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Analytical and Pharmacological Studies of Cyclophosphamide

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ABSTRACT

Pharmacokinetic and pharmacodynamic studies are an essential in order to achieve better treatment efficacy and less drug related toxicity. The kinetic studies depend on measuring the drug concentrations in body fluids. Analytical methods, sampling methods and sample preparation are important factors that affect the drug assay and hence the drug concentrations. This subsequently may alter the accuracy of pharmacokinetic and pharmacodynamic investigations. Cyclophosphamide (CPA) is an anticancer drug, which is widely used for the treatment of hematological malignancies as well as solid tumors. It is a prodrug that undergoes complicated process of metabolic activation and inactivation in the liver. Many bioanalytical assays for the quantification of CPA and its metabolites have been developed over the past decade. However, these methods have several limitations including long analysis time, sample preparation and large sample volume.

The aim of the present studies was to develop a new sensitive analytical method for cyclophosphamide and subsequently to use this method for evaluation of new sampling's technique in mice for kinetic studies of cyclophosphamide in mice.

In study I, a simple and rapid method for the determination of CPA utilizing on line sample preparation method, microextraction in packed syringe (MEPS), was developed. The quantification of CPA was carried out using liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS). The method was validated for linearity, selectivity, accuracy, precision and limits of detection and quantification. One hundred and seventy plasma samples from patients treated with CPA were analyzed with the new method and compared with the results obtained after liquid-liquid extraction (LLE) and analyzed using liquid chromatography with UV detection (LC-UV). The limit of detection (LOD) was 0.005 $\mu\text{g/mL}$ and the limit of quantification was 0.5 $\mu\text{g/mL}$ for LC-MS/MS. The accuracy of the quality control (QC) samples ranged from 91 to 106 %. The inter-day variation was within the range 5-9%, while the intra-day variation was between 1-5%. The calibration curve in plasma covered the range 0.5-150 $\mu\text{g/mL}$. The regression correlation coefficient (r) was > 0.99 for all runs. This method improved the detection limit by 100 times (0.005 $\mu\text{g/mL}$) comparing to LLE-LC-UV (0.5 $\mu\text{g/mL}$). This new method also reduced the sample handling and the analysis time by several folds compared to LC-UV detection.

In study II, we compared the pharmacokinetics of CPA using serial bleeding from the tail vein with that obtained from conventional retro-orbital sinus bleeding in mice. CPA was administered intraperitoneally to two groups of mice and blood samples were collected at different time points. For the first group ($n=6$), 20 μL blood samples were collected from the tail vein by serial bleeding. In the second group, 3 animals were killed at each time point and blood was collected from both tail vein and retro-orbital sinus. CPA was analyzed using LC-MS/MS. No significant differences in estimated pharmacokinetic parameters including AUC, T_{max} , C_{max} and half-life were observed when comparing serial- with retro-orbital bleeding. This indicates that serial sampling using tail vein in mice can offer better alternative to retro-orbital bleeding.

The present results show that LC-MS/MS in combination with serial sampling in mice can be a good alternative method to be used for early preclinical kinetic studies. However, more studies are needed to evaluate different drug categories.

List of Publications

- I. Rana Said, Zuzana Hassan, Moustapha Hassan and Mohamed Abdel-Rehim. Rapid and sensitive method for determination of cyclophosphamide in patients plasma samples utilizing microextraction by packed sorbent online with liquid chromatography-tandem mass spectrometry (MEPS-LC-MS/MS). *Journal of Liquid Chromatography & Related Technologies*, 31: 683–694, 2008.
- II. Rana Said, Mohamed Abdel-Rehim, Behnam Sadeghi, Suleiman Al-Hashemi, Zuzana Hassan and Moustapha Hassan. Cyclophosphamide Pharmacokinetics in Mice: A Comparison between Retro Orbital Sampling versus Serial Tail Vein Bleeding. *The Open Pharmacology Journal*, 1: 22-27, 2007.

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LIST OF ABBREVIATIONS

AUC	Area under the concentration-time curve
CID	Collision –induced dissociation
CL	Clearance
C_{\max}	Maximum reached concentration
CPA	Cyclophosphamide
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
GC-MS	Gas chromatography – mass spectrometry
h	Hour
HPLC	High performance liquid chromatography
IS	Internal standard
LC	Liquid Chromatography
LLE	Liquid-liquid extraction
LLOQ	The low limit of quantification
LOD	Limit of detection
MS/MS	Tandem mass spectrometry
MRM	Multiple reaction monitoring
MEPS	Microextraction by packed sorbent
4-OHCPA	4-Hydroxy cyclophosphamide
PD	Pharmacodynamics
PK	Pharmacokinetics
QC	Quality control sample
UV	Ultra violet detector
RSD	Relative standard deviation
SPE	Solid phase extraction
$t_{1/2}$	Elimination half-life
V_{ss}	Distribution volume at the steady state
v/v	Volume/volume

1 Introduction

1.1 Cancer

Cancer is not a single disease but a number of heterogeneous diseases in which the cells proliferate abnormally. Some cancers are highly invasive and may build metastasis in other tissues. The disease can occur in all ages, but the risk increase with age. Almost all cancers types are characterized by abnormalities in the genetic material of the transformed cells [1]. Several factors were reported to cause these abnormalities, such as tobacco smoke, radiation, chemicals, or infectious agents. Death caused by cancer account for about 13% of all death cases around the world [2].

Cancer is a disease as old as the human history. There are some evidences showing that cancer existed in prehistoric time in spite that it seems that the disease belongs to the modern world. The oldest description of cancer known as “Edwin Smith papyrus” was written in Egypt (3000-1500) years BC. Other documents from the old Egyptian time were found with a description of both diagnosis and surgical treatment for cancer. Hippocrates was the first to use the term *carcinoma* (the Greek name for crab) in describing non-ulcer forming and ulcer-forming tumors. He recognized the differences between sarcomas (fleshy tumors) and carcinomas (crab legs tumors).

Progress on understanding and treating cancer was slow. In 1761, Giovanni Morgagni was the first to perform autopsies to relate disease to the pathologic findings postmortem.

In 1896, Wilhelm Conrad Roentgen discovered the X-ray. He used the term X-ray in a lecture, shortly after that systems were developed to use x-rays for diagnostic purposes.

In 19th century Rudolf Virchow "the founder of cellular pathology" established the scientific basis for the modern pathologic study of cancer where a precise diagnosis was made based on the tissues removed by the surgeon [3].

In 1953, the discovery of DNA structure was a breakthrough in our understanding of cell biology. The technical and methodological development initiated studies in cellular and molecular biology, including gene mapping, carcinogenesis, angiogenesis, and signal transduction. These studies improved the understanding of cancer events at molecular level and led to new treatments based on this knowledge.

1.2 Chemotherapy

The simple definition for the term chemotherapy implies the use of chemical substances to treat a disease. The most common chemotherapeutic agents used in cancer therapy are referred to as cytostatics due to cytotoxic effects. Based on their mechanisms of action, cytostatic drugs are divided in several groups. Many cytostatics affect DNA synthesis or its function, and thus, interfere with the cell cycle. Based on their action, chemotherapy agents can be classified as cell-cycle specific (effective during certain phases of cell cycle) or cell-cycle nonspecific (effective during all phases of cell cycle).

In general, cytostatics are classified to the following groups:

- Alkylating agents including platinum compounds and nitrosoureas
- Antimetabolites
- Antitumour antibiotics
- Mitosis inhibitors including vinca alkaloids and taxanes
- Topoisomeras inhibitors including epipodophyllotoxins and camptothecanes
- Enzymes and random synthetics
- Hormonal agents

1.3 Alkylating agents

Alkylating agents are the oldest group of cytostatics and they are still the corner stone for cancer therapy. Their use in cancer treatment started in early 1940's. Several alkylating agents are derivatives of the nitrogen mustard. Nitrogen mustard is an analogue to sulfur mustard that was use as a chemical weapon during WWI and WWII. Chlorambucil, cyclophosphamide, lomustine, melphalan, procarbazine, thiotepa and busulfan are examples of commonly used alkylating agents. Alkylating agents act through the alkylation of DNA, however, they differ in their activity against different tumor types. These agents act directly on the DNA and prevent cell division by cross-linking DNA, that leads to DNA breaks or abnormal base pairing, and subsequently to cell death [4]. Alkylating agents are generally considered to be cell cycle phase non-specific as they kill the cell in various phases of the cell cycle. They are more effective in treating slow-growing cancers, but are less effective on rapidly growing cells [5]. Alkylating agents are cancerogenic and mutagenic which can also lead to secondary cancers such as acute myeloid leukemia, years after the therapy.

1.3.1 Mechanism of action

Several alkylating agents form strong electrophiles through the formation of carbonium ion intermediates. This results in the formation of covalent binding by alkylation of nucleophiles moieties. The cytotoxic effects are directly related to the alkylation of DNA through the nitrogen-7 atom of guanine although other moieties are also alkylated. The formation of one covalent bond with nucleophiles can result in mutagenesis or teratogenesis, but the formation of these bonds through cross linking can produce cytotoxicity [4, 5].

1.3.2 Cyclophosphamide

Cyclophosphamide and the related alkylating agent ifosfamide were developed by Norbert Brock. Cyclophosphamide is widely used anticancer and immunosuppressive agent. It is a prodrug that undergoes complicated process of metabolic activation and inactivation. Approximately 70-80% is activated by the cytochrome P450 (CYP) enzyme system to form 4-hydroxycyclophosphamide (4-OHCPA), which is in equilibrium with its tautomer aldophosphamide. Different members of CYP isoenzymes are involved in the bioactivation of cyclophosphamide. The main CYP that is involved in the bioactivation of cyclophosphamide is CYP2B6. 4-OHCPA is unstable and decomposes into phosphoramidate mustard (PM) and acrolein. PM is a bifunctional alkylating agent and is considered to be the ultimate alkylating metabolite. In contrast to 4-OHCPA, PM cannot enter target cells, and therefore only the intracellular formed PM fraction is considered to be cytotoxic. Furthermore, 4-OHCPA and aldophosphamide are deactivated by an oxidative reaction to 4-ketocyclophosphamide and carboxyphosphamide, respectively, in which alcohol and aldehyde dehydrogenases are involved. A second route of CPA elimination is side-chain oxidation mediated by CYP3A4, leading to the formation of the inactive metabolite 2-dechloroethylcyclophosphamide and an equimolar amount of chloroacetaldehyde [6].

1.4 Pharmacokinetics and pharmacodynamics (PK and PD)

Drug efficacy depends on the concentration of the drug at the site of action. Pharmacokinetic studies are designed to determine the fate of the drug in the body while pharmacodynamic studies are designed to determine the relation between the drug concentration and its effect. Kinetic studies are as rule necessary in order to optimize the treatment to reach maximum efficacy and safety [7].

Kinetic and dynamic studies are essential to establish therapeutic schedules and to be used for therapeutic drug monitoring and dose adjustment. This is important when using drugs with a narrow therapeutic window when the relation between plasma concentrations and therapeutic and /or toxic effects has been established [8].

Pharmacokinetic investigations are important issue in pre clinical evaluation of new candidate drugs prior to entering clinical trials. Pharmacokinetics describes mathematically the fate of the drug in the body and is useful for comparing laboratory animal strains and species in order to extrapolate this data to human [9]. Cautions have to be considered when such extrapolation is made especially when it concern young patients [10,11]

1.5 Animal models in PK/PD studies

Animal models provide basic knowledge about the biochemical fate of drug biotransformation and is a relevant physiological system for evaluation of drug metabolism [9]. Many *in vitro* systems and models are now available for prediction human drug metabolism and preclinical pharmacokinetic studies in the new drug discovery process but these models have a number of limitations and can not replace the animal studies [12].

Mice and rats are mainly used to evaluate pharmacokinetics and pharmacodynamics. Mice are the species of choice for toxicological evaluation and this is due to low cost, minimal holding space, relatively short life span and recently, since the mouse genome has been explored [12, 13].

An important requirement for reliable pharmacokinetic parameters is the proper blood sampling procedure since pharmacokinetic evaluation required blood sampling over several time points [9]. Several blood sampling techniques for rodent studies are available for investigational purpose. However, there is no standard practice since many factors contribute to the choice of sampling technique. These factors include animal species, frequency of blood collection, the required sample volume, easiness of the method and the physiological status of the animal [12].

Usually high number of animals is needed for kinetic studies because of several samples at different time points and relatively large blood volumes for analysis are needed [14].

Furthermore, the high number of animals used for concentration-time curve may lead to high standard deviation in the data and interpretation difficulties due to the differences in the expression of drug metabolising enzymes and genetic polymorphism[14].

One of the conventional sampling method is retro-orbital sinus bleeding. This technique has widely been used since large blood volume can be obtained; however, its use is limited to few sampling occasions (two time points per mouse) which mean that several animals are required for each concentration-time curve. Moreover, this method requires appropriate anesthesia, monitoring and veterinary care in case of unexpected complications. In general, this method of sampling is invasive and not accepted ethically [9, 15, 16].

Another used sampling method, which does not require anesthesia or surgical preparation of the animal, is the tail-artery or –vein bleeding technique. This is simple method, which consist of a precise incision on the mouse tail and then “milking” the blood sample. This method has not been used frequently since small blood volume can be obtained at each sampling point. However, the development of sensitive and selective analytical methods that require only small blood volumes may bring this method to the spot light again [9, 14, 15].

Several institutions and industry researchers have started to replace the retro orbital sinus sampling method with serial bleeding to avoid the ethical problems connected to sinus bleeding and the variation issues due to the high number of animals used [17].

1.6 Analytical techniques for effective pharmacokinetics studies

Proper and accurate analytical methods are the basis of pharmacokinetic and pharmacodynamic investigations. The determination of drugs and their metabolites in biological samples have several analytical problems including selectivity and sensitivity. Therefore, the analytical methods have to be reproducible and sensitive in order to provide accurate determination of the drugs and /or metabolites in biological matrices.

High performance liquid chromatography (HPLC) is one of the most used analytical techniques. HPLC is a universal technique which may be utilized for the analysis of a wide range of substances with different chemical and physical properties. HPLC sensitivity can be increased dramatically when combined with mass spectrometry (MS). The combination of LC-MS fulfills most of the analytical needs for laboratories involved in drug testing and toxicology, this is due to the recent development in high sample throughput in combination with high sensitivity and selectivity [18].

Tandem mass spectrometry (MS/MS) is now an established powerful analytical technique and it has attained a predominant role over all other techniques. This is due to high sensitivity,

specificity, and speed attainable with MS/MS beside that the technique requires less amount of matrices and shorter operating time [19]. The recent development in MS has introduced sample analysis that can be carried out on atmospheric pressure using chemical ionization source which is an important factor for analyzing drugs and their metabolites. These in combination with the development in LC that allow the analysis of crude plasma samples have improved LC-MS/MS technique. The key step in the development of LC-MS as a routine technique is the introduction of collision –induced dissociation (CID) with a second quadrupole for the analysis of the fragments generated. In principle, the first quadrupole is to select the molecular ion (parent) of the targeted analyte, which is then headed to the collision cell. By the collision with the argon molecules the parent ions are disintegrated in to several typical ions called (daughter ions). This system offers essential advantages over GC-MS and HPLC with conventional detection techniques [18]. The LC-MS/MS can be used for quantitative determination of plasma samples in pharmacokinetic studies. LC–MS/MS techniques frequently provide selective, sensitive and quantitative results often with reduced sample preparation steps and shorter analysis time compared to other commonly used techniques [20].

1.7 Sample preparation

Biological samples can not usually be assayed directly. Preparation in term of cleaning up the sample from endogenous compounds is required. The biological fluids contain a number of endogenous compounds and some of them in high concentrations. These interfere with the analysis and may also lead to blockage of the analytical columns [21].

There are several ways for sample pretreatment:

- Liquid-liquid extraction (LLE) is especially suitable for lipophilic compounds since the analyte is transferred from the aqueous matrix to an organic phase.
- Protein precipitation is the simplest technique as it only involves the addition of precipitating solvent followed by subsequent centrifugation. This technique is not always sufficient since it leaves many matrix constituents that may interfere with the assay by ion suppression and contaminate the system when LC-MS/MS assays are used.
- Solid phase extraction (SPE) is versatile technique for sample pretreatment but it is labor –intensive combined with complex procedure [21].

The on line extraction technique is a different high-throughput approach that has remarkably speed up the analysis by LC-MS/MS [22].

- Micro Extraction by Packed Sorbent (MEPS) is a new development in the field of sample preparation by SPE (solid phase extraction) [23-25]. It has the same function as SPE with some differences:
 - I. It requires small sample volumes, 10 μ L may be sufficient, and moreover the amount of solvents needed for analysis is reduced.
 - II It is fully automated-extraction and injection performed on line using one syringe on LC or GC autosampler.

1.8 Cyclophosphamide analysis and kinetics

In the past, lack of accurate analytical method for the determination of cyclophosphamide and its metabolites was an obstacle for the assessment of the clinical pharmacology of the drug. Recent massive improvement concerning sensitive and specific analytical techniques facilitates the measurement of the drug and/or its metabolites to provide adequate data on its pharmacokinetics and pharmacodynamics. However, there is still a lack of knowledge on cyclophosphamide efficacy and toxicity in several therapy schedules. This may be due to the genetic variations in cyclophosphamide activation and inactivation and/ or drug-drug interactions [26].

New bioanalytical assays for the accurate quantification of cyclophosphamide and its metabolites have been developed over the past decade.

Techniques such as gas chromatography (GC), liquid chromatography(LC), nuclear magnetic resonance spectrometry (NMR), GC-mass spectrometry (GC-MS), liquid chromatography –mass spectrometry (LC-MS) were used to quantify cyclophosphamide in biological samples [27].

Extensive pharmacokinetics analysis on cyclophosphamide has been reported. The elimination half life of cyclophosphamide is reported to be within the range 5-9 hours over large concentration range [28]. The plasma half life was reported to be shorter in children compared to the adults. The total systemic clearance of cyclophosphamide is reported to range from 4-5 L/h. The volume of distribution (V_d) of 30-50 L [29]. High inter individual variability in cyclophosphamide kinetics is well documented which may be due to variability and polymorphism in drug metabolizing enzymes involved in cyclophosphamide metabolism.

2 AIMS OF THE STUDY

The objectives of this thesis were to develop high throughput and sensitive analytical method and reliable sampling technique for studies on cyclophosphamide pharmacokinetics.

2.1 Specific aims of the study

- To develop a new analytical method for cyclophosphamide detection using LC-MS/MS utilizing on line sample preparation by using MEPS.
- To evaluate the reliability of serial bleeding technique compared to conventional sampling methods for studies on cyclophosphamide kinetics in mice.

3 Materials and Methods

3.1 Chemicals

Acetonitrile, methanol, formic acid and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). All solvents were of HPLC analytical grade.

Cyclophosphamide was purchased from Sigma (St.Louis, MO, USA). Ifosfamide was purchased from ASTA Medica AG (Frankfurt, Germany).

3.2 Animals

Female MRI mice were obtained from Scanbur (Sollentuna, Sweden). Animals were housed in acclimatized room at constant temperature and humidity on 12-hours light and dark cycle.

Animals were fed standard pellet and water *ad libitum*. Animals weight was within the range 30-40g. Animals were kept for one week for acclimation prior to experiment.

All experiments described here were carried out following the protocol approved by The Animal Ethical Committee at the Karolinska Institutet. The study was designed according to the guidelines of the Committee on the Care and Use of Laboratory Animals.

3.3 Analytical procedure

3.3.1 High performance liquid chromatography UV-detection

The liquid chromatographic (LC) system consisted of Shimadzu pump LC10AD (Kyoto, Japan) and CMA/240 autosampler with a 50 μ L sample loop, variable wave length, UV-detector, Milton Roy Spectro-Monitor 3100 (PA, USA), and CSW Chromatography Station integration system.

The liquid chromatographic analysis was run using isocratic mode. The mobile phase consisted of acetonitrile and 0.05 M phosphate buffer (20:80, v/v); the flow rate was 0.4 mL/min.

Phenomenex LC column, polar-RP 80 A (3.0 x 150 mm, 4 μ m) was used for separation. The analysis time was 20 min. The data were processed using the integration system.

3.3.2 Liquid chromatography and tandem mass spectrometry

Instrumentation

The LC system consisted of two Shimadzu pumps LC 10AD, an autosampler, CTC-Pal, (CTC Analytics AG, Zwingen, Switzerland). A guard column Optiguard (C₈, 10x1 mm) was purchased from Optimize Technologies (Oregon City, USA). The separation was performed using Zorbax

(50x2.1 mm, SB- C₈, 3.5 μm) column obtained from Agilent (CA., USA). The gate valve between the liquid chromatography and the mass spectrometer was Valco C4W valve (Valco Instruments, Houston, USA).

MEPS-LC-MS/MS: The MEPS syringe (100 μL syringe, C₂-sorbent) was obtained from SGE analytical (Melbourne, Australia).

All the experiments were carried out using a triple quadrupole mass spectrometric instrument Micromass QUIZ-SPRAY (Waters Corporation, Manchester, UK) provided with a Z-electrospray interface.

The ESI mass spectrometer run in the positive ion mode with a capillary voltage at 3.1 kV, cone voltage at 27 V, extractor at 5 V, RF lens at 0.2 V, source block and desolvation temperatures at 150 °C and 300 °C, respectively. Nitrogen was used both as drying (400 L/h), and nebulizing gases (20 L/h), the vacuum was 2.10^{-5} in the mass analyzer and 2.10^{-3} in the collision cell. Argon was used as collision gas and collision energy was 22 eV. The gases were obtained from ScanGas (Stockholm, Sweden). The data were collected and processed using MassLynx version 3.4, and all calculations were based on peak area ratios. The scan mode was multiple reaction monitoring (MRM) using precursor ion at (M+1) m/z (m/z: 261 and 261) and after collisional dissociation. The product ions 140 and 154 were obtained and used for quantification of cyclophosphamide and the internal standard.

Chromatographic condition

A gradient mode was used with mixer volume of 0.1 mL. Mobile phase A was 0.1% formic acid in water and acetonitrile 90:10 (v/v) and mobile phase B contained 0.1% formic acid in water and acetonitrile 20:80 (v/v). The gradient started from 0% of phase B up to 80 % from 1 to 4 min and then from 4 to 5 min isocratic at 80% of phase B and at 6.1 min phase B was set at 0% again. The flow rate was 150 μL/min and the sample volume was 40 μL.

3.4 Sample preparation

3.4.1 Liquid –liquid extraction for LC-UV

The blood samples were centrifuged for 5 min at 3000 g. The plasma was kept at -20 °C after the separation. To 0.5 mL of the plasma, ifosfamide (50 μL) was added as internal standard. Volume of 0.25 mL was extracted in 1.5 mL ethyl acetate, vortexed for 15 minutes followed by centrifuging at 3000 g for 10 min. The organic phase was then transferred in to new tube and

evaporated to dryness. The residue was dissolved in 100 μL of mobile phase and 30 μL were injected into HPLC.

3.4.2 Microextraction by packed sorbent (MEPS) for LC-MS/MS

The MEPS sorbent (C2) was manually conditioned with 50 μL methanol followed by 50 μL of water. After that, the syringe was connected to the autosampler and the spiked plasma sample (25 μL) was withdrawn into the syringe by the autosampler. The sorbent was then washed once with 100 μL of water/methanol 95:5 (v/v) to remove proteins and other interferences. The analytes were then eluted by 30 μL methanol/water 95:5 (v/v) directly into the LC injector.

3.5 Standard curve and controls

Calibration curves for HPLC-UV were prepared with known concentrations of cyclophosphamide diluted in plasma or water. Each calibration curve consisted of nine points covering the range from 0.5 $\mu\text{g/mL}$ to 150 $\mu\text{g/mL}$. The plasma used for the calibration curve was collected and pooled from different subjects. Quality controls (QC) consisted of human plasma spiked with known concentrations of cyclophosphamide obtained from stock solutions which were not used for the standard curve preparation. The QC samples were analyzed at the same time as the standards, patient samples and blank samples.

For MEPS, 50 μL plasma sample containing IS (40 $\mu\text{g/mL}$) was diluted with water (1:4) before extraction. For the calibration curve preparation, a stock solution of cyclophosphamide (2.0 mg/mL) and iphosphamide (I.S, 1.0 mg/mL) in distilled water. From this stock solution different diluted solutions were prepared, 50.0 μL of each diluted solution was added to 0.5 mL plasma to cover arrange of concentration between 0.5-150 $\mu\text{g/mL}$ followed by addition of 20.0 μL internal standard. For the quality control samples the concentrations were 20, 75 and 120 $\mu\text{g/mL}$.

3.6 The design of the animal studies (study II)

Cyclophosphamide was administered at a dose of 100 mg/kg intraperitoneally. The blood samples were collected at 0, 0.25, 0.5, 1, 2, 3, 4, 5 and 6 hours. The animals were divided in two groups according the sampling technique: group 1 consisted of 6 mice for serial bleeding and group 2 consisted of 27 mice for retro orbital bleeding.

In group 1, blood samples were collected serially from the tail vein. Each mouse was placed in a restrainer and the tail was swabbed with alcohol. A sharp surgical blade was then used to make

an incision over the tail vein located approximately 2-3 cm from the tip of the tail by applying light pressure from the base to the end. Blood was collected using a syringe containing 80 μ L of 0.5 M EDTA. Then, pressure was applied on the incision site for 10 second to stop the bleeding allowing the blood to clot.

In the second group, blood samples were collected through retro orbital bleeding and tail vein simultaneously. A glass capillary tube was inserted in to the retro –orbital sinus venous plexus and blood was allowed to flow via the capillary into a collection tube. Isoflurane was used to anesthetize animals prior to blood collection. Three animals were sampled at each time point. The blood samples obtained from the eyes were divided in two fractions, whole blood and plasma. The blood samples from tail vein were collected using the same technique as above. The whole blood was used for comparison between groups.

3.7 Calculations and statistics

The pharmacokinetic analysis was performed using WinNonlin compartmental modeling analysis version 5.2 (Pharsight, CA, USA). The pharmacokinetic parameters were obtained by fitting the blood concentration-time data to one-compartment model. The following parameters were obtained for comparison of PK: area under concentration-time curve (AUC), maximum reached concentration (C_{max}), half-life ($t_{1/2}$) and distribution volume (V_{ss}). Non-parametric statistics were used to analyze the means and variances of each parameter ($p < 0.05$ was considered significant).

4 Results

4.1 Analytical methods

Two analytical methods were used for the detection of cyclophosphamide both plasma and whole blood.

4.1.1 High performance liquid chromatography (UV detection)

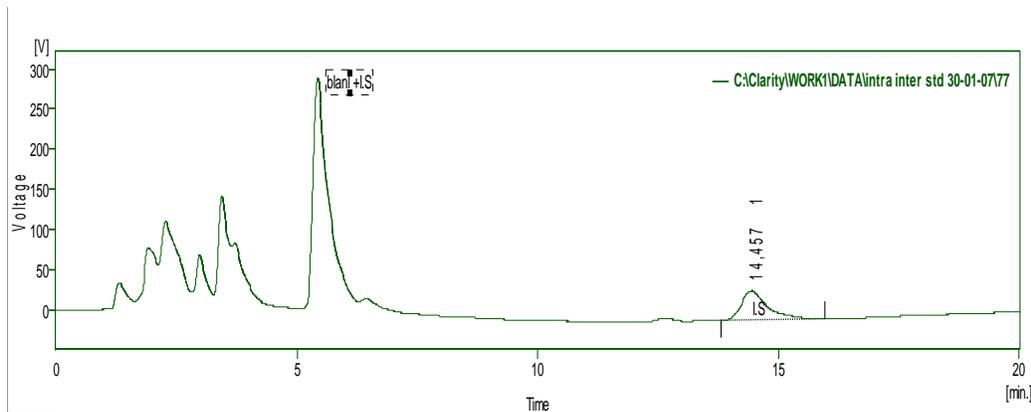
An HPLC validated method was used to detect and quantify cyclophosphamide.

The retention time for cyclophosphamide using this method was 16.5 min with run time analysis equal to 20 min (Figure 1). The calibration curve was linear within the range 1-150 μ g/mL in plasma samples. The coefficients correlation factor (r) were 0.995 ($n=3$)

The lowest detectable quantity LLOQ for cyclophosphamide was 5 µg/mL (with precision of less than 20% and accuracy of less than 15%). The between-batch mean accuracy ranged from 84 to 98% using LLE-LC-UV.

The data of between-batch variation of the precision were in the range 4.0–11% for LLE-LC-UV. The precision is determined by the percentage of the relative standard deviation (RSD) of the between-batch variations at three different concentration levels (QC samples). The precision (RSD) within batch variation were in the range 4.3-13%

(A)



(B)

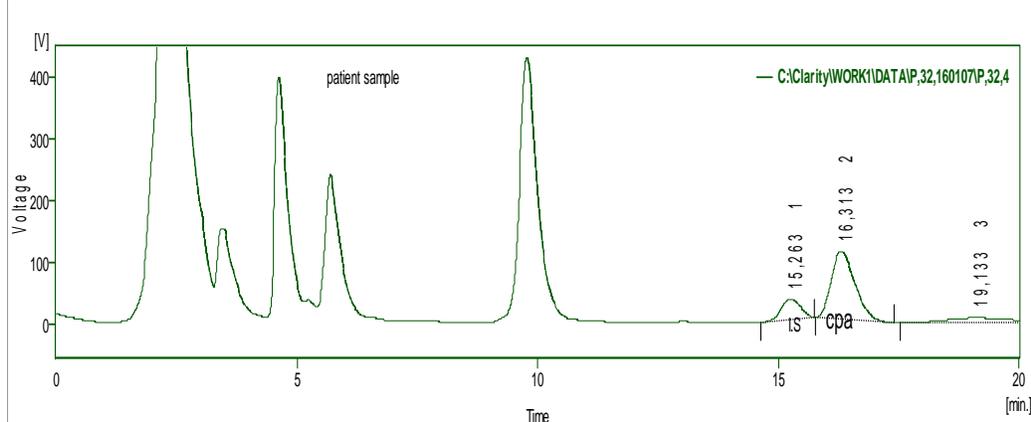


Figure 1: Representative chromatograms with UV detection obtained from (A) blank human plasma sample containing IS. (B) patient plasma sample and I.S.

4.1.2 Liquid chromatography and tandem mass spectrometry

The retention time for cyclophosphamide was 5 minutes (Figure2). MRM was obtained using the precursor ion (m/z : 261), after the collisional dissociation the product ions m/z :140 and 154 were used for quantification of cyclophosphamide and the internal standard.

Microextraction by packed sorbent was used for sample extraction. Cleaning of the sorbent was carried out using 4x250 μ L elution solution followed by 4x250 μ L of the washing solution between every extraction. This step is important to decrease memory effects and also functioned as conditioning step before the next extraction. The sorbent was used for 100-150 times and then discarded.

The calibration curves in plasma was linear within the range 0.5-150 μ g/mL with correlation coefficient $r = 0.999$.

The LOD and LLOQ were 0.005 μ g/mL and 0.5 μ g/mL, respectively.

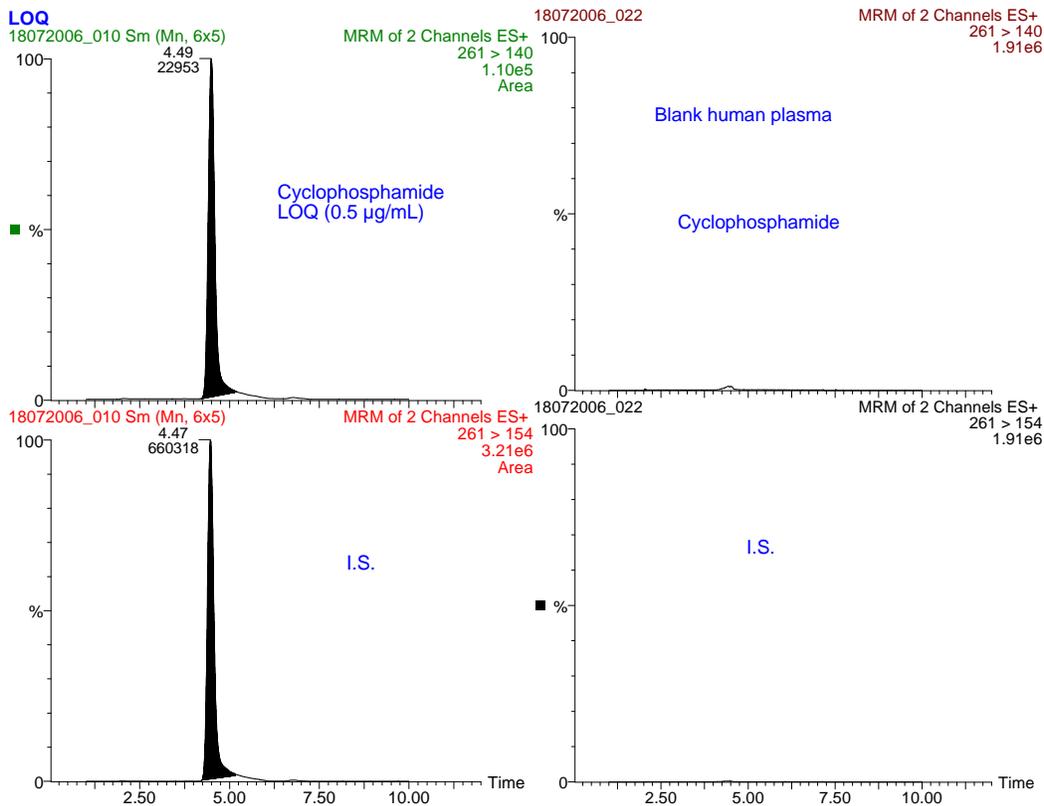


Figure2: Representative chromatograms of mass spectrometric detection obtained from (left chromatograms) human plasma spiked with cyclophosphamide (upper) 0.5 μ g/mL (LLOQ) and I.S. (lower); (right chromatograms) blank plasma sample.

4.1.3 Application of the method

One hundred and seventy patient samples were analyzed by LC-UV and LC-MS/MS.

The ratio between the plasma concentrations analyzed using LC-MS/MS and LC-UV was 1.02 ± 0.11 ranged from 0.81-1.19 (Figure 3).

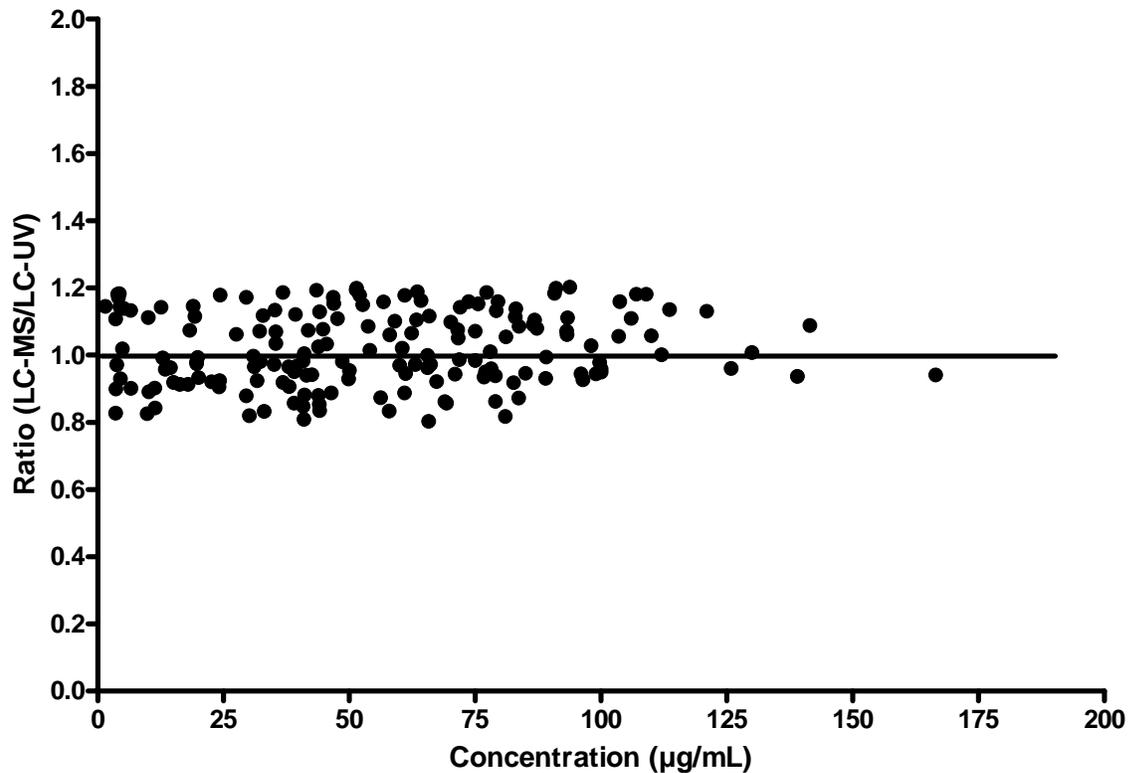


Figure 3: The ratio of patient plasma concentrations obtained by LC-MS/MS / LC-UV plotted against concentrations obtained using LC-MS/MS.

4.2 Cyclophosphamide pharmacokinetics in mice

Figure 4 shows the blood concentration-time curves obtained after the administration of cyclophosphamide. In Figure 4A the blood concentration-time curve obtained after serial bleeding is presented, while Figure 4B shows the kinetics following retro orbital bleeding. The parameters (AUC , $t_{1/2}$, C_{max} , Cl , V_{ss}) were obtained with two different blood sampling methods, tail-vein and retro-orbital bleeding following IP dose of cyclophosphamide (Table 1).

There was no significant difference in the elimination half-life between the results obtained from retro orbital bled mice and serially tail-bled mice. The values ranged between 0.49-0.52 h. The

AUC estimated from whole blood concentration-time curve after serial bleeding (68.9 $\mu\text{g}\cdot\text{hr}/\text{mL}$) was not significantly different from that obtained from retro-orbital bleeding (61.3 $\mu\text{g}\cdot\text{hr}/\text{mL}$). No significant differences in AUCs obtained from whole blood or plasma after retro orbital bleeding and whole blood obtained from tail vein bleeding taken from the same animal (61.3, 86.5 and 56.6 $\mu\text{g}\cdot\text{hr}/\text{mL}$, respectively) were observed.

C_{max} was found to be 109 $\mu\text{g}/\text{mL}$ after serial bleeding showing tendency ($p=0.09$) to be higher than that seen after retro orbital bleeding. The C_{max} calculated from tail vein sampling in group 2 was 79 $\mu\text{g}/\text{mL}$. Moreover, there were no differences observed in C_{max} using plasma or whole blood.

The apparent distribution volumes showed no significant differences between kinetics calculated after different bleeding methods (tail vein and retro orbital sinus, 44 and 60 mL, respectively). This also was found for the volume of distribution obtained from plasma samples collected using retro-orbital bleeding or whole blood in tail vein within the same animal (51 mL and 65 mL, respectively).

Clearance values varied from 63 mL/hr in tail vein group to 88 mL/hr in group 2. No significant differences were found between clearances obtained after different bleeding techniques.

Table 1. Pharmacokinetic parameters of cyclophosphamide calculated in both plasma and blood after different sampling procedures.

	Group A	Group B		
	Serial bleeding	Several animals per time point		
	Tail vein	Retro-orbital sinus	Tail vein	Retro-orbital sinus
	Whole blood	Whole blood	Whole blood	Plasma
PK parameters	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD
AUC ($\mu\text{g}/\text{mL}\cdot\text{h}$)	68.9 \pm 10.5	61.3 \pm 9.8	56.6 \pm 13.8	68.5 \pm 12.6
C_{max} ($\mu\text{g}/\text{mL}$)	108.8 \pm 18.2	81.3 \pm 11.2	78.7 \pm 24.6	97.4 \pm 19.2
$t_{1/2}$ (h)	0.45 \pm 0.08	0.52 \pm 0.05	0.51 \pm 0.08	0.49 \pm 0.07
Cl (mL/h)	63 \pm 6	80 \pm 9	88 \pm 18	72 \pm 10
V_{ss} (mL)	44 \pm 9	60 \pm 6	65 \pm 15	51 \pm 8

Elimination half-life ($t_{1/2}$), area under the concentration-time curve (AUC), maximum reached concentration (C_{max}), clearance (CL) and distribution volume at the steady state (V_{ss}).

Figure 4A

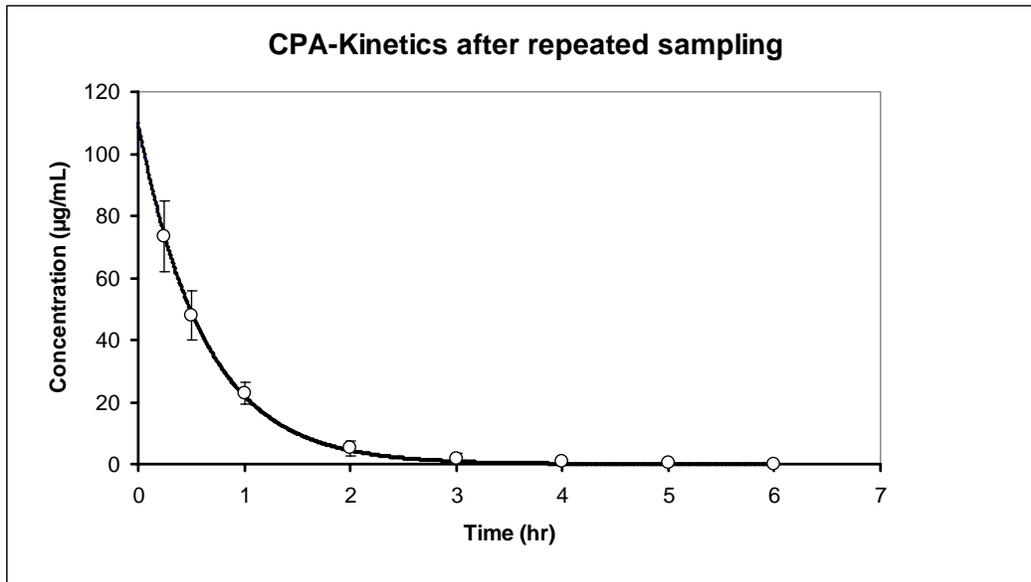


Figure 4B

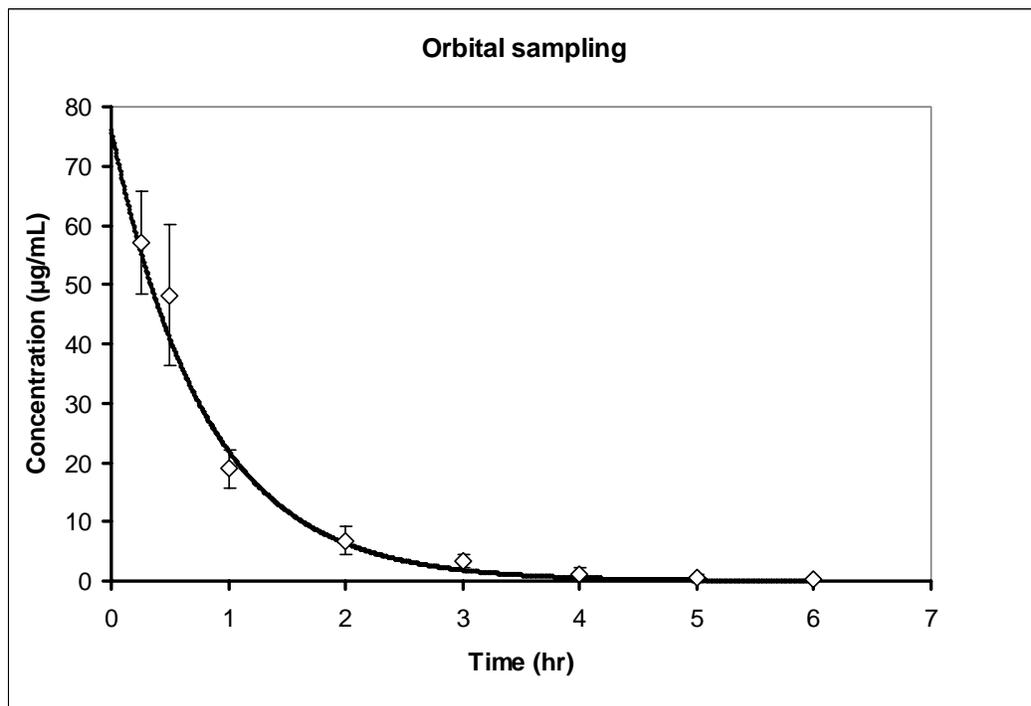


Figure 4: Concentration versus time curve obtained after the administration of cyclophosphamide (100 mg/kg): (A) After serial bleeding from the tail vein in 6 mice, (B) after orbital bleeding (3 mice in each time point).

5 Discussion

Pharmacokinetic studies are important to determine the fate of drugs in the body, while pharmacodynamic investigations are important to determine the efficacy and safety of the drug. Both in combination can be used to individualize the treatment of patients. This is of special importance in cancer therapy since many cytostatics have narrow therapeutic window. For accurate kinetic studies, highly selective and sensitive analytical methods are required. However, sample volumes and sample preparation can be major limitations and must be considered. In the present studies we used powerful technique LC-MS/MS to develop a new sensitive and rapid method to detect cyclophosphamide utilizing on line sample preparation (MEPS). This combination provides sensitive detection method and on line sample preparation requires small sample volume. Moreover, both sample preparation and the analysis time decreased significantly. We validated this method in terms of linearity, selectivity and sensitivity. The method was used in the second study, to determine cyclophosphamide pharmacokinetics in mice comparing different sampling techniques, i.e. serial tail vein bleeding technique and conventional retro-orbital sampling technique. We compared these two sampling techniques in mice using pharmacokinetics parameters for cyclophosphamide after intraperitoneal administration. This study has shown that there are no significant differences between data obtained using these two sampling techniques. Thus, serial tail vein bleeding has the advantage of fewer animals to be used for kinetics studies. This in turn will minimize inter-individual variation. Moreover, this technique is less expensive, no anesthesia is required, it is easy to handle and stress free for the animal. We hope that this method will be adopted in the future to reduce number the animals required for the pharmacokinetic study.

Cyclophosphamide has been chosen for this study since it is one of the most widely used anticancer agents, which is used in the treatment of hematological malignancies as well as solid tumors. Some more reasons for choosing CPA are the well documented pharmacokinetics and the stability of the drug which facilitate the validation of the new sampling technique.

Developing LC-MS method for cyclophosphamide detection (study I)

Several analytical methods have been developed such as GC-MS and LC-MS to quantify cyclophosphamide. However, many of them are connected with a number of problems including, large sample volume, and long analysis time and labor sample preparation. The demand for online sample preparation combined with high-throughput application in bioanalysis has increased during the recent years.

In the present investigation, we developed robust, miniaturized and fully automated method for CPA analysis based on-line sample preparation utilizing micro extraction in packed syringe [23-25] (Figure 5). This method does not require any work up procedure. This method may be the starting point for further work to optimize quantification for CPA metabolites, since CPA is a prodrug that needs to be activated to exert its toxic effect. Such a method can be of great advantage for therapeutic drug monitoring [19].

Several researchers have developed analytical methods using LC-MS technique in plasma and urine as well as for environmental samples and they have applied these assays in clinical and experimental settings [30-33]. However, these methods were shown to have some limitations such as the need for derivatization and long time work up (Table 2). The important feature in our method is the small sample volume that is required to perform the analysis (50 μ L). This can be compared with other published methods that require sample volume between 100-1000 μ L. Thus, this is an advantage facilitating pharmacokinetic studies and/or therapeutic drug monitoring in young children. One more advantage for the new method is the short analysis time which is important for clinical practice. More than 150 samples from patients treated with CPA were analyzed liquid chromatography with UV detection. These results were compared with these obtained using new method LC-MS/MS. No significant difference was observed between the methods and ratio between the methods was 1.02 ± 0.11 and ranged from 0.81-1.19. This indicate that the new method is accurate and with less than 20% variation compared to LC-UV.



Figure 5. Micro extraction by packed sorbent (MEPS)

Type of analysis	Type of sample	Extraction time	Run time analysis	Limit of detection	Limit of quantification	Ref
LC/MS-MS	Gloves	No extraction	4 min	1.95 ng/ml	31.6 ng/ml	15
LLE/HPLC-UV	Patient plasma sample	90 min	20 min	0.5µg/ml	5.0 µg/ml	16
LC-MS/MS	Patient Plasma sample	150 min	9.2 min	20.0 ng/ml	50.0 ng/ml	17
LC-MS/MS	Mice Plasma sample	70 min	6.5	-	-	18
LC-MS/MS	Urine samples	60 min	4.1	0.05 ng/ml	0.2 ng/ml	19

Table 2. Comparison between different analytical methods for CPA detection

Cyclophosphamide pharmacokinetics in mice (study II)

Since the animal studies play an important role in the preclinical and toxicological evaluation, the animal protocols used in the development of new chemical entities especially aiming the treatment of cancer has been reviewed world wide [27].

Several blood sampling methods are available, however, there is no unified practice adopted through pharmaceutical research. Many factors contribute to decision about the chosen technique. Easy, reliable and reproducible methods without negative effect on the pharmacokinetic profiles of the substances studied are preferred. Moreover, for some specific drugs, the disturbances of the physiological condition of the animal are of a great importance [34, 35].

In our study we have compared serial bleeding, using one animal, for whole concentration–time curve with retro-orbital sampling using several animals per each time point in mice. We also compared the blood concentrations of CPA obtained using retro-orbital and tail vein sampling from the same animal.

According to our results, we found that there are no differences between these two techniques when the pharmacokinetic parameters were compared. Several authors have expressed their concern that the serial bleeding technique may affect the body temperature and hence the pharmacokinetics. However, in our study, we have proved that this assumption is not valid for CPA. More studies are warranted using different drug categories and different routes of administration together with tail vein sampling technique before a final conclusion can be made [34, 35].

Further investigations should be done to examine the physiological status of the animals [9, 35] despite that the technique causes minimal trauma for the animal.

6 Conclusions

Tandem mass spectrometry (MS/MS) in combination with on line sample preparation using MEPS is a powerful analytical technique that provides high sensitivity, specificity, small blood volume used for the analysis and short analysis time which is beneficial for clinical practice as well as preclinical investigations.

We also conclude that serial bleeding via tail vein can be a good alternative method to the conventional sampling methods such as retro orbital or cardiac puncture sampling since this method requires less number of animals, no anesthesia and is less expensive.

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