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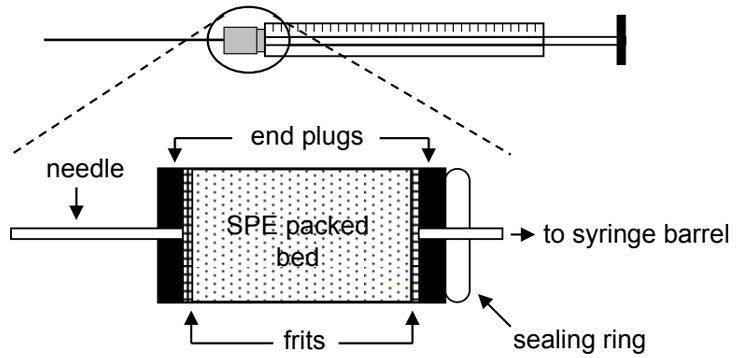
APPLICATION OF NEW TECHNOLOGY: MEPS AND LC-MS/MS FOR DETERMINATION OF THERAPEUTIC DRUGS



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Karolinska Institutet

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APPLICATION OF NEW TECHNOLOGY: MEPS AND LC-MS/MS FOR DETERMINATION OF THERAPEUTIC DRUGS

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To my parents

ABSTRACT

Bioanalysis most often requires an extraction procedure to isolate the target compounds from a complex matrix. The aim of this work was to evaluate microextraction in packed syringe (MEPS) performance as a sample preparation technique by developing new analytical methods for different categories of compounds utilizing MEPS in combination with LC-MS/MS. The overall goal was to provide high throughput methods that can offer automation, on-line coupling to mass spectrometry and short sample preparation time.

MEPS is a new sample preparation method representing a miniaturization of solid phase extraction (SPE) technique that can be fully automated. Sample preparation is performed on the packed bed. Less sorbent material is used with MEPS; about 1 mg of sorbent is inserted into a syringe needle (100-250 µL) as a plug and thus less solvents are needed. Different categories of compounds have been used to evaluate the performance of MEPS on-line with LC and tandem mass spectrometry (MS/MS), cytostatic, local anaesthetics, immunosuppressive drugs and one anaesthetic.

In study I, the first on-line quantification method for cyclophosphamide was developed and in study II it was the first quantification method for cyclophosphamide from whole blood. This method was applied to test tail vein sampling method from mice by studying the cyclophosphamide pharmacokinetic in mice. In study III, local anaesthetics were quantified from whole blood for the first time, using only 25 µL sample volume. In study IV an on-line method for immunosuppressive drugs was developed which can be implemented in clinical laboratories. Remifentanil in study V was quantified from whole blood utilizing only 20 µL sample volume which might facilitate pharmacokinetic studies on pediatric patients.

Full method validation has been performed according to the FDA guidelines. Sensitive methods have been obtained with small sample volume. The accuracy and precision results were in agreement with the acceptable ranges. Matrix effect was investigated and found to be reduced comparing to other sample preparation methods. Method comparison was done with other well established sample preparation techniques.

Further work on MEPS is recommended regarding applying this technique to more drugs and their metabolites, exploring other application areas such as environmental and food analysis, and modifying the device in a way that can reduce clogging and speed up the extraction process.

LIST OF PUBLICATIONS

I. Said Rana, Hassan Zuzan, Hassan Moustapha, Abdel-Rehim, Mohamed
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 Technologie. 2008,31: 683-694*

II. Mohamed Kamel, Rana Said, Aziza Elbeqqali, Fatma Bassyouni, Mohamed Abdel-Rehim
On-Line Determination of Cyclophosphamide in Blood Samples Utilizing Microextraction by Packed Sorbent and Liquid Chromatography Tandem Mass Spectrometry (MEPS-LC-MS/MS). *The Open Spectroscopy Journal. 2009,3: 26-30*

III. Said Rana, Kamel Mohamed, El-Beqqali Aziza, Abdel-Rehim Mohamed
Microextraction by packed sorbent for LCMS/MS determination of drugs in whole blood samples. *Bioanalysis. 2010, 2: 197-205*

IV. Rana Said, Anton Pohanka, Mohamed Abdel-Rehim, Olof Beck
Determination of four immunosuppressive drugs in whole blood using MEPS and LC-MS/MS allowing automated sample work-up and analysis. *Submitted to Therapeutic Drug Monitoring*

V. Rana Said, Anton Pohanka, Olof Beck, Maria Andersson, Mohamed Abdel-Rehim
Determination of remifentanil in human plasma by liquid chromatography and tandem spectrometry utilizing micro extraction in packed syringe (MEPS) as sample preparation. *Manuscript*

Related publications not included in the thesis

Said R, Abdel-Rehim M, Sadeghi B, Al-Hashemi S, Hassan Z, Hassan M, Cyclophsophamide pharmacokinetics in mice: A Comparison between retro orbital sampling versus serial tail vein bleeding. *The Open Pharmacology Journal.2007, 1:* 22-27

Ilse Dubbelboer, Anton Pohanka, Rana Said, Olof Beck
Quantification of Tacrolimus and its Three Demethylated Metabolites in Human Whole Blood using LC-ESI-MS/MS. *Manuscript*

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LIST OF ABBREVIATIONS

C2	Ethyl silica
C8	Octyl silica
C18	Octadecyl silica
CE	Capillary electrophoresis
CPA	Cyclophosphamide
CYP	Cytochrome P450
CYS	Cyclosporine
ESI	Electrospray ionization
EVE	Everolimus
GC	Gas chromatography
HPLC	High performance liquid chromatography
IS	Internal standard
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLOQ	The lowest limit of quantification
LOD	Limit of detection
LPME	Liquid phase microextraction
MEPS	Microextraction in packed syringe
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PK	Pharmacokinetics
PP	Protein precipitation
Q	Quadrupole
QC	Quality control samples
RF	Radio frequency
RP-LC	Reversed-phase liquid chromatography
RSD	Relative standard deviation
SIR	Sirolimus
SME	Supported membrane extraction
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Selected reaction monitoring
t½	Elimination half-life
TAC	Tacrolimus
UV	Ultraviolet

1 INTRODUCTION

The measurement of drugs and metabolites in biological samples provides information that plays an important role in toxicokinetic and pharmacokinetic studies, and in therapeutic drug monitoring (TDM). It is often of great importance that results are rapidly obtained and that high numbers of samples can be processed (*1*). Biological samples can not usually be assayed directly but require some preparation before instrumental analysis. The complexity of the sample preparation procedure needed is dependent on the nature of the matrices. Biological fluids such as whole blood, urine and plasma are complex and contain a large number of endogenous compounds, some of them in high concentrations. The sample preparation procedure should effectively isolate the analytes from matrix compounds (*2*).

Bioanalytical methods are many times used for determination of low concentrations of analyte in complex matrices. As a consequence, these methods need to be sensitive, precise, selective and robust. The bioanalytical method consists of two main components. The first is sample preparation, which consists of the techniques used to extract the compounds from the biological fluids such as plasma, serum or urine and whole blood before analysis. The second component is detection of the compound, often by chromatographic separation from other interfering components left in the sample extract.

Recently, developments in the field of bioanalysis have offered new methods for sample preparation as well as for chromatographic separation and detection (*3*). In order to process high numbers of samples, techniques allowing for fully automated systems are needed. In the following text, a short overview on the sample preparation by conventional and newly developed techniques and chromatographic separation and detection will be given.

1.1 SAMPLE PREPARATION

Chromatographic separation and detection techniques often receive the main attention in method development. Timewise, however, the sample preparation is a bottle-neck and can consume about 80% of the analysis time in some bioanalytical methods (*4-5*).

The need for same-day reporting of results for large numbers of biological samples makes high-throughput bioanalysis essential. With the advent of combined liquid chromatography-mass spectrometry (LC-MS), which offers possibilities for developing high through-put and rapid applications, an increase of attention and focus on sample preparation has taken place (*4, 6*). Sample preparation should be considered as an integrated part of the analytical procedure (*5*). Key features when developing sample preparation procedures are automation, integration, on-line and miniaturization (*6-7*).

New trends in sample preparation have appeared, such as sample preparation in 96-well plates and the automation of conventional methods for solid phase extraction (SPE), protein precipitation (PP) and liquid-liquid extraction (LLE).

Also more recent approaches are now available such as liquid-liquid microextraction (LLME), solid phase microextraction (SPME) and microextraction by packed sorbent (MEPS) (*4-5*)**.**

These new approaches both offer some advantages but also suffer from limitations, which explain why the conventional off-line sample preparation methods are still used in routine laboratories.

1.2 PROTEIN PRECIPITATION

Sample preparation by protein precipitation (PP) is often used in combination with LC-MS in bioanalysis (*8*). PP can be used to prepare plasma and blood samples. It is a simple method based on precipitating the proteins and then analysing the supernatant after centrifugation. It can be used both for hydrophobic and hydrophilic compounds. Acidic reagents can be used for precipitation, such as trichloroacetic acid and perchloric acid. However, these strong acids may cause stability problems for compounds that are not stable at low pH. Organic solvents such as methanol, acetone and acetonitrile are alternatives to these acids (*4, 9-10*). Methanol is preferred because it can produce a clearer supernatant suitable for direct injection.

Another common type of precipitation for proteins is salt-induced precipitation. As the salt concentration of a solution is increased, proteins aggregate and precipitate from the solution (*11*).

PP is a rapid and simple sample preparation method, but suffers from the drawback that endogenous compounds other than proteins, such as phospholipids, are still present in the supernatant, which causes matrix effects in the MS analysis (*12*).

1.3 LIQUID-LIQUID EXTRACTION

LLE is a traditional method for sample preparation in bioanalysis (*13*). The principle behind the separation is the distribution of sample components between two immiscible liquids (aqueous and organic). The analyte is extracted from the aqueous phase into the organic phase. Thereafter, the organic layer is transferred and evaporated. Different aspects determine the choice of organic solvents such as:

- distribution coefficient
- density difference between liquid phases
- boiling point, vapor pressure
- flammability, toxicity

The main advantages of LLE methods are large sample capacity, clean extracts and an easy approach to sample concentration. The limitations are: use of environmentally harmful organic solvents, time consuming procedure, difficult to automate, risk for poor recovery and problems with formation of emulsions.

Traditional LLE has been developed and modified in recent years by introduction of liquid phase microextraction (LPME) (*14-16*), single drop-liquid phase micro extraction (LPME) and supported membrane extraction (SME) (*4, 17-19*).

1.4 SOLID PHASE EXTRACTION

Solid phase extraction (SPE) was introduced in the early 1970's and was derived from materials developed for reversed-phase chromatography. The principle of SPE is similar to LLE in that it is based on the partitioning of the analytes between two phases (*6, 20*). The target analytes should have stronger affinity to the solid phase than the matrix components (adsorption step) (*6-7*). In modern SPE, the sorbent is packed between two fritted disks in a polypropylene cartridge and the liquid phase is passed through the cartridge (*21*). SPE cartridges are available in sizes containing 10 mg to 10g of sorbent. The most commonly used materials are silica-based with chemically bonded functional groups or highly cross-linked polymers such as styrene-

divinylbenzene and polymethacrylate. These cartridges are designed for single use in order to eliminate carry-over problems (*6*).

The principal experimental procedure is the following (*4, 6*):

1- Activation the surface of the sorbent by passing of proper solvents (3-5 bed volumes)
2- Application of sample (adsorption step)
3- Washing to remove unwanted interfering matrix components
4- Elution of the analytes

There exists a wide range of SPE sorbents such as non-polar, polar and ion exchange. This classification is based on the chemical character of the functional group attached to the silica. As a consequence, the polarity of the solvent used depends on the chosen solid phase sorbent.

For reversed-phase SPE, the stationary phase is usually silica bonded with alkyl and/or aryl functional groups. C18 and C8 are the most common sorbents used. The packing materials used for SPE and liquid chromatography are similar but particles used in SPE are larger (*4, 6*). Sample preparation by SPE is commonly used in bioanalysis (*4, 6, 22*). It can be performed off-line with automation and on-line integrated with the chromatographic system (*23-26*).

1.5 SOLID PHASE MICROEXTRACTION

Solid-phase microextraction (SPME) was introduced in the beginning of 1990s by Arthur and Pawliszyn (*27*). This innovative and solvent free technology was developed to meet the demand for on-line sample preparation methods (*28-30*). SPME consists of a silica fiber coated with a liquid polymer or a solid sorbent or a combination of both. In this technique the coated fiber is exposed to the liquid sample for a specific period of time. There are several types of SPME device being used, coated fibers, vessels, steel bars, disks and coating on the inside of the tubes. The most popular device is the coated fiber type (*4, 28-32*).

SPME has been used in applications for analysis of flavors, fragrances and in the forensic field. SPME is usually used together with gas chromatography (GC) (*4, 33*). This technique has some disadvantages such as limited capacity and high cost.

1.6 MICROEXTRACTION IN PACKED SYRINGE

MEPS is a new technique and represents an attempt of SPE miniaturisation using much reduced sorbent. It is based on using a syringe with a special needle as an extraction device, Figure 1. Sample preparation takes place on the packed bed which can be packed or coated to provide selective sampling. MEPS was developed in the AstraZeneca research laboratory in Sweden to meet the need for high-throughput analysis (*34-36*). MEPS was first demonstrated in two patents issued 2003 (*35-36*). The first scientific publication appeared in 2004 (*34*), about using MEPS for determination of local anesthetics by using both LC and GC for chromatographic detection.

MEPS can be connected on-line to LC, GC and capillary electrophoresis without further modification. In MEPS the sorbent is inserted directly into the needle. The MEPS sorbent cartridge can be used for over 100 injections depending on the sample matrix. Another important feature is that MEPS can handle sample volumes starting from 10 µL and up to 1000 µL.

MEPS can be used both manually and fully automated (the sampling, extraction and injection are on-line).

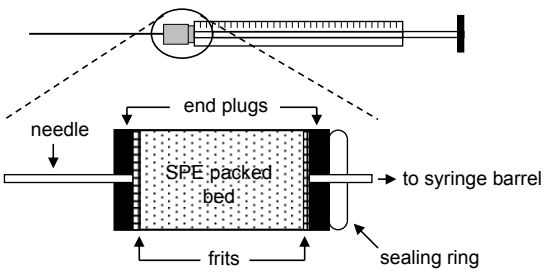


Figure 1. Schematic picture of a MEPS syringe with integrated sorbent.

The biological fluid is diluted before sample loading. For plasma the dilution factor is typically 5, while for whole blood it is typically 25 (*37*). Thereafter, the sample is loaded by withdrawing and ejecting in the sample vial or ejecting to the waste. The loading step can be repeated many times to increase recovery of the analyte. Step two is the washing of the sorbent, to remove proteins and other unwanted material. The third

step is the elution with organic solvent directly to the LC or GC injector, step four. Step five is washing A and B and takes place after elution to eliminate the carry-over and conditioning the material for further use. Wash A is usually the elution solution and wash B is the washing solution used after sample loading, for a schematic presentation of the MEPS procedure, see Figure 2.

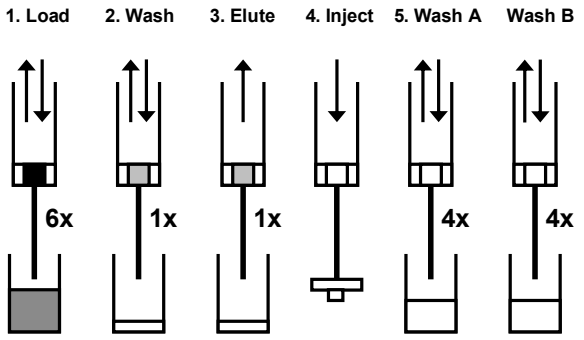


Figure 2. The five steps involved in the sample preparation by microextraction in packed syringe (MEPS). Number of iterations is taken from the protocol used in paper IV.

1.6.1 Operation of MEPS

In MEPS, multiple sampling can be performed (draw-eject). Recovery from MEPS can be affected by different factors, mainly the used sorbent and the influence of number of extraction cycles. The extraction recovery of MEPS can be calculated as the following:

$$\text{Recovery} = \frac{\text{Response of extract}}{\text{Response of direct injection of the same concentration}} \times 100$$

Extraction efficiency can be investigated by number of sample loading needed to reach the required sensitivity. During the method development investigation of the number of loading cycles that is needed for the required sensitivity should be performed.

Carry-over is an expected problem when using MEPS. This phenomenon can lead to bad accuracy and precision (*5, 37-38*). Carry-over should be estimated for every procedure. The degree of carry-over depends partly on the chemical nature of the

analyte and can be generated from adsorption in the syringe or generated in the LC system.

1.6.2 Application of MEPS

This technique has been successfully used to extract wide range of drugs and their metabolites from different biological matrices such as plasma (*34, 38-51*), urine (*44, 46, 52-58*) and blood (*59-62*). Other application have been investigated like polycyclic aromatic hydrocarbons in water (*63*), wine (*64*) and hair (*65*). MEPS has been connected on-line to LC and GC mass spectrometry (*5, 34, 37-39, 41-43, 51, 53-54, 63*).

The technique can replace most existing SPE methods by scaling the reagents and sample volume. Some review articles are available about MEPS technique discussing the factors affecting the performance of MEPS and the recent advances in MEPS (*5, 37-38, 66*).

1.7 ANALYTES

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. 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 concentration and therapeutic and or toxic effect has been established. In this thesis different categories of compounds were chosen to be a model for MEPS performance evaluation. Developing high throughput analysis utilizing small sample volume method for these compounds will be of good value in pediatric pharmacokinetic studies and TDM of these compounds in children.

1.7.1 Cyclophosphamide

Cyclophosphamide, an alkylating agent, is widely used as an anticancer and immunosuppressive agent, Figure 3. Alkylating agents are the oldest group of cytostatics and they are still the corner stone for cancer therapy (*67-70*). Their use in cancer treatment

started in the early 1940's. Cyclophosphamide is a prodrug that undergoes a 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). (*69, 71-74*). The given doses are between 2-200 mg/day and t½ about 7 hours.

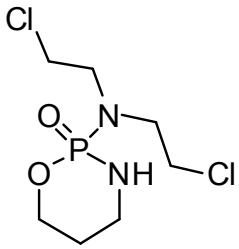


Figure 3. Cyclophosphamide structure.

1.7.2 Anaesthetics

Local anaesthetics consist of two families: aminoamide and aminoester local anaesthetics. They produce profound anaesthesia for several hours.

1.7.2.1 Lidocaine

Lidocaine (2-(diethylamino)-N-(2,6-dimethylphenyl) ethanamide) was the first member of the aminoamide-type local anaesthetics group, Figure 4. It acts by blocking the fast voltage gated sodium (Na+) channels in the cell membrane of the neurons, thereby blocking the neurotransmission. Lidocaine is applied topically or by local injection, is absorbed rapidly and has a rapid onset of action. Approximately 90% of the dose will be metabolized in the liver, mainly by CYP1A2 (and to a minor extent by CYP3A4), to pharmacologically active metabolites. The t½ of lidocaine is approximately 1.5–2 hours (*75-77*) and the given doses are between 50-300 mg iv.

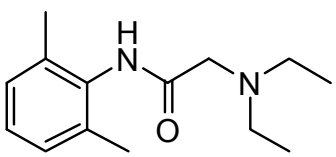


Figure 4. Lidocaine chemical structure.

1.7.2.2 Bupivacaine

Bupivacaine is another member of the aminoamide type local anaesthetics group, Figure 5. It is administered for local anaesthesia including infiltration, nerve block, epidural, and intrathecal anaesthesia (*76*). It is the most toxic among all of the anaesthetic drugs. The main use of bupivicaine is to prevent post-operative pain after surgical procedures. The given doses are 25-150 mg epidural with t½ between 2.4-3.6 hours (epidural).

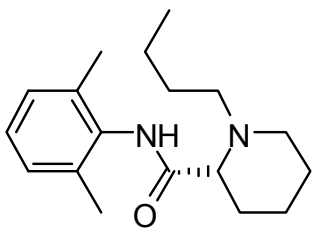


Figure 5. Bupivacaine chemical structure

1.7.2.3 Ropivacaine

Ropivacaine is a local anaesthetic agent which is chemically homologous with bupivacaine, Figure 6. Ropivacaine is metabolized by CYP1A2. The elimination half-life of ropivacaine is 2-6 hours and therefore has a longer duration of action than lidocaine. Ropivacaine can also pass the blood-brain barrier and induces toxicity to the central nervous system if it reaches the systemic circulation in sufficient concentrations (*78-81*). The given doses are 75-200 mg epidural and t½ 3.6 -6 hours (epidural).

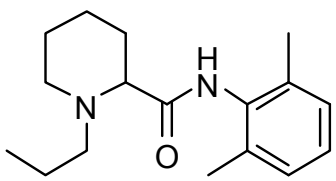


Figure 6. Ropivacaine chemical structure

1.7.3 Immunosuppressive drugs

Immunosuppressive agents are drugs used to suppress the normal activity of the immune system (*82*). They are used to prevent rejection of organs after organ transplantation and also in the treatment of autoimmune disorders. The key factor for

successful organ transplantation is effective immunosuppressive therapy. The biggest challenge for organ transplantation is meeting the need for immunosuppressant to prevent rejection, meanwhile minimizing drug-induced toxicities (*82-83*).

Immunosuppressive treatment of the transplantation patient begins with the induction phase, immediately after transplantation maintenance therapy then follows life of the allograft. Induction and maintenance strategies include choosing medicines at specific doses or at doses adjusted to achieve target therapeutic levels to give the transplantation patient long survival.

1.7.3.1 Cyclosporine

Cyclosporine was introduced to the clinic during the early 1980s, Figure 7. It was the first immunosuppressive that was subjected to therapeutic drug monitoring (TDM). TDM of cyclosporine is an essential tool in the managements of transplant recipients. Cyclosporine displays a large degree of inter-and intra- individual pharmacokinetic variability. The given doses range between 1-15 mg/kg/day with t½ between 5-20 hours.

1.7.3.2 Tacrolimus

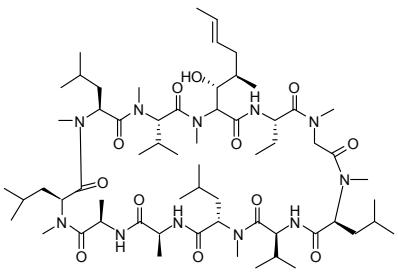
Tacrolimus, Figure 7 is a macrolide antibiotic and its active against helper T cells; preventing the production of IL-2 via calcineurin inhibition. This drug is used for maintenance immunosuppression and for saving the patients with refractory rejection under cyclosporine-based therapy. The given doses between 0.075-0.30 mg/kg/day with t½ between 12-16 hours.

1.7.3.3 Sirolimus

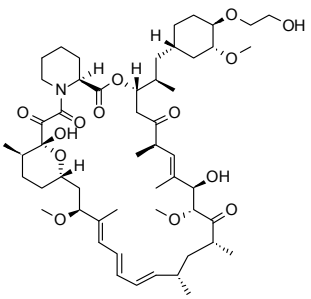
This immunosuppressive agent was approved by the FDA for prevention of kidney transplant rejection in 1999, Figure 7. Sirolimus is a macrolide antibiotic that binds to the FK-binding protein, its mechanism of action is via the mTOR. It inhibits G1- to S-phase cell division and, by that, cell proliferation. This agent is used for maintaining immunosuppression and to prevent rejection. The given doses are 2-6 mg/day with t½ 62 hours.

1.7.3.4 Everolimus

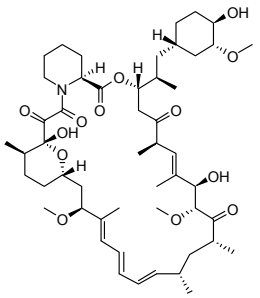
Everolimus is a rapamycin analogue, Figure 7. In cancer cells, everolimus inhibits mTOR, a protein that acts as a central regulator of tumor cell division, cell metabolism and blood vessel growth. Everolimus is a once-daily oral therapy that provides continuous inhibition of mTOR. It is used as a complementary immunosuppressant to cyclosporine-tacrolimus based protocols in transplant recipients. The given dose is 1.5 mg/day with t½ of 30 hours.



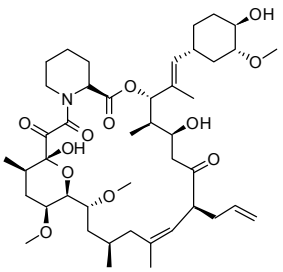
Cyclosporine



Everolimus



Sirolimus



Tacrolimus

Figure 7. Chemical structures of the immunosuppressive drugs.

1.7.4 Remifentanil

Remifentanil is a synthetic opioid and a fentanyl analogue, Figure 8. It has approximately the same potency as fentanyl but remifentanil has unique pharmacokinetic characteristics: a very rapid achievement of the peak effect of an intravenous dose, a consistently rapid elimination of the drug with complete cessation of its effects within five to ten minutes after discontinuing its administration (*84-87*).

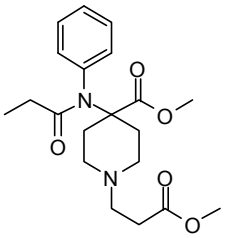


Figure 8. Remifentanil chemical structure

1.8 LIQUID CHROMATOGRAPHY

The Russian botanist, Mikhail S. Tswett is the father of chromatography and he started his pioneer work early 1900s in separating compounds (pigments), extracted from plants by using packed columns (*88*). Tswett used open glass columns with calcium carbonate and aluminum particles and noticed different coloured bands and related it to the separation of the compounds. The separation technique was named chromatography being obtained from the Greek words chroma, (colour) and graph, meaning colour writing (*89*).

Modern HPLC is considered a further development of Tswetts original chromatographic principle. Solvents of different compositions make up the mobile phase, and the particles are the stationary phase (*90-91*). The separation of the compounds is due to partitioning between the stationary phase and the mobile phase. It was not until 1970s, that commercial liquid chromatography instruments appeared. Before that, different techniques had been used e.g. open-column chromatography, paper chromatography, and thin layer chromatography. These were only used for qualitative analysis and were suffering from lack of separation power between similar compounds.
By the 1980s, the HPLC technique was well established and is still used for many bioanalytical applications. Much advancement in column material and design has taken place, and computers and automation were added to the HPLC systems (*89, 92*).

1.8.1 Ultra performance liquid chromatography

The underlying principles behind the recent development of "ultra performance" LC (UPLC) is based on the van Deemter equation that describes the relationship between the flow rate and the theoretical plate height (the height equivalent to a theoretical plate, HETP). Since the particle size is one of the variables, using a column with smaller sorbent particles will enhance the chromatographic performance. If the particle size decreases to less than 2.5 μm, the efficiency will increase substantially even at high flow rate. In practice sub 2 μm particle columns are used for this purpose. These columns generate a higher back pressure so they must be connected to an instrument that can deliver solvents at

the high pressure and at constant flow. Commercial systems are available for UPLC. This technique offer shorter analysis time and better sensitivity in addition to increased separation power (*93-96*).

1.8.2 Monolithic columns

Monolithic columns exhibit high separation efficiency at high flow rate and are not made of packed particles. A special feature of monolithic material is the high porosity. This feature gives the column the ability for low flow resistance at high speed of the mobile phase, which enhances the rate of the mass transfers of the sample molecules through the beds of the stationary phase. Monolithic material can be made of both organic and inorganic polymers (*97*).

1.8.3 Detection in chromatography

1.8.3.1 Photometric detection

The photometric detector is a common LC detector and measures the amount of light absorbed at selected wavelength of the visible or UV region. The main features of this detector are universal applicability and easiness of use. Photometric detectors have some limitations towards compounds that do not posses a UV chromophore (i.e. aromatic rings or double bonds). Lack of selectivity and limited qualitative information makes UV-detectors less suitable to use for identification purposes (*98*).

1.8.3.2 Mass spectrometry detection

Mass spectrometry is related to gas-phase separation of ionised species according to their mass-to charge ratio (*m/z*) ratio. This type of detection offers high sensitivity and selectivity. Mass spectrometry in bioanalysis has progressed dramatically through the last twenty years.

The basic components of the mass spectrometer are an inlet device, an ion source, ion separation system (mass analyzer) and detector. Electrospray ionisation (ESI) is the interface that was used with the equipment for the generation of results for this thesis. In the ESI the analyte solution passes through the electrospray needle that is kept at a high potential. Charged droplets are sprayed from the needle with surface charge of the same polarity equal to the charge on the needle. Droplet formation is assisted by the nebulizer gas, usually nitrogen. Also neutral compounds can be transferred to ionic form by de-protonation, protonation or cationisation and can thus be analysed by MS (*99*), Figure 9.

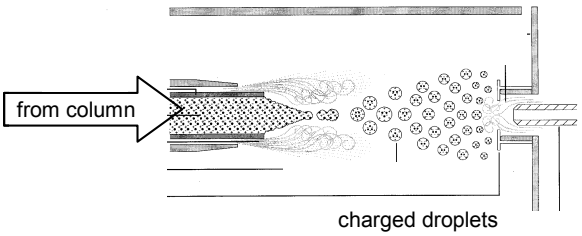


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Figure 9. Diagram of an electrospray ionization source.

There are three steps involved in transferring ions from solution to gas phase

- Dispersal of fine spray from a region of a strong electrical field
- Solvent evaporation by heat or dry gas or both
- Ions evaporating from the surface of the droplet, droplets shrink until they reach the level where the surface tension can not stand the charge. At this point the droplets disintegrate and produce smaller droplets that can disintegrate further.

The quadrupole mass analyser consists of four parallel rods that have fixed DC and alternating RF potentials applied to them. The length and the diameter of the rods determine the mass range and the resolution of the equipment. Ions produced in the source of the instrument are focused and passed along the middle of the quadrupole filter. Their motion will be influenced by the electric field so that only ions with a given *m/z* interval will be in resonance and pass onto the detector.

The selective power of MS can be increased if the quadrupole filters are linked together in series, tandem mass spectrometry. (Q1) and (Q3) quadrupoles serve as mass filters, while (Q2) quadrupole function as a collision cell where fragmentation by high energy collisions take place, Figure 10. The ions passed through Q1 are named precursor (or parent) ions. The ions resulted from the fragmentation are called product (or daughter) ions (*99-101*). Selecting a particular ion or ions to monitor will increase the sensitivity; this process is called selected reaction monitoring (SRM).

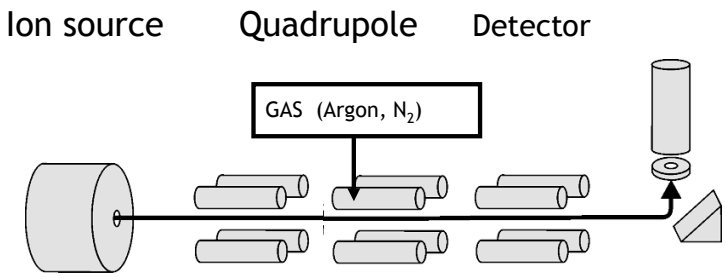

Figure 10. A triple quadrupole ESI mass spectrum.

The introduction of MS/MS systems in bioanalytical work started already in the late 1980s when flow injection analysis FIA-MS/MS methods were introduced for neonatal screening assays (*8, 102*). Tandem mass spectrometry (MS/MS) is now an established powerful analytical technique and it has attained a predominant role over other techniques (*100, 103-104*). The combination of LC-MS/MS fulfils most of the analytical needs for laboratories involved in laboratory medicine, both in research and routine laboratories. Within the last ten years the sensitivity of the instruments has improved significantly. This has been possible by optimizing the process of atmospheric pressure ion generation and transfer of ions into the high vacuum area of the instrument (*105*). The major innovation was applying the ion spray orthogonal to the main axis of the mass spectrometry.

An LC-MS/MS-system offers some advantages over GC-MS in the clinical laboratories, since it is applicable also to polar and large molecules. For the routine applications in the TDM field, methods for quantification of immunosuppressant drugs, the new generation of anticonvulsive and antipsychotic dugs has been developed (*8*). There is an increasing interest to replace existing methods in pharmacology, endocrinology and toxicology with LC-MS/MS. However, implementation of LC/MS-MS methods still require substantial amount of expertise. LC-MS/MS has the potential to improve the performance of laboratory medicine and replace the predominant methods using immunoassays. This will need full automation and development of high-throughput applications (*8, 102*).

2 AIMS

The aim of the present work was to explore MEPS as sample preparation method by developing on-line and automated quantitative methods for therapeutic drugs that combine sample preparation with LC-MS/MS

More specifically, the aims were:

- Develop a new LC-MS/MS analytical method for cyclophosphamide determination in plasma.
- Develop a new LC-MS/MS analytical method for cyclophosphamide detection in whole blood to facilitate *in vivo* studies.
- Develop a new LC-MS/MS analytical method for lidocaine, ropivacaine and bupivacine detection in whole blood.
- Develop a new LC-MS/MS analytical method for determination of immunosuppressive drugs in whole blood.
- Develop a new LC-MS/MS analytical method for remifentanil detection in plasma to be employed for neonatal clinical studies.

3 MATERIAL AND METHODS

3.1 SUBSTANCES

Study (I) and (II)

Cyclophosphamide was purchased from Sigma Chemical Company (St. Louis, MO). Ifosfamide was used as the internal standard and obtained from ASTA Medica AG (Frankfurt, Germany).

Study (II)

Lidocaine-d3 (IS) was supplied by Department of Medicinal Chemistry, AstraZeneca, Södertälje, Sweden.

Study (III)

Lidocaine, ropivacaine, bupivacaine and lidocaine-d3 (IS) were supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje, Sweden).

Study (IV)

Cyclosporine and everolimus were provided by Novartis (Basel, Switzerland) and everolimus also purchased from Fluka, sirolimus was provided by Wyeth (Madison, NJ), tacrolimus was provided by Astellas Pharma Inc (Tokyo, Japan). The internal standards ascomycin (ASC) and cyclosporine D (CYD) were purchased from Alexis Biochemicals (San Diego, CA).

Study (V)

Remifentanil hydrochloride and the internal standard remifentanil-$^{13}C_6$ hydrochloride (IS) were purchased from Toronto Research Chemicals (North York, Canada).

3.2 REAGENTS

All substances and chemicals were of analytical grade except for, methanol and ammonium formate in study (IV) which were of LC-MS grade. The water used in the analysis for all the studies was Reagent Grade Milli-Q Plus water (Millipore Co, Bedford, MA).

3.3 BIOLOGICAL SAMPLES

Study (I)

Blank human plasma was obtained from AstraZeneca blood bank. Patient samples were obtained from patients who had been subjected to allogenic bone marrow transplantation at Karolinska Huddinge hospital.

Study (II)

Blank mouse blood was obtained from AstraZeneca, Södertälje

Study (III)

Blank human blood was obtained from AstraZeneca blood bank.

Study (IV)

Blank human blood used for calibration and controls were purchased from the Karolinska University Hospital blood bank. Patient samples were obtained from decoded left-over patient blood samples from the TDM service at the Clinical Pharmacology Department.

Study (V)

Blank plasma was obtained from Karolinska University Hospital blood bank.

Patient samples used for method comparison were decoded left-over patient blood samples from the TDM service at the Clinical Pharmacology Department.

3.4 SAMPLE PREPARATION

The MEPS conditions for different analytes and matrices (human plasma in study I and V; mouse blood in study II and human blood in study III and IV) were optimized to the protocols presented below. In all studies, the MEPS was handled by a CTC-PAL autosampler. All new MEPS cartridges were manually activated and equilibrated with 50 µL methanol followed by 50 µL of water before being used. A compilation of the MEPS approaches is found in Table 1.

Table 1. Compilation of the MEPS protocols used in Study I-IV

Study	Compound type	Analyte	Matrix/ dilution factor	Sorbent	Sampling vol; No of sample loadings	Wash/ equilibration steps n/n	Samples/ cartridge
I	Cytostatic	Cyclophosphamide	Plasma/5	C2	25 µL;1	4/4	100-150
II	Cytostatic	Cyclophosphamide	Blood/25	C2	100 µL;4	4/4	40-100
III	Local anaesthetics	Lidocaine, Ropivacaine Bupivacaine	Blood/25	C18	100 µL;3	3/3	~50
IV	Immunosuppressive	Cyclosporine, Everolimus, Sirolimus Tacrolimus	Blood/25	C8	100 µL;6	4/4	100-120
V	Anaesthetics	Remifentanil	Plasma/9	M1(mixed mode)	50 µL;4	4/4	~50

3.5 INSTRUMENTS AND MATERIAL

Study I

MEPS-LC-MS/MS: Zorbax (SB-C8, 50×2.1mm, 3.5µm) column obtained from Agilent (Santa Clara, CA) was used with Gradient HPLC system.

Triple quadrupole mass spectrometric instrument Micromass QII-spray (Waters Corporation, Manchester, UK) equipped with a Z-electrospray interface operated in positive ion mode.

LLE-LC-UV: HPLC isocratic mode was used, using Phenomenex LC column, polar-RP 80A (3.0 ×150 mm, 4µm).

The detector was Milton Roy Spectro Monitor 3100 (Ivyland, PA) and the UV detection wavelength was 195 nm.

Study II

Ace C18 (100×2.1 mm, 3µm) column obtained from Advanced Technologies (Aberdeen, Scotland) was used with HPLC gradient mode.

Triple quadrupole mass spectrometric instrument Micromass QII-spray (Waters Corporation, Manchester, UK) equipped with a Z-electrospray interface operated in positive ion mode was used.

Study III

Gradient HPLC mode was used using Zorbax SB-C8 (50×2.1mm, 3.5µm) column obtained from Agilent (Santa Clara, CA).

Triple quadrupole mass spectrometric instrument Micromass QII-spray (Waters Corporation, Manchester, UK) equipped with a Z-electrospray interface operated in positive ion mode.

Study IV and V

Kinetex C18 column (50×2.1mm, 2.6µm) obtained from Phenomenex (Torrance, CA) was used as analytical column; Hypersil Gold C8 (10×2.1 mm) obtained from Thermo Scientific was used as guard column with gradient HPLC system.

Triple quadrupole mass spectrometer (TSQ Quantum, Thermo Scientific, Waltham, MA) equipped with electrospray ionisation source (ESI).

4 RESULTS

4.1 DESIGN AND METHOD DEVELOPMENT

4.1.1 Study I

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)

A MEPS procedure for cyclophosphamide quantification in plasma was developed by testing different sorbents, C2, C8, C18 and polystyrene. The highest recovery was obtained with C2, when plasma sample was diluted four times with water. Washing solution volume was optimized. 30 µL of 95:5 methanol - water was used to elute cyclophosphamide. Ifosphamide was used as internal standard since it is a cyclophosphamide structural isomer. LLE-HPLC-UV was used as reference method.
The analysis was based on a C8 analytical column. Cyclophosphamide and the internal standard were co-eluted and no cross-talk was observed between the SRM channels. The retention time for cyclophosphamide was 4.45 min with a total analysis time of 5.0 min. Detection and quantification was performed using SRM.

LLE-HPLC-UV
This reference method was based on previous published methods utilizing LC-UV with modification of the mobile phase and the column (*106-107*). Different types of C8 columns were tested for better separation. Liquid extraction was used for sample preparation. The sample preparation time was 45 min. The retention time for cyclophosphamide using this method was 16.5 min. The analysis time was 30 min, this was due to the presence of a peak eluting after cyclophosphamide.

4.1.2 Study II

On-Line Determination of Cyclophosphamide in Blood Samples Utilizing Microextraction by Packed Sorbent and Liquid Chromatography Tandem Mass Spectrometry (MEPS-LC-MS/MS).

A prerequisite for method development was the possibility to use 20 µL blood for sample analysis in order to facilitate pharmacokinetic experiments in mouse by using serial tail sampling.

Based on the previous study, MEPS-C2 sorbent was chosen for cyclophosphamide quantification in the diluted blood, with a dilution factor of 25. The IS was $^{2}H_{3}$-lidocaine. Pumping the sample 4×100 µL was enough to gain the required sensitivity. The carry-over was eliminated by four washing cycles of both the elution solution followed by the washing solution. Gradient HPLC was used. The scan mode was SRM using the proton adduct as precursor ion $[M+H]^{+}$.

4.1.3 Study III

Microextraction by packed sorbent for LC–MS/MS determination of drugs in whole blood samples.

Local anaesthetics in this study were quantified from whole blood samples. The method was developed to handle small sample volumes. In the study MEPS-C18 was used to extract the local anaesthetic compounds from whole blood while C8 guard and analytical columns were used. Only water was used for washing to prevent analyte leakage. Due to the basic nature of the analytes the elution solution was methanol-water containing 0.25% ammonium hydroxide. Three washing cycles of elution and washing solution were capable of reducing the carry-over to 0.02%. The packing bed was using for about 50 samples before it was discarded. The scan mode was SRM using the proton adduct as precursor ion $[M+H]^{+}$.

4.1.4 Study IV

Determination of four immunosuppressive drugs in whole blood using MEPS and LC-MS/MS allowing automated sample work-up and analysis.

The chromatographic system was based on a 50 mm reversed-phase C18 column and used a gradient of methanol: 0.1% formic acid from 43% to 98% in 2.5 min. A C8 guard column was used to protect the chromatographic column and did not prolong retention. In this system, which was developed to obtain a shorter time of analysis, the analytes and internal standards were not fully separated. The first eluting substance was ASC (1.60 min), which was used as internal standard for EVE, SIR and TAC. These eluted within 0.1 min after ASC and overlapped chromatographically. CYS eluted slightly before its internal standard CYD and these also overlapped chromatographically.

Detection and quantification was performed using the SRM, selective detection was achieved in the mass spectrometer by detecting products ions formed from the ammoniated molecules. No signs of interference due to cross-talk between the SRM channels were observed. Formic acid and ammonium formate were added to both mobile phase A and B and the concentrations were optimized for sensitivity and chromatographic performance.
The MEPS procedure was developed by manual operation and was then transferred to the CTC-PAL platform.

4.1.5 Study V

Determination of remifentanil in human plasma by liquid chromatography and tandem spectrometry utilizing micro extraction in packed syringe (MEPS) as sample preparation

MEPS was used to quantify remifentanil in whole plasma utilizing only 20 µL. For development of the MEPS procedure a generic SPE protocol for basic compound extraction was followed using different sorbent materials. Different percentages of methanol were tested for washing and elution to obtain the highest recovery. The different sorbents tested were C2, C4, C8, C18 and M1 (mixed C8 and cation exchange). The highest recovery was obtained with M1 sorbent, Figure 11. The method development was done manually using spiked plasma with 20 ng/mL of remifentanil.

Remifentanil-$^{13}C_6$ was available commercially and chosen as internal standard to provide best possible quantification. A reversed-phase chromatographic system was selected using a Kinetex C18 column in order to achieve a rapid analysis time and a low back pressure.

The mass spectrometric detection was performed with SRM in order to achieve high selectivity and sensitivity. Monitoring conditions were optimized for remifentanil and the IS. Positive electrospray ionisation was chosen, which gave the protonated molecules as major species and these were selected as precursor ions. A number of intense product ions were observed.

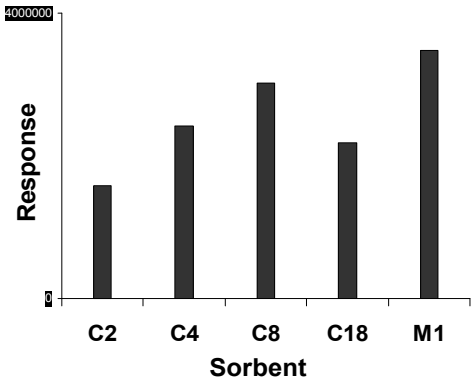

Figure 11. Comparison between different MEPS sorbents on the recovery (%) of remifentanil.

4.2 METHOD VALIDATION

4.2.1 Linearity

For all studies linearity was assessed by calibration curves generated from several concentration levels for each analyte. The calibration curve was constructed by linear regression of the analyte/IS peak area ratio against the concentration. The calibration curve used $1/x^2$ as weighing factor. The validation results of the linearity for all the studied analytes are listed in Table 2.

Table 2. Summary of the linearity results for all the studied analytes.

Analyte	Unit	Linearity range	r^2
CPA plasma	µg/mL	0.50-150	0.999, n=6
CPA blood	µg/mL	0.10-100	>0.99, n=3
Lidocaine	nmol/L	10-10 000	0.996, n=3
Ropivacaine	nmol/L	10-10 000	0.999, n=3
Bupivacaine	nmol/L	10-10 000	0.995, n=3
CYS	ng/L	3-1500	0.999, n=6
EVE	ng/L	0.5-50	0.998, n=6
SIR	ng/L	0.5-50	0.994, n=6
TAC	ng/L	0.5-50	0.993, n=6
Remifentanil	ng/L	0.05-50	0.999, n=6

4.2.2 Accuracy and precision

Accuracy and precision were studied by running three or four levels of QC samples with replicates for each concentration during several days. Each day new calibration curve was prepared. The accuracy and precision results are summarised for all the analytes in Table 3.

Table 3. Summary of accuracy and precision results for all the studied analytes.

Study	**Analyte**	**Accuracy range**	**Precision, CV% range**	
			Intra-day (n=6), %	Inter-day (n=18), %
I	Cyclophosphamide in plasma	95-106	1.0-5.0	5.0-9.0
II	Cyclophosphamide in blood	102-110	2.0-7.0	4.0-6.0
III	Lidocaine	90-109	1.0-3.0	2.0-7.0
III	Ropivacaine	85-97	1.0-3.0	2.0-12.0
III	Bupivacaine	89-97	2.0-5.0	5.0-12.0
IV	Cyclosporine	102-103	4.2-6.2	5.1-7.1
IV	Tacrolimus	103-106	2.0-8.4	7.4-9.1
IV	Sirolimus	102-108	3.8-5.0	6.8-8.9
IV	Everolimus	103-109	3.5-11.7	6.3-13.7
V	Remifentanil	97.8-102	4.4-6.3	6.3-11.3

4.2.3 Limit of detection and quantification

Lowest limit of quantification (LLOQ) was defined as the lowest point in the standard curve with precision not exceeding 20%. Limit of detection (LOD) was estimated at a signal to noise ratio of 3. This concentration was reached by dilution the lowest point of

the standard curve with blank plasma. Limit of detection and quantification for all the analytes are summarised in Table 4.

Table 4. Summary of the LOD and LOQ results for all the studied analytes

Study Number	Analyte	LOD	LOQ
I	Cyclophosphamide in plasma µg/mL	0.005	0.50
II	Cyclophosphamide in blood µg/mL	0.005	0.10
III	Lidocaine, nmol/	3.5	10.0
III	Ropivacaine, nmol/l	3.5	10.0
III	Bupivacaine, nmol/l	3.5	10.0
IV	Cyclosporine, ng/ml	0.9	3.00
IV	Tacrolimus, ng/ml	0.15	0.50
IV	Sirolimus, ng/ml	0.15	0.50
IV	Everolimus, ng/ml	0.15	0.50
V	Remifentanil, ng/ml	0.02	0.05

4.2.4 Selectivity

Using MEPS as sample preparation provided selective methods, in study I, II and III selectivity was investigated by analysing different blank blood samples with and without adding IS. LC-MS/MS analysis showed no interferences from endogenous compounds, see Figure 12. In study IV the selectivity was studied by running three different blank samples while monitoring the transitions for the four analytes moreover for each of the analyte, 20 blood samples from patients not receiving the respective drug were analysed for interfering peaks. No interferences were detected. Also in study V the selectivity was tested by analysing 20 randomly selected patient samples from TDM laboratory for patients treated with antiepileptic drugs.

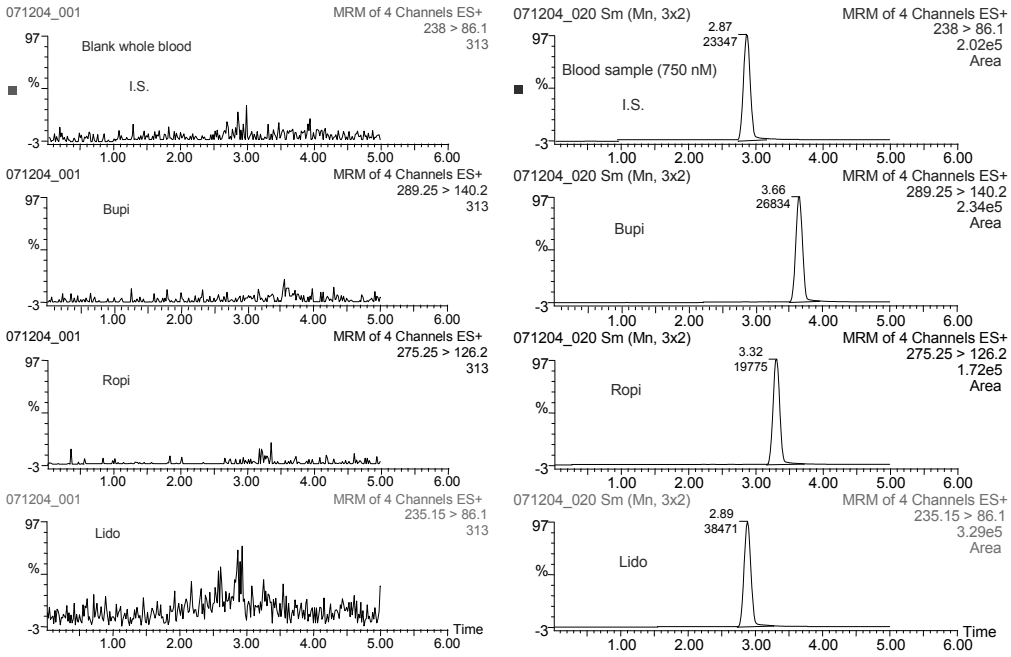

Fig 12. Representative chromatograms with mass spectrometric detection obtained from blank blood sample (left) and from human blood spiked with lidocaine, ropivacaine, bupivacaine (750 nmol/L each) and IS (right).

4.2.5 Matrix effect

Two approaches were followed to investigate the matrix effect; post column infusion and post-extraction spike methods in study IV (Figure 13) and study V (Figure 14). No significant matrix effect was observed at the retention time of the analytes. In study II no significant matrix effect (ion suppression) at cyclophosphamide retention time was observed through post column infusion experiment.

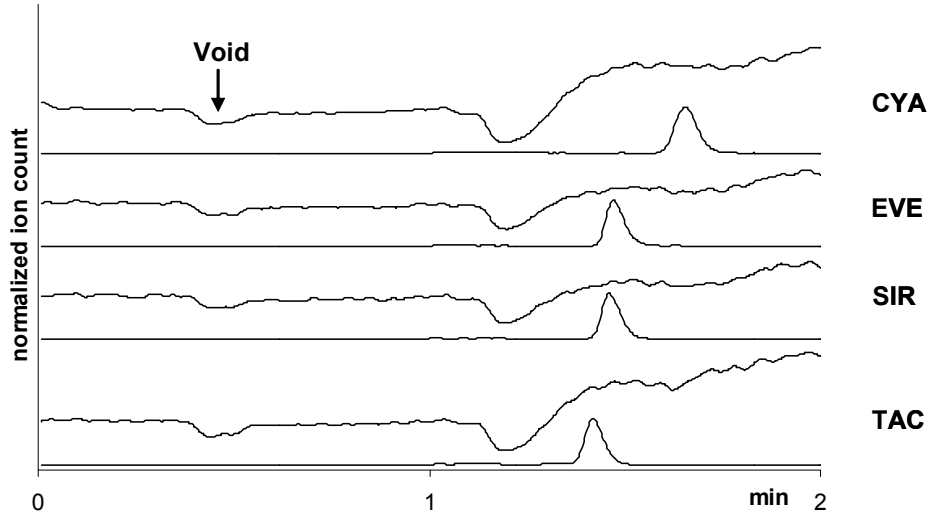

Figure 13. Matrix effect after injecting a blank blood extract for study IV. The analytes were infused post column at a flow rate of 10 μL/min. For comparison the retention time of the analytes are shown in the lower traces.

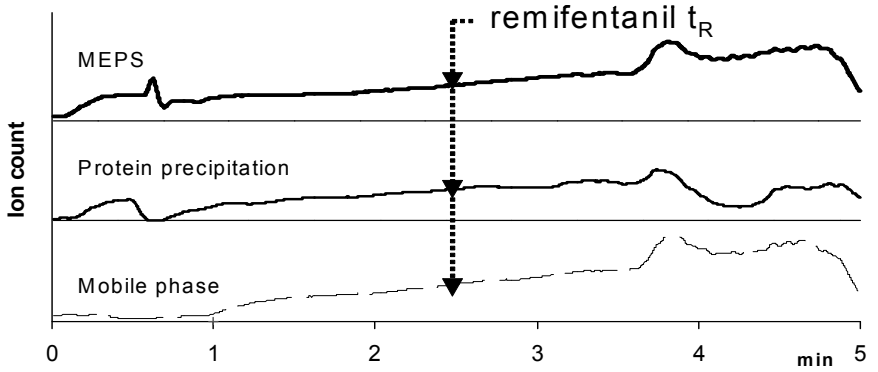

Figure 14. Results from post column infusion experiment in study V.

4.2.6 Carry-over

Carry-over was investigated by running a blank sample or elution solution after the highest standard concentration. The carry-over was reduced by washing the sorbent a number of times with washing solution followed by elution solution. The measured carry-over was between 0.02-0.40% among all the investigated analytes.

4.2.7 Method comparison and application of the methods

In study I method comparison was made between MEPS-LC-MS/MS and LC-UV for cyclophosphamide determination in plasma (Figure 15). 170 patient samples were analysed by both methods with mean ratio between 1.02±0.11 ranges from 0.81-1.19.

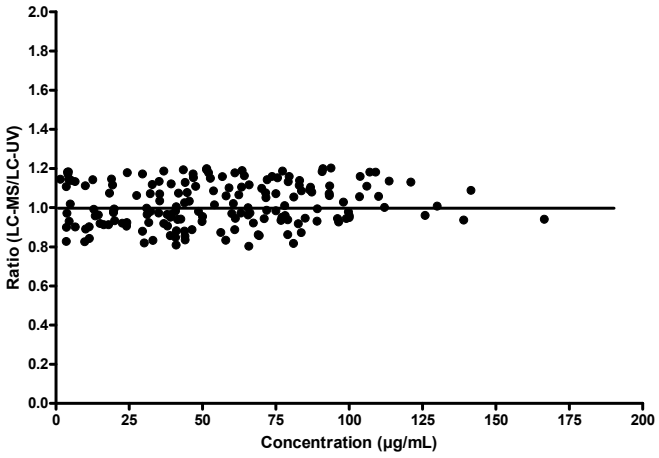

Figure 15. The ratio of patient plasma concentrations obtained by LC-MS/MS / LC-UV plotted against concentrations obtained using LC-MS/MS.

In study IV, method comparison was done between MEPS-LC-MS/MS, protein precipitation LC-MS/MS and immunoassay methods. The ratio (immunoassay/MEPS) were as the following:

CYS: 1.24±0.41 (r^2 =0.97, n=20), EVE: 1.48± 0.23(r^2 =0.84, n=20), SIR: 0.97± 0.11 (r^2 =0.98, n=20) and TAC: 1.36 ± 0.41 (r^2 =0.95, n=20).

The comparison for MEPS-LC-MS/MS and protein precipitation LC-MS/MS showed that the new MEPS method results were in agreement with protein precipitation LC-MS/MS (Figure 16).

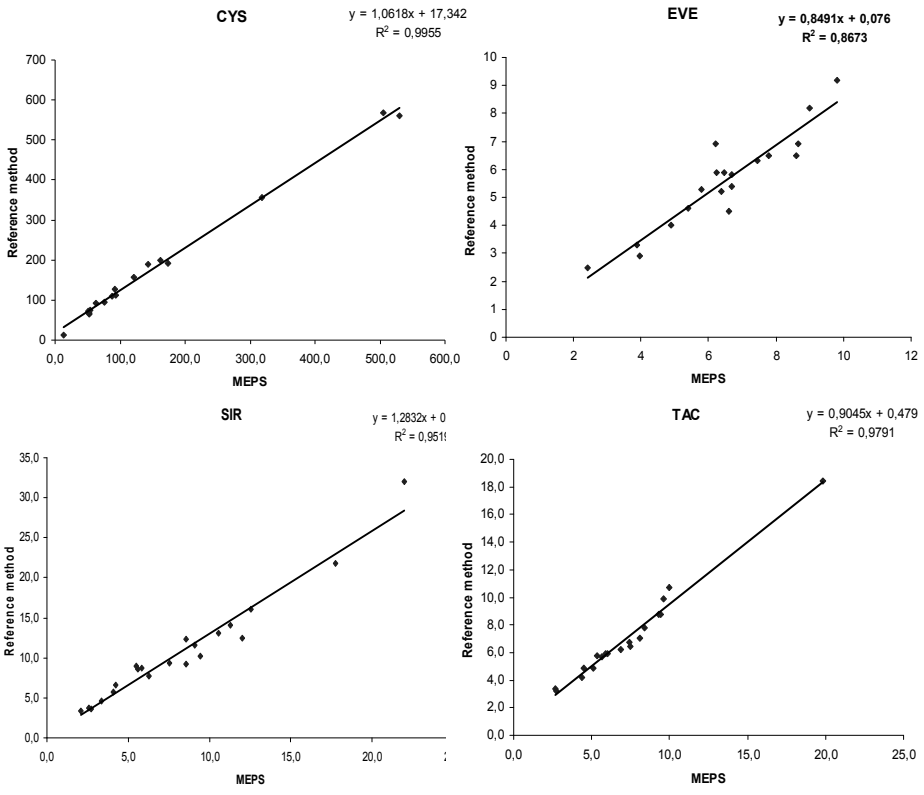

Figure 16. Method comparison between MEPS and the reference method protein precipitation (n=20 for each).

4.2.8 Extraction recovery and extraction efficiency

Extraction recovery for MEPS in study II and III was calculated by comparing the peak area of extracted QC samples (low and high) with peak area obtained from standards prepared in mobile phase, this experiment was done off-line. The extraction recovery was over 90% (Figure17).

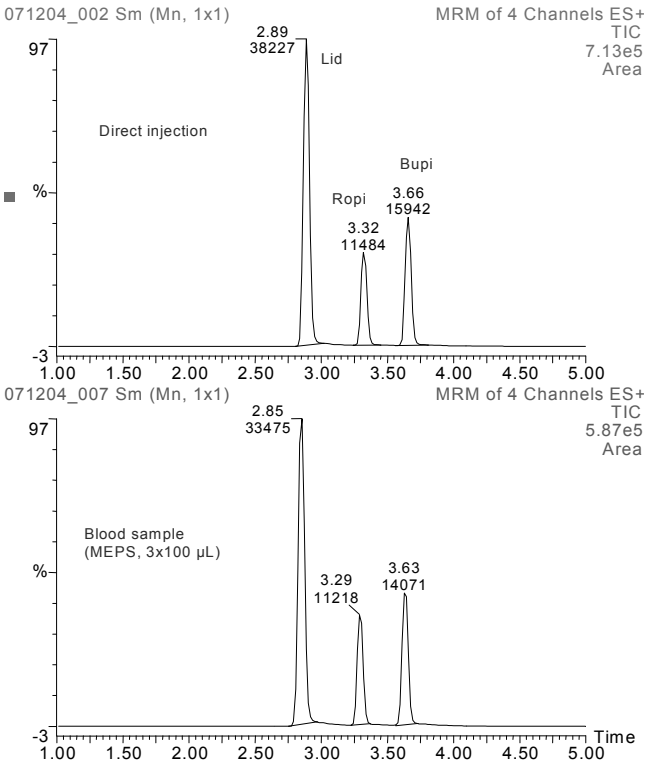

Figure 17. Comparison of mass chromatogram of lidocaine (1000nmol/l), ropivacaine (500 nmol/l) and bupivacaine (500nmol/l) obtained by direct injection (in the mobile phase, top) and after extraction from blood with MEPS (below).

Extraction efficiency was evaluated in study IV and V by testing multiple loading cycles (1-10) on the highest standard concentration sample, using new sample for each testing loading cycle, this experiment was done off-line. In study IV six sample loadings were satisfactory for the needed sensitivity (Figure 18), while in study V, four sample loadings were needed to get sufficient sensitivity (Figure 19).

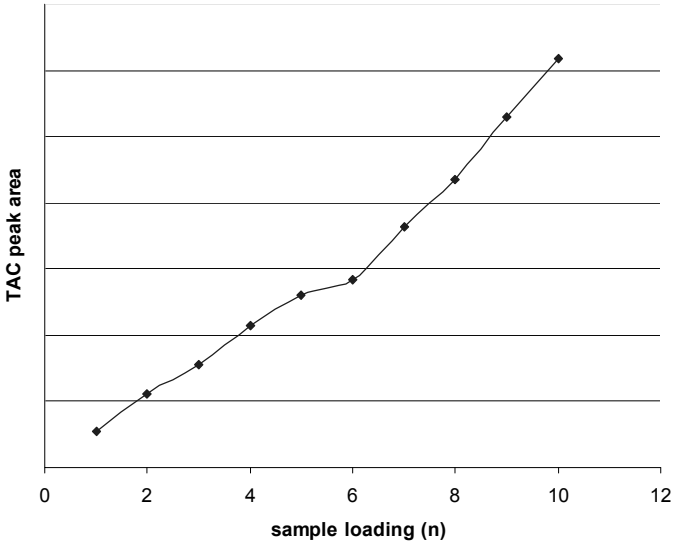

Figure 18:. Tacrolimus response (as peak area) as a function of the number of MEPS extraction cycles.

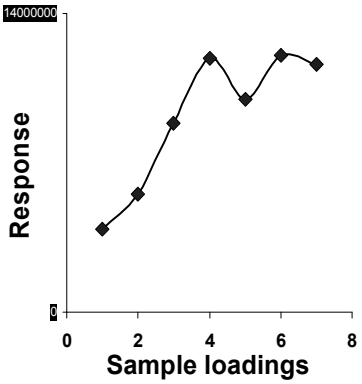

Figure 19. Remifentanil mass spectrometry response (peak area) as a function of the number of MEPS loading cycles.

5 DISCUSSION

In bioanalysis, a large fraction of the total analysis time is related to the sample preparation step. Therefore there is a need for new sample preparation techniques that will enable time effective high throughput analytical methods to be developed. The general aim of this work was to investigate and evaluate MEPS as new on-line sample preparation technique by developing new analytical methods for different categories of compounds utilizing MEPS in combination with LC-MS/MS. It has been proposed that MEPS has some potential advantages such as reducing the matrix effect, reducing the quantity of used solvents, reducing sample volume, enabling automation and standardization techniques that simplify method development (*3, 36-37*). This work confirmed that MEPS is a useful sample preparation technique for bioanalysis in combination with LC-MS/MS.

5.1 EVALUATION OF THE PERFORMED STUDIES

In study I and II, methods for determination of cyclophosphamide were developed for human plasma and mouse blood. The method for human plasma was used for the analysis of a large number of patient samples demonstrating the capability of using MEPS for automation. The accuracy of the results was confirmed by comparison with an established reference method. The MEPS LC-MS/MS method had higher sensitivity and used a reduced sample volume.

For determination of cyclophosphamide in mouse blood the MEPS procedures were slightly modified. A smaller sample volume was used (20 µL instead of 50 µL) in combination with a five times higher dilution before loading. This was needed to avoid clogging of the cartridge. Another modification was the elution solution to provide a stronger response. The method of study II was used to study different blood sampling techniques for pharmacokinetics studies in mice (*108*), animal models provide basic knowledge about the biochemical fate of drug biotransformation and it is a relevant physiological system for evaluation of drug metabolism (*109*). Mice are the species of choice for toxicological evaluation (*110*). Usually large number of mice is needed for kinetic studies since samples at many time points are needed and also relatively large blood volumes. Reducing the number of mice needed is possible by changing the traditional blood sampling procedure, from retro-orbital sinus to tail vein bleeding technique. The main limitation of using mice is that only small blood volume can be

obtained at each sampling point, so the development of sensitive analytical methods that require small blood volume is of great value to facilitate pharmacokinetics studies (*111*).

In study III three local anaesthetics were determined in human blood. The study further explored the potential of MEPS utilizing blood as specimen. The procedure used 25 µL of blood and the validation demonstrated that a well functioning and automated method for determination of lidocaine, bupivacaine and ropivacaine was successfully developed.

In study IV the MEPS-LC-MS/MS technique was used for determination of four immunosuppressive drugs in whole blood. These drugs are commonly monitored in different clinical laboratories using immunoassays, but these methods suffered from lack of specificity of certain test systems results in a method deviation (bias) (*84*). There are an increasing number of laboratories, who have switched to LC-MS/MS, for immunosuppressant quantification. This is an important application in the field of TDM with a high demand on sample throughput. TDM needs to deliver a precise and accurate measurement of an administrated drug to be a successful tool (*112*). Our method was compared with both immunochemical methods and a LC-MS/MS procedure based on PP as sample preparation method. The accuracy of the MEPS method was confirmed by acceptable agreement with the reference method. The difference to immunochemical method was confirmed in previous work (*113*). The benefit of using MEPS for routine determination of immunosuppressive drugs with LC-MS/MS is the possibility of automation besides a cleaner extract. This method is ready to be used as a new analytical platform for TDM in clinical routine environment.

Study V was specifically aimed at reducing the sample volume for determination of remifentanil in plasma from newborns. The sample volume was successfully reduced from 50 µL (using PP) to 20 µL, which is a significant difference. The limited total blood volume that can be taken is one of the biggest practical limitations in clinical pharmacokinetics studies on infants and children (*114*). For pediatric patients, the quantity of the withdrawn blood is limited by patient's age, size and underlying diseases. This is emphasis the necessity for developing accurate analytical methods that can handle small sample volume (*115-116*).

5.2 MATRIX EFFECT AND AUTOMATION USING MEPS

Influence from matrix is common in ESI-LC-MS method since interfering compounds might co-elute with the analytes. Other ionisation techniques used in LC-MS also suffer from matrix effects but to lesser extent (*117-118*). Matrix effects seen in LC-MS are related to the competition of charges between the analyte and co-eluting interfering substances, which will affect the efficiency analyte ionisation (*119-121*).

Problems with matrix effect are common when using PP as sample preparation method. Sample preparation with PP is common in LC-MS methods. This is due to the high selectivity of the detection that allows PP procedures to be used for many applications (*12, 90, 95*). In previous publications, the MEPS technique was demonstrated to reduce salt and phospholipid concentrations significantly compared to PP (*37-38*). In study IV and V the matrix effect was investigated. Two approaches were used to assess the matrix effect; qualitative and quantitative. The qualitative approach consists of post-column infusion, in which the analyte is constantly infused after the analytical column, while injecting a blank matrix extract (*122*). In the quantitative approach the response of the analyte in a reference solution (mobile phase) is compared to the response of the analyte spiked into a blank matrix extract (*38, 123*). The results from study IV and V confirmed that MEPS reduces the influence from matrix as compared with PP.

A key feature of MEPS is the possibility of automation, which is more difficult when using SPE. This is due to the larger elution volumes usually needed necessitating a concentration step. The automation of SPE is possible by using special pipetting robots or by using switching valves (*6*) In MEPS the syringe is used both for extraction and injection. The direct injection of the eluent is possible because of the limited elution volume needed.

The potential of fully automated systems makes MEPS an attractive method in combination with LC-MS/MS. By using the highly selective LC-MS/MS technique, the full chromatographic separation of analytes is not required and thus gives shorter analysis times. Automation of the entire analytical system is essential for developing LC-MS/MS to make it competitive with immunoassay analyzers commonly used in clinical chemistry and medical laboratory.

5.3 FACTORS AFFECTING MEPS PERFORMANCE

Composition of the washing solution

The role of the washing solution in MEPS is the same as in SPE to selectively remove the interferences from the sorbent while retaining the analytes. In method development the washing step should be designed to avoid more than a 5% analyte loss. Following this rule, in study I, II and IV the washing solution consisted of 95:5% (water: methanol), while in study III only water was used as washing solution. The type of interaction between the analyte and the sorbent is mainly based on hydrophobic. A better cleaning can be obtained by repetitive washings but this will prolong the time needed for the procedure and may increase the risk for leakage (*38*). The design of the washing procedure is a trade-off between recovery and degree of washing (*38*).

In study V where mixed mode sorbent was used the washing solution consisted of 5% methanol in aqueous 0.1% formic acid in order to retain remifentanil on the ion exchange functionality.

MEPS and carry-over

Carry-over is expected to occur when using MEPS and must always be considered. This phenomenon depends on several factors such as used sorbent, the type of interaction between the analyte and the sorbent, design of the procedure and the design of the hardware.

In study IV and V the carry-over was tested by running blank samples after the highest standard concentration, while in study I, II and III the carry-over was tested by running elution solution after the highest standard concentration. In all the five studies the level of carry-over (<0.4%) was less that 0.5% which is the maximum carry-over proposed for automated systems (*124*).

Carry-over related to MEPS can vary between applications (*37*). MEPS has a small quantity of sorbent, which can be easily and effectively washed online while the previous sample is still running. The between sample washing procedure must be optimized with regard to carry-over. Usually the elution solution is sufficient for washing. It is rather the number of washing cycles that are optimized during the method development. In the present studies the numbers of washing cycles were 3 or 4. In addition to washing the sorbent also the injector must be cleaned between injections.

Also there are some approaches to reduce carry-over by better design of the MEPS syringe. One of the approaches is that the sample is no longer in contact with the syringe but it is aspirated into a holding loop. The syringe only acts as an aspirator and dispenser.

Extraction efficiency

In study IV and V the effect of sample loading replicates on the extraction efficiency was investigated (Figure18). There was increase in amount of extracted analyte response (peak area) when increasing the number loading replications.

The time for interaction between the sorbent and the sample is critical so the speed of sample loading should be considered. Method developing procedures was done manually for all the studies. A slight variation within the results was noticed due to operator performance since it is difficult to maintain the same speed of sample loading. A hand-held automated analytical syringe will help to overcome this problem and has been presented by SGE.

The elution solution

Since small amount of sorbent is used in MEPS, the elution step can be achieved by using a small volume of elution solution which can be injected directly into the chromatograph. The elution solution must be able to displace the analytes from the sorbent with maximum 50 μL volume. For MEPS methanol is a common organic modifier (*37*). In study III where the analyte was a weak base a mixture of methanol and water was used containing 0.25% ammonium hydroxide as elution solution. This solution was capable to break the nonpolar interaction between the analyte and the sorbent. In study V where the type of interaction is ionic, the pH should be two units above the pk_a value. For this purpose the solution consisted of methanol and water 90:10 (v/v) containing 3% ammonium hydroxide.

MEPS sorbents

Sorbents used in MEPS are the same as in SPE so many of the existing SPE methods can be replaced by rescaling and modification.

In paper I and II, C2 was chosen for cyclophosphamide analysis since it gave the highest recovery. This might be due to the fact that the C2 sorbent is the most polar among the tested sorbents (C18, C8 and C4) and that the analyte is slightly polar and more easily desorbed. In paper IV, C8 sorbent was chosen for immunosuppressive quantification as it produced the highest recovery among the tested sorbents (C18, C8, C4 and C2). In paper V where the analyte is basic, mixed mode sorbent was used (cation exchange, C8) for remifentanil quantification. This choice was made after testing different sorbents. The mixed mode sorbent is believed to produce cleaner extracts.

Through this work it was noticed that the performances of MEPS cartridge was affected by the number of processed samples. This was noticed especially in low concentration samples. In study IV two types of C8 sorbents were used. One was the commercially available C8 and the other was modified C8 sorbent with wider needle to reduce the back pressure. This needle type reduced clogging and resulted in a prolonged the life time of the sorbent, making it possible to inject 120 blood samples before replacement. For plasma samples, MEPS sorbent can be used for up to 120 samples.

5.4 MEPS AND INVESTIGATED APPLICATIONS

Cyclophosphamide

There are several publications about cyclophosphamide quantification utilizing LC-MS/MS, LC-UV and GC-MS with different adopted sample preparation methods (*68, 125-131*). A comparison among these methods is summarized in Table 5. In this study we presented for the first time on-line sample preparation method for cyclophosphamide quantification that utilize only 50 µL sample volume which is considered to be the smallest sample volume among other published methods. Study II is the first published method for cyclophosphamide quantification in blood.

Table 5. Comparison between several cyclophosphamide measuring methods

Analysis method	Extraction method/ sample volume	Matrix	Extraction time (min)	Analysis time (min)	Limit of detection ng/mL	Method ref
LC-MS/MS	MEPS (50 µL)	mouse plasma	1	4.5	5.0	Study I
LC-MS/MS	PP (250 µL)	mouse plasma	35	2.0	3.1	(*126*)
LC-MS/MS	PP (100 µL)	human plasma	20	10.0	20.0	(*129*)
LC-MS/MS	SPE (450 µL)	human plasma	20	9,2	15.0	(*128*)

Local anaesthetics in whole blood

Local anaesthetics have been quantified in urine, serum and plasma (*56, 76, 78, 132-142*). This is the first method to quantify local anaesthetics in blood. Good sensitivity was obtained with 25 µL sample volume needed. The sample preparation was simple and automated. Mass spectrometry was first used for local anaesthetics determination in 2000 (*42*). SPE was used as sample preparation in some of the methods (*140, 143*). These methods require large sample volume and the procedures were done off-line.

Immunosuppressive in whole blood

LC-MS/MS has been used for detection of immunosuppressive drugs in whole blood with different methods of sample preparation (*12, 82, 112, 144-155*). Quantification of immunosuppressant is recommended to be in whole blood (*156*) due to the large distribution in erythrocytes; 95% for TAC (*157*) and SIR (*158*), between 40-60% for CYS (*159*) and about 75% for EVE (*160*). Most published sample preparation methods are PP and SPE. The main advantages for using MEPS in immunosuppressive quantification are; fully automated system, highly sensitive method using only 50 µL sample volume. Also cleaner extracts can be obtained with MEPS which is a benefit in a routine laboratory.

Remifentanil

Bioanalysis of remifentanil is challenging, since remifentanil is subjected to hydrolysis catalyzed by tissue and circulating esterases (*84-86, 161*). Several methods have been published for remifentanil quantification in whole blood (*162-167*) (*162, 168-170*) and

plasma (*171-172*) LLE sample preparation method was mainly used in the previous methods. LLE method required long time working procedure besides using large quantity of solvents moreover, these procedures were not automated. Also solid phase extraction was used either as main sample preparation method or after using PP. Those methods required 250-500 µL sample volume and the procedures were performed offline. By using MEPS as sample preparation method, sample volume needed was only 20 µL and high sensitivity achieved with total analysis time of five minutes with automated sample preparation procedures.

6 CONCLUSION

An increasing interest in sample preparation has been generated by introducing new extraction techniques that addressed the need for automation and miniaturization. Microextraction by packed syringe MEPS is a promising sampling technique especially when used combined with LC-MS/MS.

- The procedures were fully automated with processing time as low as one min per sample (study I).
- In application to blood samples, the sorbent could be used for more than one hundred samples before the extraction recovery was reduced.
- The MEPS technique can be used for a wide range of compounds and requires a small sample volume. In addition, less amount of solvent is needed than for traditional SPE.
- MEPS can be applied to many of the of the existing SPE methods.
- The use of MEPS is effective to reduce the matrix effect and thus provide more precise and accurate methods

7 FUTURE PERSPECTIVES

Developing of sample preparation in term of miniaturization, automation and introduction of new selective solid phase will continue. Future work should be focused on applying this technique to other drugs and their metabolites. Exploring a new range of applications in different areas such as environmental and food analysis will add new applications to this technique abilities.

Using MEPS with sorbents such as antibodies and molecularly imprinted polymers (MIPs) can potentially increase selectivity.

Better design of the extraction device will facilitate sampling, separation and quantification. In this thesis we have used modified hardware in term of wider needle. Further work should be done to enhance the MEPS performance by trying to reduce the back pressure and thus prevent clogging. Also the autosampler that is used with MEPS should be modified in term of the used software and the applied washing mechanism. Modifying the software will speed up the extraction process and thus more throughput analysis can be obtained and by working on the washing procedures carry-over problem can be eliminated.

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9 REFERENCES

1. Marquet, P. (1999) [Therapeutic monitoring: analytic, pharmacokinetic and clinical aspects], *Acta Clin Belg Suppl 1*, 2-12.

2. Mullett, W. M. (2007) Determination of drugs in biological fluids by direct injection of samples for liquid-chromatographic analysis, *J Biochem Biophys Methods 70*, 263-273.

3. Berna, M. J., Ackermann, B. L., and Murphy, A. T. (2004) High-throughput chromatographic approaches to liquid chromatographic/tandem mass spectrometric bioanalysis to support drug discovery and development, *Analytica Chimica Acta 509*, 1-9.

4. Novakova, L., and Vlckova, H. (2009) A review of current trends and advances in modern bio-analytical methods: chromatography and sample preparation, *Anal Chim Acta 656*, 8-35.

5. Blomberg, L. G. (2009) Two new techniques for sample preparation in bioanalysis: microextraction in packed sorbent (MEPS) and use of a bonded monolith as sorbent for sample preparation in polypropylene tips for 96-well plates, *Anal Bioanal Chem 393*, 797-807.

6. Hennion, M. C. (1999) Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography, *J Chromatogr A 856*, 3-54.

7. Pawliszyn, J. (2003) Sample preparation: quo vadis?, *Anal Chem 75*, 2543-2558.

8. Vogeser, M., and Seger, C. (2008) A decade of HPLC-MS/MS in the routine clinical laboratory--goals for further developments, *Clin Biochem 41*, 649-662.

9. Ma, J., Shi, J., Le, H., Cho, R., Huang, J. C., Miao, S., and Wong, B. K. (2008) A fully automated plasma protein precipitation sample preparation method for LC-MS/MS bioanalysis, *J Chromatogr B Analyt Technol Biomed Life Sci 862*, 219-226.

10. Shakleya, D. M., Jansson, L. M., and Huestis, M. A. (2007) Validation of a LC-APCI-MS/MS method for quantification of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyraline (EMDP) in infant plasma following protein precipitation, *J Chromatogr B Analyt Technol Biomed Life Sci 856*, 267-272.

11. Zellner, M., Winkler, W., Hayden, H., Diestinger, M., Eliasen, M., Gesslbauer, B., Miller, I., Chang, M., Kungl, A., Roth, E., and Oehler, R. (2005) Quantitative validation of different protein precipitation methods in proteome analysis of blood platelets, *Electrophoresis 26*, 2481-2489.

12. Bogusz, M. J., Enazi, E. A., Hassan, H., Abdel-Jawaad, J., Ruwaily, J. A., and Tufail, M. A. (2007) Simultaneous LC-MS-MS determination of cyclosporine A, tacrolimus, and sirolimus in whole blood as well as mycophenolic acid in plasma using common pretreatment procedure, *J Chromatogr B Analyt Technol Biomed Life Sci 850*, 471-480.

13. Silvestre, C. I., Santos, J. L., Lima, J. L., and Zagatto, E. A. (2009) Liquid-liquid extraction in flow analysis: A critical review, *Anal Chim Acta 652*, 54-65.

14. Rasmussen, K. E., Pedersen-Bjergaard, S., Krogh, M., Ugland, H. G., and Gronhaug, T. (2000) Development of a simple in-vial liquid-phase microextraction device for drug analysis compatible with capillary gas chromatography, capillary electrophoresis and high-performance liquid chromatography, *J Chromatogr A 873*, 3-11.

15. Pedersen-Bjergaard, S., and Rasmussen, K. E. (2005) Bioanalysis of drugs by liquid-phase microextraction coupled to separation techniques, *J Chromatogr B Analyt Technol Biomed Life Sci 817*, 3-12.

16. Lambropoulou, D. A., and Albanis, T. A. (2007) Liquid-phase micro-extraction techniques in pesticide residue analysis, *Journal of Biochemical and Biophysical Methods 70*, 195-228.

17. Jonsson, J. A., and Mathiasson, L. (2000) Membrane-based techniques for sample enrichment, *J Chromatogr A 902*, 205-225.

18. Jönsson, J. Å., and Mathiasson, L. (1992) Supported liquid membrane techniques for sample preparation and enrichment in environmental and biological analysis, *TrAC Trends in Analytical Chemistry 11*, 106-114.

19. Jönsson, J. Å., and Mathiasson, L. (2001) Memrane extraction in analytical chemistry, *Journal of Separation Science 24*, 495-507.

20. Henion, J., Brewer, E., and Rule, G. (1998) Sample preparation for LC/MS/MS: analyzing biological and environmental samples, *Anal Chem 70*, 650A-656A.

21. van Hout, M. W., de Zeeuw, R. A., and de Jong, G. J. (1999) Coupling device for desorption of drugs from solid-phase extraction-pipette tips and on-line gas chromatographic analysis, *J Chromatogr A 858*, 117-122.

22. Boos, K.-S., and Grimm, C.-H. (1999) High-performance liquid chromatography integrated solid-phase extraction in bioanalysis using restricted access precolumn packings, *TrAC Trends in Analytical Chemistry 18*, 175-180.

23. Kitchen, C. J., Musson, D. G., and Fisher, A. L. (2004) Column-switching technique for the sensitive determination of ertapenem in human cerebrospinal fluid using liquid chromatography and ultraviolet absorbance detection, *J Chromatogr B Analyt Technol Biomed Life Sci 799*, 9-14.

24. Heinig, K., and Bucheli, F. (2003) Ultra-fast quantitative bioanalysis of a pharmaceutical compound using liquid chromatography-tandem mass spectrometry, *J Chromatogr B Analyt Technol Biomed Life Sci 795*, 337-346.

25. Holm, A., Molander, P., Lundanes, E., Ovrebo, S., and Greibrokk, T. (2003) Fast and sensitive determination of urinary 1-hydroxypyrene by packed capillary column switching liquid chromatography coupled to micro-electrospray time-of-flight mass spectrometry, *J Chromatogr B Analyt Technol Biomed Life Sci 794*, 175-183.

26. Souverain, S., Rudaz, S., and Veuthey, J. L. (2004) Restricted access materials and large particle supports for on-line sample preparation: an attractive approach for biological fluids analysis, *J Chromatogr B Analyt Technol Biomed Life Sci 801*, 141-156.

27. Hawthorne, S. B., Miller, D. J., Pawliszyn, J., and Arthur, C. L. (1992) Solventless determination of caffeine in beverages using solid-phase microextraction with fused-silica fibers, *J Chromatogr 603*, 185-191.

28. Musteata, F. M., and Pawliszyn, J. (2007) In vivo sampling with solid phase microextraction, *J Biochem Biophys Methods 70*, 181-193.

29. Xie, W., Pawliszyn, J., Mullett, W. M., and Matuszewski, B. K. (2007) Comparison of solid-phase microextraction and liquid-liquid extraction in 96-well format for the determination of a drug compound in human plasma by liquid chromatography with tandem mass spectrometric detection, *J Pharm Biomed Anal 45*, 599-608.

30. Musteata, F. M., and Pawliszyn, J. (2007) Bioanalytical applications of solid-phase microextraction, *TrAC Trends in Analytical Chemistry 26*, 36-45.

31. Hutchinson, J. P., Setkova, L., and Pawliszyn, J. (2007) Automation of solid-phase microextraction on a 96-well plate format, *J Chromatogr A 1149*, 127-137.

32. Setkova, L., Risticevic, S., Linton, C. M., Ouyang, G., Bragg, L. M., and Pawliszyn, J. (2007) Solid-phase microextraction-gas chromatography-time-of-flight mass spectrometry utilized for the evaluation of the new-generation super elastic fiber assemblies, *Anal Chim Acta 581*, 221-231.

33. Krogh, M., Johansen, K., Tonnesen, F., and Rasmussen, K. E. (1995) Solid-phase microextraction for the determination of the free concentration of valproic acid in human plasma by capillary gas chromatography, *J Chromatogr B Biomed Appl 673*, 299-305.

34. Abdel-Rehim, M. (2004) New trend in sample preparation: on-line microextraction in packed syringe for liquid and gas chromatography applications. I. Determination of local anaesthetics in human plasma samples using gas chromatography-mass spectrometry, *J Chromatogr B Analyt Technol Biomed Life Sci 801*, 317-321.

35. M.Abdel-Rehim. (2003) *AstraZeneca Application, Current Patents Gazette, week 0310 WO 03019149 77*.

36. M.Abdel-Rehim.(March 2003) *PCT,World Intellectual Property Organization, International Publication*

37. Abdel-Rehim, M. (2009) Recent advances in microextraction by packed sorbent for bioanalysis, *J Chromatogr A*.

38. Altun, Z., and Abdel-Rehim, M. (2008) Study of the factors affecting the performance of microextraction by packed sorbent (MEPS) using liquid scintillation counter and liquid chromatography-tandem mass spectrometry, *Anal Chim Acta 630*, 116-123.

39. Abdel-Rehim, M., Andersson, A., Breitholtz-Emanuelsson, A., Sandberg-Stall, M., Brunfelter, K., Pettersson, K. J., and Norsten-Hoog, C. (2008) MEPS as a rapid sample preparation method to handle unstable compounds in a complex matrix: determination of AZD3409 in plasma samples utilizing MEPS-LC-MS-MS, *J Chromatogr Sci 46*, 518-523.

40. Said, R., Hassan, Z., Hassan, M., and Abdel-Rehim, M. (2008) 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.

41. Altun, Z., Abdel-Rehim, M., and Blomberg, L. G. (2004) New trends in sample preparation: on-line microextraction in packed syringe (MEPS) for LC and GC

applications Part III: Determination and validation of local anaesthetics in human plasma samples using a cation-exchange sorbent, and MEPS-LC-MS-MS, *J Chromatogr B Analyt Technol Biomed Life Sci 813*, 129-135.

42. Abdel-Rehim, M., Bielenstein, M., Askemark, Y., Tyrefors, N., and Arvidsson, T. (2000) High-performance liquid chromatography-tandem electrospray mass spectrometry for the determination of lidocaine and its metabolites in human plasma and urine, *J Chromatogr B Biomed Sci Appl 741*, 175-188.

43. Abdel-Rehim, M., Altun, Z., and Blomberg, L. (2004) Microextraction in packed syringe (MEPS) for liquid and gas chromatographic applications. Part II--Determination of ropivacaine and its metabolites in human plasma samples using MEPS with liquid chromatography/tandem mass spectrometry, *J Mass Spectrom 39*, 1488-1493.

44. El-Beqqali A, K. A., Abdel-Rehim M,. (2007) Microextraction in packed syringe / liquid chromatography /electrospray tandem mass spectrometry for quantification of acebutolol and metoprolol in human plasma and urine samples., *J. Liq. Chromatogr. & Relat. Technol. 30*, 575-586.

45. Chaves, A. R., Leandro, F. Z., Carris, J. A., and Queiroz, M. E. (2010) Microextraction in packed sorbent for analysis of antidepressants in human plasma by liquid chromatography and spectrophotometric detection, *J Chromatogr B Analyt Technol Biomed Life Sci 878*, 2123-2129.

46. Altun, Z., Blomberg, L. G., Jagerdeo, E., and Abdel-Rehim, M. (2006) Drug Screening Using Microextraction in a Packed Syringe (MEPS)/Mass Spectrometry Utilizing Monolithic-, Polymer-, and Silica-Based Sorbents, *Journal of Liquid Chromatography & Related Technologies 29*, 829-839.

47. Abdel-Rehim, M., Andersson, L. I., Altun, Z., and Blomberg, L. G. (2006) Microextraction in Packed Syringe Online with Liquid Chromatography-Tandem Mass Spectrometry: Molecularly Imprinted Polymer as Packing Material for MEPS in Selective Extraction of Ropivacaine from Plasma, *Journal of Liquid Chromatography & Related Technologies 29*, 1725-1736.

48. Abdel-Rehim, M., Dahlgren, M., Blomberg, L., Claude, S., and Tabacchi, R. (2006) Microextraction in Packed Syringe (MEPS) Utilizing Methylcyanopropyl–Silarylene as Coating Polymer for Extraction of Drugs in Biological Samples, *Journal of Liquid Chromatography & Related Technologies 29*, 2537-2544.

49. Abdel-Rehim, M., Hassan, Z., Skansem, P., and Hassan, M. (2007) Simultaneous Determination of Busulphan in Plasma Samples by Liquid Chromatography-electrospray Ionization Mass Spectrometry Utilizing Microextraction in Packed Syringe (MEPS) as On-line Sample Preparation Method, *Journal of Liquid Chromatography & Related Technologies 30*, 3029-3041.

50. Abdel-Rehim, M., Skansen, P., Vita, M., Hassan, Z., Blomberg, L., and Hassan, M. (2005) Microextraction in packed syringe/liquid chromatography/electrospray tandem mass spectrometry for quantification of olomoucine in human plasma samples, *Analytica Chimica Acta 539*, 35-39.

51. Vita, M., Skansen, P., Hassan, M., and Abdel-Rehim, M. (2005) Development and validation of a liquid chromatography and tandem mass spectrometry method for determination of roscovitine in plasma and urine samples utilizing on-line sample preparation, *J Chromatogr B Analyt Technol Biomed Life Sci 817*, 303-307.

52. Abdel-Rehim, M. A., Y.; Norsten-Ho¨o¨g, C.; Petterson, K.J.; Halldin, M. (2006) Quantification of 4-OH-2, 6-xylidine and its conjugate in human urine samples utilising microextraction in packed syringe online with liquid chromatography and electrospray tandem mass spectrometry (MEPS-LC-MS/MS). *J. Liq. Chromatogr. & Rel. Technol. 29*, 2413-2424.

53. Abdel-Rehim, M., Dahlgren, M., and Blomberg, L. (2006) Quantification of ropivacaine and its major metabolites in human urine samples utilizing microextraction in a packed syringe automated with liquid chromatography-tandem mass spectrometry (MEPS-LC-MS/MS), *J Sep Sci 29*, 1658-1661.

54. El-Beqqali, A., Kussak, A., and Abdel-Rehim, M. (2007) Determination of dopamine and serotonin in human urine samples utilizing microextraction online with liquid chromatography/electrospray tandem mass spectrometry, *J Sep Sci 30*, 421-424.

55. El-Beqqali, A., and Abdel-Rehim, M. (2007) Quantitative analysis of methadone in human urine samples by microextraction in packed syringe-gas chromatography-mass spectrometry (MEPS-GC-MS), *J Sep Sci 30*, 2501-2505.

56. Jagerdeo, E., and Abdel-Rehim, M. (2009) Screening of cocaine and its metabolites in human urine samples by direct analysis in real-time source coupled to time-of-flight mass spectrometry after online preconcentration utilizing microextraction by packed sorbent, *J Am Soc Mass Spectrom 20*, 891-899.

57. Morales-Cid, G., Cardenas, S., Simonet, B. M., and Valcarcel, M. (2009) Fully automatic sample treatment by integration of microextraction by packed sorbents into commercial capillary electrophoresis-mass spectrometry equipment: application to the determination of fluoroquinolones in urine, *Anal Chem 81*, 3188-3193.

58. Abdel-Rehim, M., Askemark, Y., Norsten-Höög, C., Pettersson, K.-J., and Halldin, M. (2006) Quantification of 4-OH-2,6-Xylidine and its Conjugates in Human Urine Samples Utilising Microextraction in Packed Syringe On-line with Liquid Chromatography and Electrospray Tandem Mass Spectrometry (MEPS-LC-MS/MS), *Journal of Liquid Chromatography & Related Technologies 29*, 2413-2424.

59. Said R, A.-R. M., Sadeghi B, Al-Hashemi S, Hassan Z, Hassan M,. (2007) Cyclophsophamide pharmacokinetics in mice: A Comparison between retro orbital sampling versus serial tail vein bleeding., *The Open Pharmacology Journal 1*, 22-27.

60. Said R, H. Z., Hassan M, Abdel-Rehim M,. (2008) 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) J. Liq. Chromatogr. & Relat. Technol., 2008;31: 683-694., *J. Liq. Chromatogr. & Relat. Technol. 31*, 683-694.

61. Rana Said, A. P., Mohamed Abdel-Rehim, Olof Beck. (2010) Determination of four immunosuppressive drugs in whole blood using MEPS and LC-MS/MS allowing automated sample work-up and analysis, *submitted*.

62. Mohamed Kamel, R. S., Aziza Elbeqqali, Fatma Bassyouni, Mohamed Abdel-Rehim (2009) On-Line Determination of Cyclophosphamide in Blood Samples Utilizing Microextraction by Packed Sorbent and Liquid Chromatography Tandem Mass Spectrometry (MEPS-LC-MS/MS) *The Open Spectroscopy Journal 3*, pp.26-30.

63. El-Beqqali, A., Kussak, A., and Abdel-Rehim, M. (2006) Fast and sensitive environmental analysis utilizing microextraction in packed syringe online with gas chromatography-mass spectrometry: determination of polycyclic aromatic hydrocarbons in water, *J Chromatogr A 1114*, 234-238.

64. Jönsson, S., Hagberg, J., and van Bavel, B. (2008) Determination of 2,4,6-Trichloroanisole and 2,4,6-Tribromoanisole in Wine Using Microextraction in Packed Syringe and Gas Chromatography−Mass Spectrometry, *Journal of Agricultural and Food Chemistry 56*, 4962-4967.

65. Miyaguchi, H., Iwata, Y. T., Kanamori, T., Tsujikawa, K., Kuwayama, K., and Inoue, H. (2009) Rapid identification and quantification of methamphetamine and amphetamine in hair by gas chromatography/mass spectrometry coupled with micropulverized extraction, aqueous acetylation and microextraction by packed sorbent, *Journal of Chromatography A 1216*, 4063-4070.

66. Hiroyuki, K. (2005) Recent Advances in Solid-Phase Microextraction and Related Techniques for Pharmaceutical and Biomedical Analysis, *Current Pharmaceutical Analysis 1*, 65-84.

67. Binotto, G., Trentin, L., and Semenzato, G. (2003) Ifosfamide and cyclophosphamide: effects on immunosurveillance, *Oncology 65 Suppl 2*, 17-20.

68. Slattery, J. T., Kalhorn, T. F., McDonald, G. B., Lambert, K., Buckner, C. D., Bensinger, W. I., Anasetti, C., and Appelbaum, F. R. (1996) Conditioning regimen-dependent disposition of cyclophosphamide and hydroxycyclophosphamide in human marrow transplantation patients, *J Clin Oncol 14*, 1484-1494.

69. Moore, M. J. (1991) Clinical pharmacokinetics of cyclophosphamide, *Clin Pharmacokinet 20*, 194-208.

70. Brock, N. (1989) Oxazaphosphorine cytostatics: past-present-future. Seventh Cain Memorial Award lecture, *Cancer Res 49*, 1-7.

71. Hassan, M., Nilsson, C., Olsson, H., Lundin, J., and Osterborg, A. (1999) The influence of interferon-alpha on the pharmacokinetics of cyclophosphamide and its 4-hydroxy metabolite in patients with multiple myeloma, *Eur J Haematol 63*, 163-170.

72. Hassan, M., Ljungman, P., Ringden, O., Hassan, Z., Oberg, G., Nilsson, C., Bekassy, A., Bielenstein, M., Abdel-Rehim, M., Georen, S., and Astner, L. (2000) The effect of busulphan on the pharmacokinetics of cyclophosphamide and its 4-hydroxy metabolite: time interval influence on therapeutic efficacy and therapy-related toxicity, *Bone Marrow Transplant 25*, 915-924.

73. Clarke, L., and Waxman, D. J. (1989) Oxidative metabolism of cyclophosphamide: identification of the hepatic monooxygenase catalysts of drug activation, *Cancer Res 49*, 2344-2350.

74. Baumann, F., and Preiss, R. (2001) Cyclophosphamide and related anticancer drugs, *J Chromatogr B Biomed Sci Appl 764*, 173-192.

75. Eiriksson, C., and Brogden, R. N. (1984) Lorcainide. A preliminary review of its pharmacodynamic properties and therapeutic efficacy, *Drugs 27*, 279-300.

76. Cuvillon, P., Nouvellon, E., Ripart, J., Boyer, J. C., Dehour, L., Mahamat, A., L'Hermite, J., Boisson, C., Vialles, N., Lefrant, J. Y., and de La Coussaye, J. E. (2009) A comparison of the pharmacodynamics and pharmacokinetics of

bupivacaine, ropivacaine (with epinephrine) and their equal volume mixtures with lidocaine used for femoral and sciatic nerve blocks: a double-blind randomized study, *Anesth Analg 108*, 641-649.

77. Meral, G., Tasar, F., Sayin, F., Saysel, M., Kir, S., and Karabulut, E. (2005) Effects of lidocaine with and without epinephrine on plasma epinephrine and lidocaine concentrations and hemodynamic values during third molar surgery, *Oral Surg Oral Med Oral Pathol Oral Radiol Endod 100*, e25-30.

78. Arvidsson, T., Askemark, Y., and Halldin, M. M. (1999) Liquid chromatographic bioanalytical determination of ropivacaine, bupivacaine and major metabolites, *Biomed Chromatogr 13*, 286-292.

79. Ekstrom, G., and Gunnarsson, U. B. (1996) Ropivacaine, a new amide-type local anesthetic agent, is metabolized by cytochromes P450 1A and 3A in human liver microsomes, *Drug Metab Dispos 24*, 955-961.

80. Arvidsson, T., and Eklund, E. (1995) Determination of free concentration of ropivacaine and bupivacaine in blood plasma by ultrafiltration and coupled-column liquid chromatography, *J Chromatogr B Biomed Appl 668*, 91-98.

81. Halldin, M. M., Bredberg, E., Angelin, B., Arvidsson, T., Askemark, Y., Elofsson, S., and Widman, M. (1996) Metabolism and excretion of ropivacaine in humans, *Drug Metab Dispos 24*, 962-968.

82. Kahan, B. D., Keown, P., Levy, G. A., and Johnston, A. (2002) Therapeutic drug monitoring of immunosuppressant drugs in clinical practice, *Clin Ther 24*, 330-350; discussion 329.

83. Dirks, N. L., Huth, B., Yates, C. R., and Meibohm, B. (2004) Pharmacokinetics of immunosuppressants: a perspective on ethnic differences, *Int J Clin Pharmacol Ther 42*, 701-718.

84. Westmoreland, C. L., Hoke, J. F., Sebel, P. S., Hug, C. C., Jr., and Muir, K. T. (1993) Pharmacokinetics of remifentanil (GI87084B) and its major metabolite (GI90291) in patients undergoing elective inpatient surgery, *Anesthesiology 79*, 893-903.

85. Pitsiu, M., Wilmer, A., Bodenham, A., Breen, D., Bach, V., Bonde, J., Kessler, P., Albrecht, S., Fisher, G., and Kirkham, A. (2004) Pharmacokinetics of remifentanil and its major metabolite, remifentanil acid, in ICU patients with renal impairment, *Br J Anaesth 92*, 493-503.

86. Ross, A. K., Davis, P. J., Dear Gd, G. L., Ginsberg, B., McGowan, F. X., Stiller, R. D., Henson, L. G., Huffman, C., and Muir, K. T. (2001) Pharmacokinetics of remifentanil in anesthetized pediatric patients undergoing elective surgery or diagnostic procedures, *Anesth Analg 93*, 1393-1401, table of contents.

87. Duthie, D. J. (1998) Remifentanil and tramadol, *Br J Anaesth 81*, 51-57.

88. Engelhardt, H. (2004) One century of liquid chromatography. From Tswett's columns to modern high speed and high performance separations, *J Chromatogr B Analyt Technol Biomed Life Sci 800*, 3-6.

89. Ettre LS. (2003) MS Tswett and the invention of chromatography, *LCGC North America 21*, 458-468.

90. Horvath, C. G., Preiss, B. A., and Lipsky, S. R. (1967) Fast liquid chromatography: an investigation of operating parameters and the separation of nucleotides on pellicular ion exchangers, *Anal Chem 39*, 1422-1428.

91. Huber, J. F., and Hulsman, J. A. (1967) A study of liquid chromatography in columns. The time of separation, *Anal Chim Acta 38*, 305-313.

92. Still, W. C., Kahn, M., and Mitra, A. (1978) Rapid chromatographic technique for preparative separations with moderate resolution, *The Journal of Organic Chemistry 43*, 2923-2925.

93. Yuan, H., Wang, F., Tu, J., Peng, W., and Li, H. (2008) Determination of lovastatin in human plasma by ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry and its application in a pharmacokinetic study, *J Pharm Biomed Anal 46*, 808-813.

94. Li, L., Pabbisetty, D., Carvalho, P., Avery, M. A., and Avery, B. A. (2008) Analysis of CoQ10 in rat serum by ultra-performance liquid chromatography mass spectrometry after oral administration, *J Pharm Biomed Anal 46*, 137-142.

95. Stephanson, N., Helander, A., and Beck, O. (2007) Alcohol biomarker analysis: simultaneous determination of 5-hydroxytryptophol glucuronide and 5-hydroxyindoleacetic acid by direct injection of urine using ultra-performance liquid chromatography-tandem mass spectrometry, *J Mass Spectrom 42*, 940-949.

96. Li, F., Maguigad, J., Pelzer, M., Jiang, X., and Ji, Q. C. (2008) A novel 'peak parking' strategy for ultra-performance liquid chromatography/tandem mass spectrometric detection for enhanced performance of bioanalytical assays, *Rapid Commun Mass Spectrom 22*, 486-494.

97. Huang, M. Q., Mao, Y., Jemal, M., and Arnold, M. (2006) Increased productivity in quantitative bioanalysis using a monolithic column coupled with high-flow direct-injection liquid chromatography/tandem mass spectrometry, *Rapid Commun Mass Spectrom 20*, 1709-1714.

98. Shintani, H. (1985) Improvement of ion chromatography with ultraviolet photometric detection and comparison with conductivity detection for the determination of serum cations, *J Chromatogr 341*, 53-63.

99. Ho, C., Lam, C., Chan, M., Cheung, R., Law, L., Lit, L., Ng, K., Suen, M., and Tai, H. (2003) Electrospray ionisation mass spectrometry: principles and clinical applications, *Clin Biochem Rev 24*, 3-12.

100. de hoffmann, c., stroobant (1999) *mass spectrometry principles and applications*, wiley.

101. Lee, M. S., and Kerns, E. H. (1999) LC/MS applications in drug development, *Mass Spectrom Rev 18*, 187-279.

102. Morris, M. (2010) Future paths of mass spectrometry in routine clinical chemistry, *Clinica Chimica Acta 411*, 905-906.

103. Xu, R. N., Fan, L., Rieser, M. J., and El-Shourbagy, T. A. (2007) Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS, *J Pharm Biomed Anal 44*, 342-355.

104. Holcapek, M., Kolarova, L., and Nobilis, M. (2008) High-performance liquid chromatography-tandem mass spectrometry in the identification and

determination of phase I and phase II drug metabolites, *Anal Bioanal Chem 391*, 59-78.

105. Bakhtiar, R., Ramos, L., and Tse, F. L. (2001) Use of atmospheric pressure ionization mass spectrometry in enantioselective liquid chromatography, *Chirality 13*, 63-74.

106. Kensler, T. T., Behme, R. J., and Brooke, D. (1979) High-performance liquid chromatographic analysis of cyclophosphamide, *J Pharm Sci 68*, 172-174.

107. Wantland, L. R., and Hersh, S. D. (1979) High-performance liquid chromatographic assay of cyclophosphamide in raw material and parenteral dosage forms, *J Pharm Sci 68*, 1144-1146.

108. Rana Said, M. A.-R., Behnam Sadeghi, Suleiman Al-Hashemi, Zuzana Hassan, Moustapha Hassan. (2007) Cyclophosphamide Pharmacokinetics in Mice: A Comparison Between Retro Orbital Sampling Versus Serial Tail Vein Bleeding *The Open Pharmacology Journal 1*, 30-35.

109. Bazare, J., Jr., Leamons, M. L., and Young, J. F. (1981) Sampling methods for pharmacokinetic studies in the mouse, *J Pharmacol Methods 5*, 99-120.

110. Hui, Y. H., Huang, N. H., Ebbert, L., Bina, H., Chiang, A., Maples, C., Pritt, M., Kern, T., and Patel, N. (2007) Pharmacokinetic comparisons of tail-bleeding with cannula- or retro-orbital bleeding techniques in rats using six marketed drugs, *J Pharmacol Toxicol Methods 56*, 256-264.

111. Peng, S. X., Rockafellow, B. A., Skedzielewski, T. M., Huebert, N. D., and Hageman, W. (2009) Improved pharmacokinetic and bioavailability support of drug discovery using serial blood sampling in mice, *J Pharm Sci 98*, 1877-1884.

112. Seger, C., Tentschert, K., Stoggl, W., Griesmacher, A., and Ramsay, S. L. (2009) A rapid HPLC-MS/MS method for the simultaneous quantification of cyclosporine A, tacrolimus, sirolimus and everolimus in human blood samples, *Nat Protoc 4*, 526-534.

113. Yang, Z., and Wang, S. (2008) Recent development in application of high performance liquid chromatography-tandem mass spectrometry in therapeutic drug monitoring of immunosuppressants, *J Immunol Methods 336*, 98-103.

114. Davis, P. J., Wilson, A. S., Siewers, R. D., Pigula, F. A., and Landsman, I. S. (1999) The effects of cardiopulmonary bypass on remifentanil kinetics in children undergoing atrial septal defect repair, *Anesth Analg 89*, 904-908.

115. Reed, M. D. (1999) Optimal sampling theory: An overview of its application to pharmacokinetic studies in infants and children, *Pediatrics 104*, 627-632.

116. Drusano, G. L., Forrest, A., Yuen, G., Plaisance, K., and Leslie, J. (1994) Optimal sampling theory: effect of error in a nominal parameter value on bias and precision of parameter estimation, *J Clin Pharmacol 34*, 967-974.

117. Chambers, E., Wagrowski-Diehl, D. M., Lu, Z., and Mazzeo, J. R. (2007) Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses, *J Chromatogr B Analyt Technol Biomed Life Sci 852*, 22-34.

118. Marchi, I., Viette, V., Badoud, F., Fathi, M., Saugy, M., Rudaz, S., and Veuthey, J. L. (2010) Characterization and classification of matrix effects in biological samples analyses, *J Chromatogr A 1217*, 4071-4078.

119. Remane, D., Wissenbach, D. K., Meyer, M. R., and Maurer, H. H. (2010) Systematic investigation of ion suppression and enhancement effects of fourteen stable-isotope-labeled internal standards by their native analogues using atmospheric-pressure chemical ionization and electrospray ionization and the relevance for multi-analyte liquid chromatographic/mass spectrometric procedures, *Rapid Commun Mass Spectrom 24*, 859-867.

120. Gosetti, F., Mazzucco, E., Zampieri, D., and Gennaro, M. C. (2010) Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry, *J Chromatogr A 1217*, 3929-3937.

121. King, R., Bonfiglio, R., Fernandez-Metzler, C., Miller-Stein, C., and Olah, T. (2000) Mechanistic investigation of ionization suppression in electrospray ionization, *J Am Soc Mass Spectrom 11*, 942-950.

122. Bonfiglio, R., King, R. C., Olah, T. V., and Merkle, K. (1999) The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, *Rapid Commun Mass Spectrom 13*, 1175-1185.

123. Matuszewski, B. K., Constanzer, M. L., and Chavez-Eng, C. M. (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, *Anal Chem 75*, 3019-3030.

124. Rossi, D. T., and Zhang, N. (2000) Automating solid-phase extraction: current aspects and future prospects, *J Chromatogr A 885*, 97-113.

125. Ekhart, C., Gebretensae, A., Rosing, H., Rodenhuis, S., Beijnen, J. H., and Huitema, A. D. (2007) Simultaneous quantification of cyclophosphamide and its active metabolite 4-hydroxycyclophosphamide in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-MS/MS), *J Chromatogr B Analyt Technol Biomed Life Sci 854*, 345-349.

126. Silvertand, L. H., Vazvaei, F., Weigl, P., Rosing, H., Hillebrand, M. J., van Maanen, M. J., and Beijnen, J. H. (2005) Simultaneous quantification of fludarabine and cyclophosphamide in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry, *Rapid Commun Mass Spectrom 19*, 3673-3680.

127. Barbieri, A., Sabatini, L., Indiveri, P., Bonfiglioli, R., Lodi, V., and Violante, F. S. (2006) Simultaneous determination of low levels of methotrexate and cyclophosphamide in human urine by micro liquid chromatography/electrospray ionization tandem mass spectrometry, *Rapid Commun Mass Spectrom 20*, 1889-1893.

128. Baumann, F., Lorenz, C., Jaehde, U., and Preiss, R. (1999) Determination of cyclophosphamide and its metabolites in human plasma by high-performance liquid chromatography-mass spectrometry, *J Chromatogr B Biomed Sci Appl 729*, 297-305.

129. de Jonge, M. E., van Dam, S. M., Hillebrand, M. J., Rosing, H., Huitema, A. D., Rodenhuis, S., and Beijnen, J. H. (2004) Simultaneous quantification of cyclophosphamide, 4-hydroxycyclophosphamide, N,N',N''-triethylenethiophosphoramide (thiotepa) and N,N',N''-triethylenephosphoramide (tepa) in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry, *J Mass Spectrom 39*, 262-271.

130. Sadagopan, N., Cohen, L., Roberts, B., Collard, W., and Omer, C. (2001) Liquid chromatography-tandem mass spectrometric quantitation of cyclophosphamide and its hydroxy metabolite in plasma and tissue for determination of tissue distribution, *J Chromatogr B Biomed Sci Appl 759*, 277-284.

131. Malet-Martino, M., Gilard, V., and Martino, R. (1999) The analysis of cyclophosphamide and its metabolites, *Curr Pharm Des 5*, 561-586.

132. Oertel, R., Richter, K., Weile, K., Gramatte, T., Berndt, A., and Feller, K. (1993) A simple method for the determination of articaine and its metabolite articainic acid in dentistry: application to a comparison of articaine and lidocaine concentrations in alveolus blood, *Methods Find Exp Clin Pharmacol 15*, 541-547.

133. Mathieu, O., Hillaire-Buys, D., Dadure, C., Barnay, F., Mathieu-Daude, J. C., and Bressolle, F. (2006) Liquid chromatography-electrospray mass spectrometry determination of free and total concentrations of ropivacaine in human plasma, *J Chromatogr B Analyt Technol Biomed Life Sci 831*, 91-98.

134. Koehler, A., Oertel, R., and Kirch, W. (2005) Simultaneous determination of bupivacaine, mepivacain, prilocaine and ropivacain in human serum by liquid chromatography-tandem mass spectrometry, *J Chromatogr A 1088*, 126-130.

135. Reif, S., Le Corre, P., Dollo, G., Chevanne, F., and Le Verge, R. (1998) High-performance liquid chromatographic determination of ropivacaine, 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine and 2',6'-pipecoloxylidide in plasma, *J Chromatogr B Biomed Sci Appl 719*, 239-244.

136. Stumpe, M., Morton, N. S., and Watson, D. G. (2000) Determination of free concentrations of ropivacaine and bupivacaine in plasma from neonates using small-scale equilibrium-dialysis followed by liquid chromatography-mass spectrometry, *J Chromatogr B Biomed Sci Appl 748*, 321-330.

137. Jonsson, J. A., Andersson, M., Melander, C., Norberg, J., Thordarson, E., and Mathiasson, L. (2000) Automated liquid membrane extraction for high-performance liquid chromatography of Ropivacaine metabolites in urine, *J Chromatogr A 870*, 151-157.

138. Gaudreault, F., Drolet, P., and Varin, F. (2009) High-performance liquid chromatography using UV detection for the simultaneous quantification of ropivacaine and bupivacaine in human plasma, *Ther Drug Monit 31*, 753-757.

139. Engman, M., Neidenstrom, P., Norsten-Hoog, C., Wiklund, S. J., Bondesson, U., and Arvidsson, T. (1998) Determination of ropivacaine and [2H3]ropivacaine in biological samples by gas chromatography with nitrogen-phosphorus detection or mass spectrometry, *J Chromatogr B Biomed Sci Appl 709*, 57-67.

140. Koster, E. H., Wemes, C., Morsink, J. B., and de Jong, G. J. (2000) Determination of lidocaine in plasma by direct solid-phase microextraction combined with gas chromatography, *J Chromatogr B Biomed Sci Appl 739*, 175-182.

141. Oertel, R., Oertel, A., and Feller, K. (1993) A new method of blood sampling and determination of the local anesthetic agent lidocaine in dentistry, *Methods Find Exp Clin Pharmacol 15*, 131-136.

142. Sawaki, K., Okubo, M., Shimomiya, T., Tukagoshi, E., Sakai, T., Yamazaki, T., Kenmochi, M., Miyao, M., Kaneko, Y., Ichinohe, T., and Kawaguchi, M.

(2009) Evaluation of high-performance liquid chromatography and mass spectrometry method for pharmacokinetic study of local anesthetic ropivacaine in plasma, *Biomed Res 30*, 319-324.

143. Moeller, M. R., Steinmeyer, S., and Kraemer, T. (1998) Determination of drugs of abuse in blood, *J Chromatogr B Biomed Sci Appl 713*, 91-109.

144. Stettin, M., Halwachs-Baumann, G., Genser, B., Fruhwirth, F., Marz, W., and Khoschsorur, G. A. (2006) Determination of cyclosporine A in whole blood: comparison of a chromatographic method with three different immunological methods, *Talanta 69*, 1100-1105.

145. Salm, P., Taylor, P. J., and Rooney, F. (2008) A high-performance liquid chromatography-mass spectrometry method using a novel atmospheric pressure chemical ionization approach for the rapid simultaneous measurement of tacrolimus and cyclosporin in whole blood, *Ther Drug Monit 30*, 292-300.

146. Poquette, M. A., Lensmeyer, G. L., and Doran, T. C. (2005) Effective use of liquid chromatography-mass spectrometry (LC/MS) in the routine clinical laboratory for monitoring sirolimus, tacrolimus, and cyclosporine, *Ther Drug Monit 27*, 144-150.

147. Koster, R. A., Dijkers, E. C., and Uges, D. R. (2009) Robust, high-throughput LC-MS/MS method for therapeutic drug monitoring of cyclosporine, tacrolimus, everolimus, and sirolimus in whole blood, *Ther Drug Monit 31*, 116-125.

148. Korecka, M., and Shaw, L. M. (2009) Review of the newest HPLC methods with mass spectrometry detection for determination of immunosuppressive drugs in clinical practice, *Ann Transplant 14*, 61-72.

149. Koal, T., Deters, M., Casetta, B., and Kaever, V. (2004) Simultaneous determination of four immunosuppressants by means of high speed and robust on-line solid phase extraction-high performance liquid chromatography-tandem mass spectrometry, *J Chromatogr B Analyt Technol Biomed Life Sci 805*, 215-222.

150. Kirchner, G. I., Jacobsen, W., Deters, M., Christians, U., Nashan, B., Winkler, M., Vidal, C., Kaever, V., Sewing, K., and Manns, M. P. (2001) Fast quantification method for sirolimus and its major metabolites, *Transplant Proc 33*, 1091-1092.

151. Keevil, B. G., Tierney, D. P., Cooper, D. P., and Morris, M. R. (2002) Rapid liquid chromatography-tandem mass spectrometry method for routine analysis of cyclosporin A over an extended concentration range, *Clin Chem 48*, 69-76.

152. Deters, M., Kaever, V., and Kirchner, G. I. (2003) Liquid chromatography/mass spectrometry for therapeutic drug monitoring of immunosuppressants, *Analytica Chimica Acta 492*, 133-145.

153. Ceglarek, U., Lembcke, J., Fiedler, G. M., Werner, M., Witzigmann, H., Hauss, J. P., and Thiery, J. (2004) Rapid simultaneous quantification of immunosuppressants in transplant patients by turbulent flow chromatography combined with tandem mass spectrometry, *Clin Chim Acta 346*, 181-190.

154. Christians, U., Jacobsen, W., Serkova, N., Benet, L. Z., Vidal, C., Sewing, K. F., Manns, M. P., and Kirchner, G. I. (2000) Automated, fast and sensitive quantification of drugs in blood by liquid chromatography-mass spectrometry with on-line extraction: immunosuppressants, *J Chromatogr B Biomed Sci Appl 748*, 41-53.

155. Annesley, T. M., and Clayton, L. (2004) Simple extraction protocol for analysis of immunosuppressant drugs in whole blood, *Clin Chem 50*, 1845-1848.

156. Ansermot, N., Fathi, M., Veuthey, J. L., Desmeules, J., Rudaz, S., and Hochstrasser, D. (2008) Simultaneous quantification of cyclosporine, tacrolimus, sirolimus and everolimus in whole blood by liquid chromatography-electrospray mass spectrometry, *Clin Biochem 41*, 728-735.

157. Nagase, K., Iwasaki, K., Nozaki, K., and Noda, K. (1994) Distribution and protein binding of FK506, a potent immunosuppressive macrolide lactone, in human blood and its uptake by erythrocytes, *J Pharm Pharmacol 46*, 113-117.

158. Trepanier, D. J., Gallant, H., Legatt, D. F., and Yatscoff, R. W. (1998) Rapamycin: distribution, pharmacokinetics and therapeutic range investigations: an update, *Clin Biochem 31*, 345-351.

159. Lemaire, M., and Tillement, J. P. (1982) Role of lipoproteins and erythrocytes in the in vitro binding and distribution of cyclosporin A in the blood, *J Pharm Pharmacol 34*, 715-718.

160. Kovarik, J. M., Kahan, B. D., Kaplan, B., Lorber, M., Winkler, M., Rouilly, M., Gerbeau, C., Cambon, N., Boger, R., and Rordorf, C. (2001) Longitudinal assessment of everolimus in de novo renal transplant recipients over the first post-transplant year: pharmacokinetics, exposure-response relationships, and influence on cyclosporine, *Clin Pharmacol Ther 69*, 48-56.

161. Egan, T. D. (1995) Remifentanil pharmacokinetics and pharmacodynamics. A preliminary appraisal, *Clin Pharmacokinet 29*, 80-94.

162. Zhang, C., Zhang, L., Zhang, X., Yang, L., Zhai, S., and Duan, L. (2008) Population pharmacokinetics study of remifentanil in Chinese adult patients determined by an LC-MS/MS method, *Int J Clin Pharmacol Ther 46*, 477-488.

163. Haidar, S. H., Liang, Z., Selinger, K., Hamlett, L., and Eddington, N. D. (1996) Determination of remifentanil, an ultra-short-acting opioid anesthetic, in rat blood by high performance liquid chromatography with ultraviolet detection, *J Pharm Biomed Anal 14*, 1727-1732.

164. Grosse, C. M., Davis, I. M., Arrendale, R. F., Jersey, J., and Amin, J. (1994) Determination of remifentanil in human blood by liquid-liquid extraction and capillary GC-HRMS-SIM using a deuterated internal standard, *J Pharm Biomed Anal 12*, 195-203.

165. Bender, J., van den Elshout, J., Selinger, K., Broeders, G., Dankers, J., and van der Heiden, C. (1999) Determination of remifentanil in human heparinised whole blood by tandem mass spectrometry with short-column separation, *J Pharm Biomed Anal 21*, 559-567.

166. Bjorksten, A. R., Chan, C., and Crankshaw, D. P. (2002) Determination of remifentanil in human blood by capillary gas chromatography with nitrogen-selective detection, *J Chromatogr B Analyt Technol Biomed Life Sci 775*, 97-101.

167. Chism, J. P., and Rickert, D. E. (1996) The pharmacokinetics and extra-hepatic clearance of remifentanil, a short acting opioid agonist, in male beagle dogs during constant rate infusions, *Drug Metab Dispos 24*, 34-40.

168. Zhang, L. P., Yang, L., Bi, S. S., Lu, W., Zhang, X. H., Zhai, S. D., and Duan, L. P. (2009) Population pharmacokinetics of remifentanil in patients undergoing orthotopic liver transplantation, *Chin Med J (Engl) 122*, 1032-1038.

169. Haidar, S. H., Moreton, J. E., Liang, Z., Hoke, J. F., Muir, K. T., and Eddington, N. D. (1997) Evaluating a possible pharmacokinetic interaction between remifentanil and esmolol in the rat, *J Pharm Sci 86*, 1278-1282.

170. Gergov, M., Nokua, P., Vuori, E., and Ojanpera, I. (2009) Simultaneous screening and quantification of 25 opioid drugs in post-mortem blood and urine by liquid chromatography-tandem mass spectrometry, *Forensic Sci Int 186*, 36-43.

171. Bossu, E., Montinaro, A., Lecce, R., Farina, A., Suppa, E., Draisci, G., and Gostoli, G. (2006) LC-MS Determination of remifentanil in maternal and neonatal plasma, *J Pharm Biomed Anal 42*, 367-371.

172. Kabbaj, M., and Varin, F. (2003) Simultaneous solid-phase extraction combined with liquid chromatography with ultraviolet absorbance detection for the determination of remifentanil and its metabolite in dog plasma, *J Chromatogr B Analyt Technol Biomed Life Sci 783*, 103-111.