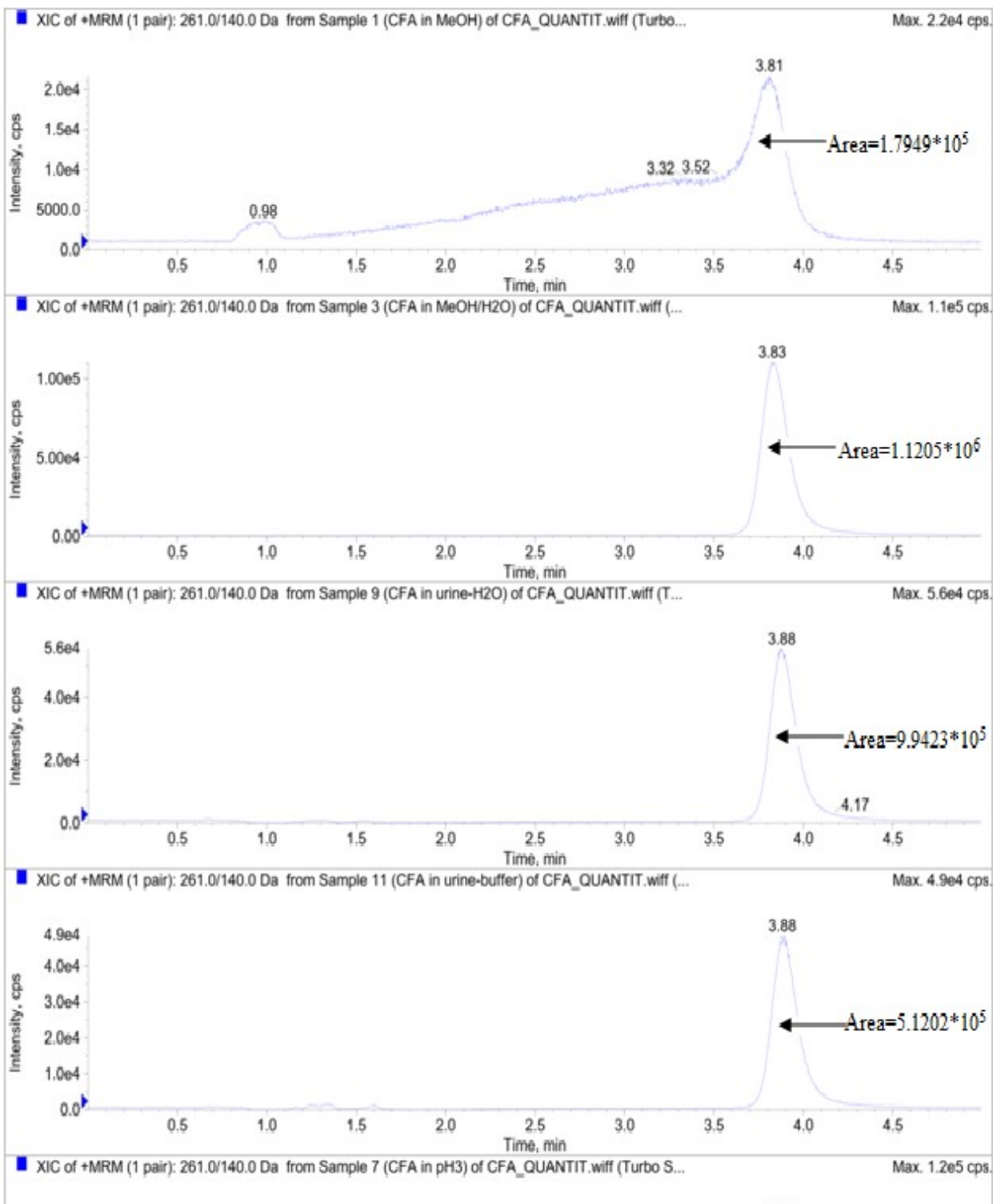


Figure 8 CP diluted in a) methanol b) methanol: water (1:1) c) mixture urine: water (1:1) pH=3 d) urine: buffer pH=3 mixture e) buffer pH=3.

5.8. Matrix effects

Urine is a complex biological material, which contains urea, creatinine, uric acid, hormones, pigments and mucins. Some of them can influence extraction process using SPE (deactivation of active points at the surface of sorbent, sorption, blocking of flow channels). Therefore, the way the urine sample is treated before sorption in SPE can be crucial for recovery of CP. Urine samples, after spiking with CP were prepared in four different ways:

- 5 mL urine + 2 mL PBS buffer (pH=7)
- 5 mL urine + 2 mL buffer (pH=3)
- 5 mL urine acidified with ammonia to pH=3.
- Urine

CP was isolated from samples using SPE and recovery of CP has been shown in figure 9.

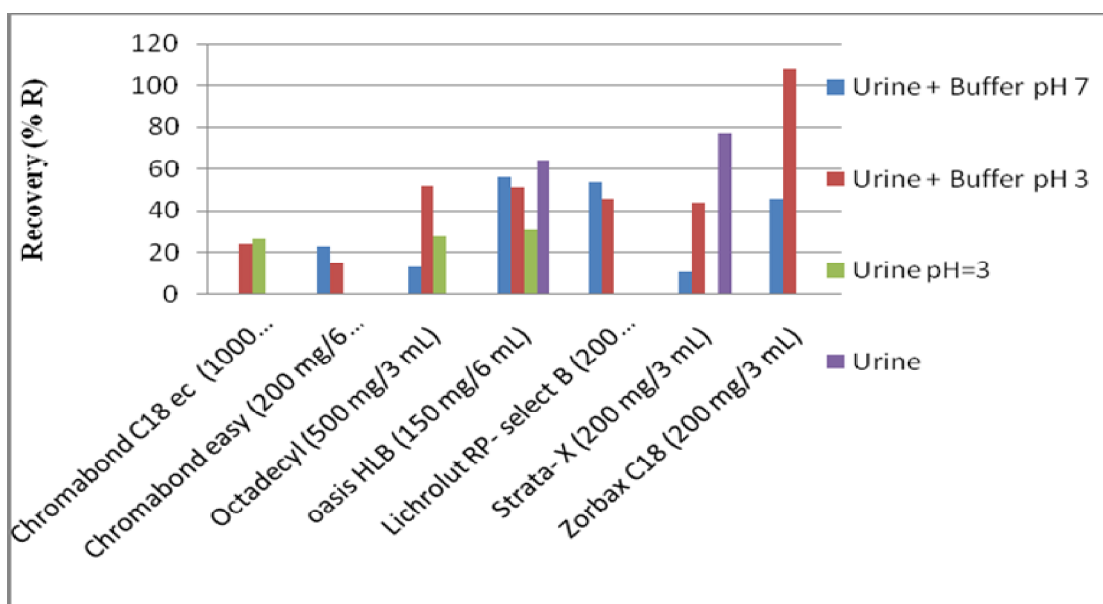


Figure 9 Influence of sample preparation of urine before SPE on recovery of CP.

No significant difference has been observed between urine samples prepared before SPE in different way, especially in case of Oasis HLB, Lichrolut RP-select B, Chromabond C18 ec and Chromabond Easy sorbent. For SPE, urine sample without pretreatment were used for carrying the experiment.

5.9. Influence of time of evaporation on recovery

A set of six different CP solutions in MeOH has been prepared. The content of CP was constant (4.68 ng/mL) but amount of MeOH was different (1 mL, 2 mL, 3mL). Solvent was removed using Turbo evaporator. Temperature was set on 40° C and gas pressure was 4 psi. Differences in solvent amount should result in differences in evaporation time. Dry residue was re-dissolved in the mobile phase (mixture MeOH: water 1:1, v/v) and analyzed. Results, in the form of recovery calculated according to CP standard solution without evaporation treated as 100 % are shown in Table 7.

Table 7 Influence of time of evaporation on recovery

Time required for sample evaporation	Recovery % (R1)	Recovery % (R2)
Approx. 20 min. (MeOH 1 mL)	91	93
Approx. 40 min. (MeOH 2 mL)	89	93
Approx. 60 min (MeOH 3 mL)	94	137

Sample having only 1 ml of methanol evaporates faster than sample containing 2 ml and 3 ml of solvent. Each 1 mL of MeOH needs approximately 20 minutes for complete evaporation. From table 7 we can conclude that there is no influence of time of evaporation on CP recovery. Recovery ranged from 89 to 94 % except in one sample where recovery is 137 %, this is due to human error.

5.10. Sorbent types

To check best sorbent seven different type of cartridge such as J.T baker (Octadecyl), Chromabond C₁₈ (Octadecyl), Strata-X from Phenomena (Polymeric surface modified styrene-divinylobenzene), Merck Lichrolut RP-select, Agilent Zorbax C18 (monomeric bonding, carbon loading 11 %), Waters Oasis HLB cartridge (hydrophilic-lipophilic balance sorbent reversed phase sorbent polymeric water- wettable reversed phase sorbent), Chromabond Easy (polar modified polystyrene- divinylbenzene copolymer with weak anion exchanger) were used.

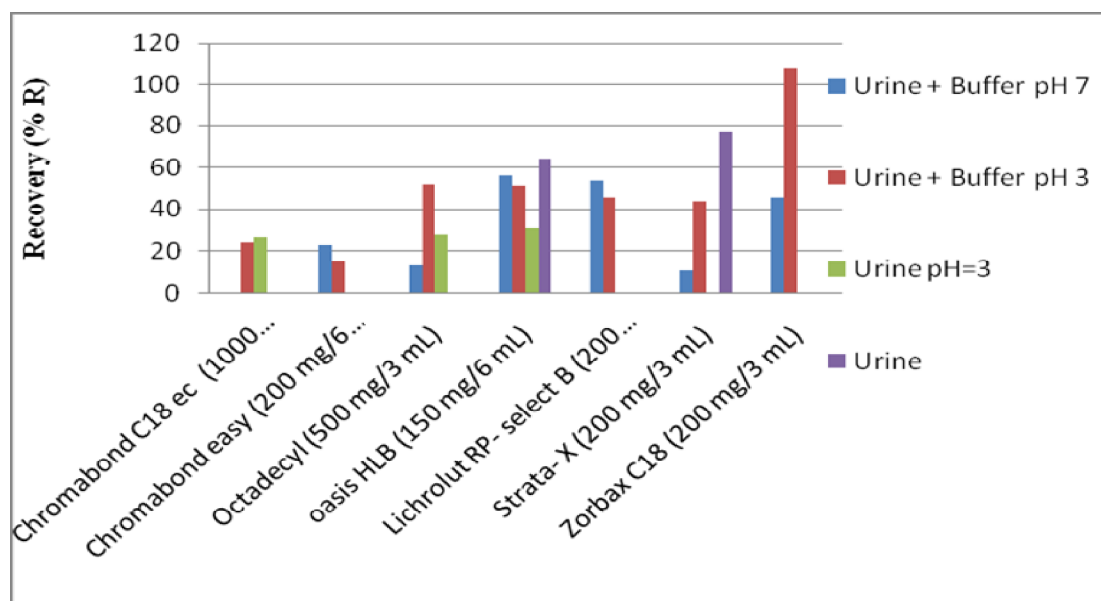


Figure 10 Influence of sorbent on CP recovery

Oasis, Strata, Merck and Zorbax give high recovery. So, analyses were carried out using Strata-X.

5.11. Influence of different eluent solvent on recovery:

Methanol, dichloromethane, methanol: water (1:1), methanol and ethyl acetate solvents were used for eluting the analyte in order to find suitable elution solvent. Elution was performed by adding 3 mL aliquots of different eluting solvent as mentioned above except methanol: water (1:1) mixture, passing them through under vacuum and collecting them separately, dried and analyzed. Whereas in case of methanol: water (1:1) elution was performed by using 1 ml and analyzed directly.

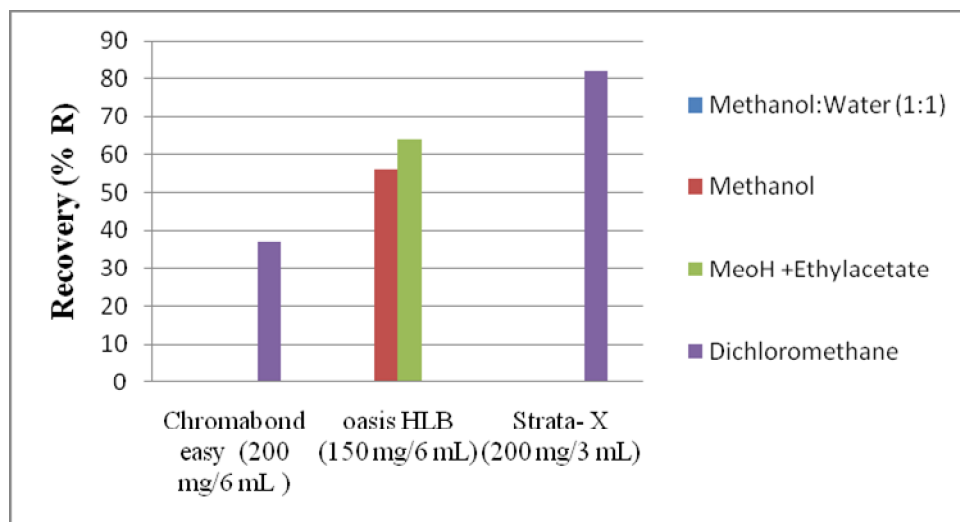


Figure 11 Influence of different eluent solvent on CP recovery.

Using methanol: water (1:1) solvent, analyte was not eluted. Whereas methanol, dichloromethane, methanol and ethyl acetate were good solvent for eluting the CP from cartridge. Among three, dichloromethane show high recovery of CP in Strata-X cartridge. Thus, dichloromethane was found suitable eluting solvent for CP analyte.

5.12. Stability study of CP solutions

In order to observe the degradation process of CP in respect to time extract from urine samples were kept at ambient temperature and after the degradation process started they were moved to refrigerator at 4 °C. All extracts and CP standard solution were dissolved in the mixture MeOH: H₂O (1:1) and were kept in transparent vials. All the samples were analyzed after every three days and recoveries were calculated (Table 8) according to results obtained the very first day of analysis (100 %).

Table 8 Degradation of CP in time

Sorbent used	Recovery [%]			
	Day 1	Day 3	Day 6	Day 9
Extract from urine sample with buffer pH=3				
Extract from Chromabond C18 ec	100	71	46	
Extract from Chromabond easy	100	73	60	53
Extract from Octadecyl	100	50	37	33
Extract from oasis HLB	100	51	37	29
Extract from Lichrolut RP- select	100	48	37	33
Extract from Strata- X	100	48	36	32
Extract from Zorbax C18	100	38	29	26
Extract from urine sample with PBS buffer pH=7				
Extract from Chromabond C18 ec	100	92	77	69
Extract from Chromabond easy	100	78	70	59
Extract from oasis HLB	100	54	43	38
Extract from Lichrolut RP- select B	100	-	-	-
Extract from Strata- X	100	50	39	35
Extract from Zorbax C18	100	59	48	43

Recovery of CP was found almost half reduced after keeping the extract at ambient temperature which represented in Figure 13 and 14. These results indicated an approximately deterioration of 50 percent in day 3 and approximately 67 percent over day 9. This information supports that both standard and samples should be analyzed as soon as possible after the samples are collected to avoid error due to loss of potency. Depending on the environmental conditions in which organic compounds are present, they can undergo changes resulting from chemical, biological or photochemical reactions. Photo degradation and biodegradation are major

degradation processes which can naturally happen. Photo degradation, as a chemical reaction that occurs under the influence of photons or light, could take place and cause decrease of CP content in the extract. We believe that bacteria presented in urine samples were removed during SPE. However, we do not have scientific proof because it was not the subject of the investigations.

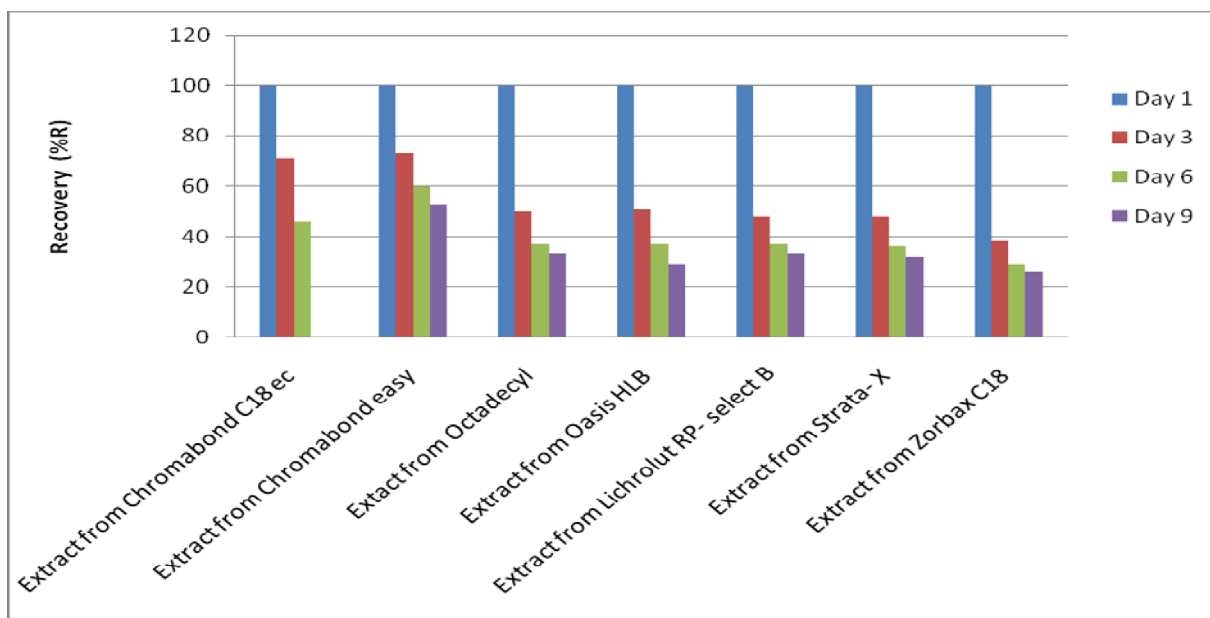


Figure 12 Stability study of extract urine (urine samples diluted with buffer pH=3).

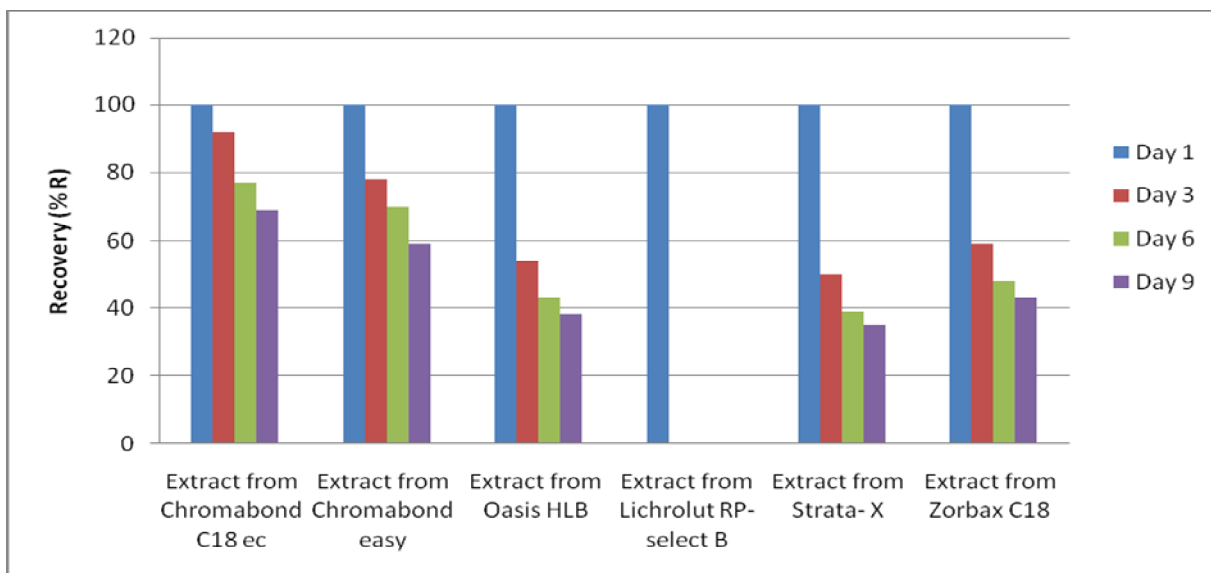


Figure 13 Stability study of extract urine (urine samples diluted with PBS buffer pH=7)

6. APPLICATION OF THE METHOD

To assess the feasibility and effectiveness of this SPE- LC-MS/MS method, urine samples of health workers were collected and analyzed. Fifteen samples were collected and store at $-80\text{ }^{\circ}\text{C}$ until analysis. Two blank samples were collected from six year boy and twenty six year old girl. Spiked sample was prepared and analyze. Chromatogram is shown below (Figure 14). The CP results were below the analytical detection limits in all samples.

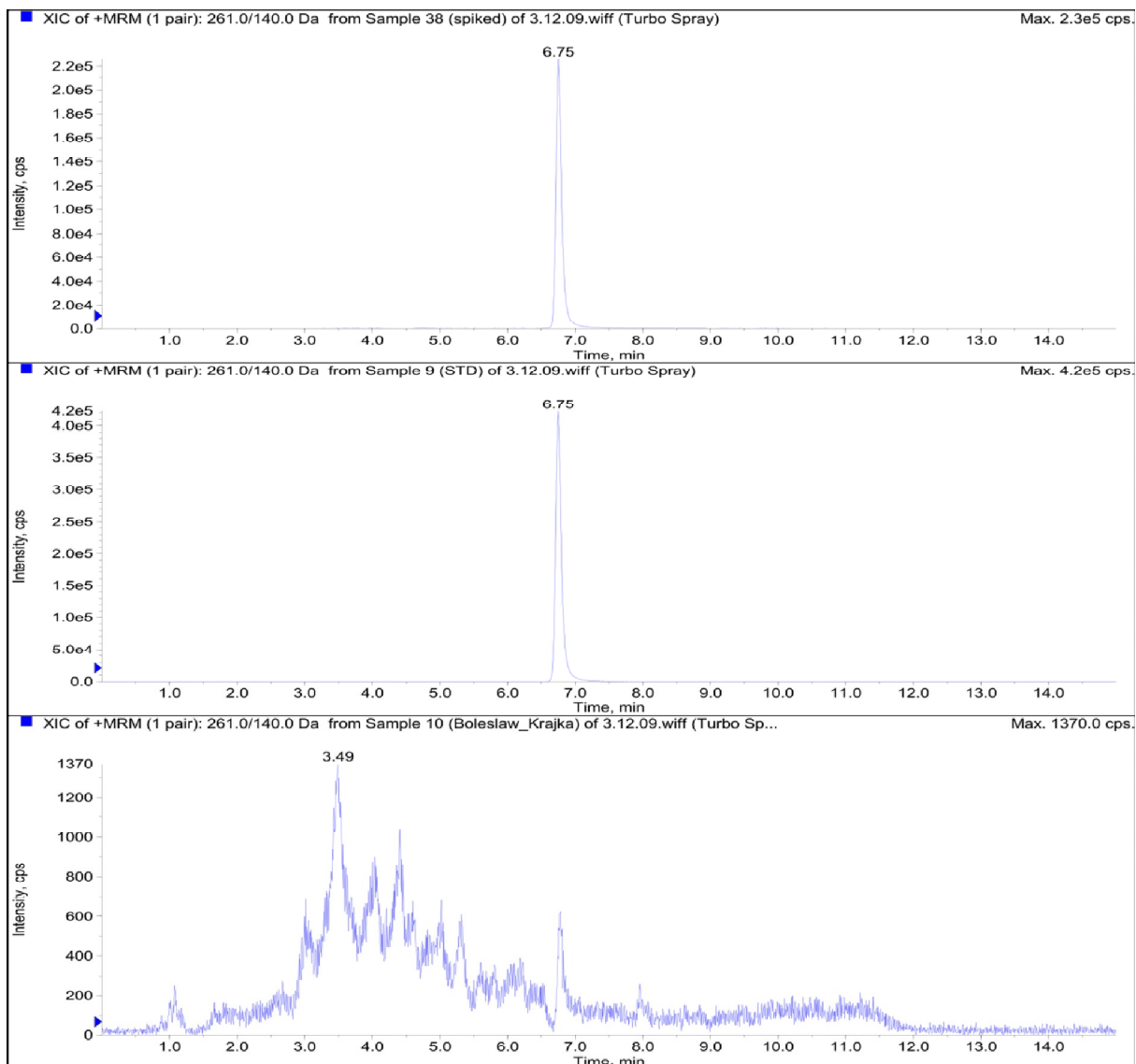


Figure 14 Chromatogram of a) spiked sample b) standard c) health worker.

7. CONCLUSION

According to IARC, CP is human genotoxic carcinogen [1, 11]. Several reports have discussed exposure to CP during occupational activities and concluded that it should be avoided because any detectable level is considered to be hazardous. In order to obtain accurate measurements of CP in urine samples SPE methods was optimized. Optimization of SPE method was carried out by changing different parameter such as different type and amount of sorbent, composition and volume of elution, washing solvent. SPE was effective with no interfering peaks from matrix compound and recovery was above 75%.

After optimizing SPE method, CP separation conditions of the HPLC-MS/MS analysis was achieve by changing different parameter such as suitable mobile phase and its composition, flow rate, scan type, polarity. LC/MS/MS detection was performed using triple quadrupole mass spectrometer working in MRM mode. The samples were prepared using SPE and analyzed using a gradient separation. Total analysis time was 15 minute. Calibration curve were made to see the instrument performance and SPE procedures which show good linearity.

A sensitive, specific and accurate HPLC/MS/MS has been developed for the analysis of CP in human urine. This SPE-LC-MS/MS method was applied for the analysis of urine samples of health workers. Fifteen samples urine was collected from Medical University of Gdansk, who were exposed with CP during their work in pharmacy. Two blank samples were collected from six year boy and twenty six year girl. In all sample CP was found below LOD.

The stability of the CP was studied. Analyses were conducted on the day prepared (day 1) and days 3, 6 and 9. These results indicated an approximately deterioration of 50 percent in day 3 and approximately 67 percent over day 9. This information supports that both standard and samples should be analyzed as soon as possible after the samples are collected to avoid error due to loss of potency.

8. SUMMARY

Biological samples urine are much more complex due to presence of proteins, salts, acids, bases and various organic compounds with similar chemistry to analytes of interest. As a result, the extraction methods for biological samples have been difficult. The main task was to remove maximum interfering substance from urine matrix and conversion of analytes into a more suitable form for injection. Therefore, optimization of SPE method was done by changing different parameter such as different type and amount of sorbent, composition and volume of elution, washing solvent. The use of 24 well SPE plate for matrix purification significantly allows the use of small urine and solvent volumes, reduces the sample preparation time and ensures a high throughput, thus allowing the routine biological monitoring of CP as indices of human exposures. In order to investigate the effectiveness of the SPE method tests were repeated on Strata-X cartridge and method showed good recovery (above 75 %) of CP.

After optimizing SPE method, CP separation conditions of the HPLC-MS/MS analysis was achieved by changing different parameter such as suitable mobile phase and its composition, flow rate, scan type, polarity. The LC separation was performed on Lichrocart® 100 RP-18 column (125 × 4 mm, particle size of 5 μm) with 1 mM formic acid with ammonia buffer pH 3 and methanol using gradient program at a flow rate 1 mL/min with total run time 15 min. A reversed phase HPLC system was interfaced with an ESI source coupled to tandem mass spectrometry. The triple quadrupole mass spectrometer was operated in positive ion mode and MRM was used for analysis of CP. LOD and LOQ were 0.27 ng/mL and 0.54 ng/mL. A sensitive, specific and accurate HPLC/MS/MS has been developed for monitoring CP in urine samples. The use of reversed phase HPLC coupled to tandem mass spectrometry has facilitated the analysis of CP in short retention time.

Fifteen urine samples were collected from medical personnel of Medical University of Gdansk, who were exposed with CP during their work in pharmacy. SPE-HPLC-MS/MS method was applied for the analysis of health workers and all urine samples from university workers has CP below analytical limit of detection.

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