UNIVERSITA' DEGLI STUDI DI MILANO

Facoltà di Farmacia

Dottorato di Ricerca in Chimica del Farmaco

Curriculum Analisi Farmaceutica, Biofarmaceutica e Tossicologica

AUTOMATION OF SAMPLE PREPARATION IN BIO-ANALYTICS FOR HIGH-THROUGHPUT, ACCURATE LC-MS/MS ANALYSIS AND LABORATORY INFORMATION MANAGEMENT SYSTEM

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XXIII Ciclo (A.A. 2007-2010)

The following work was carried out in Merck Serono SA, Colleretto Giacosa (TO).

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1. Acronyms

ADC: Anti Droplet Control

ADME: Absorption, Distribution, Metabolism and Excretion

ANSI: American National Standards Institute

AN: Analyte

API-LC–MS: Atmospheric Pressure Ionization Liquid Chromatography Mass Spectrometry

(API)-MS: Atmospheric Pressure Ionization Mass Spectrometry

APPI: Atmospheric Pressure Photochemical Ionization

APCI: Atmospheric Pressure Chemical Ionization

CAde: 2-Chloroadenine

CdA: 2-Clorodeoxyadenisina

CID: Collisionally Induced Dissociation

CYP: Cytochrome P450

CORE-Technology: Compressed O-Ring Expansion Technology

Da: Dalton

DC: Direct Current

DBS: Dried Blood Spots

DDM: Dynamic Dilution Module

DMPK: Drug Metabolism and Pharmacokinetics

DQ: Design Qualification

EA: Ethyl Acetate

ELISA: Enzyme-Linked ImmunoSorbent Assay

EQ: Equipment Qualification

ESI: Electro Spry Ionization

GALP: Good Automated Laboratory Practice

GLP: Good Laboratory Practice

GxP: Good Practice

HPLC: High Performance Liquid Chromatography

HPLC–UV: High Performance Liquid Chromatography coupled to Ultra Violet detection

HTPK: High-throughput Pharmacokinetics

HTBSP: High-Throughput Bioanalytical Sample Preparation

IQ: Installation Qualification

IS: Internal Standard

iSWAP: Integrated Solid Waste Alternative Program

LADME: Liberation, Absorption, Distribution, Metabolism and Excretion

LC: Liquid Chromatography

LC/MS: Liquid Chromatography Mass Spectrometry

LIMS: Laboratory Information Management System

LLE: Liquid-Liquid Extraction

LLOQ: Lower Limit of Quantitation

MAD: Monitoring Air Displacement

MET: Metabolite

MRM: Multi-Reaction Monitoring

MS: Mass Spectrometry

MS (2): Multiple Sclerosis

MS/MS: Tandem Mass Spectrometry

MTBE: Methyl Tert-Butil Ether

NCE: New Chemical Entity

OQ: Operational Qualification

PBPK: Physiologically Based Pharmacokinetic

PQ: Performance Qualification

PCR: Polymerase Chain Reaction

PhD: Philosophiæ Doctor

PK: Pharmacokinetic

PPT: Protein Precipitation Technique

RF: Radio Frequency

SBS: Society for Biomolecular Screening

SOP: Standard Operative Procedure

SPE: Solid Phase Extraction

SRM: Single-Reaction Monitoring

TADM: Total Aspirate and Dispense Monitoring

TCA: Trichloroacetic Acid

ULOQ: Upper Limit of Quantitation

WI: Work Instruction

2. Acknowledgments

I would like to express my sincere gratitude towards my supervisors Prof. Marina Carini and Dr. Luca Maria Barbero for their help and support, and for giving me the opportunity to work and study at the Merck Serono S. A., Italy.

To Dr. Luca Maria Barbero goes out a very special thank his expertise, understanding and patience, added considerably to my experience. I appreciate his vaste knowledge and skill in many scientific and not areas.

My thanks also to personnel involved collaboration together with colleagues and support staff.

I am particularly grateful to Dr.ssa Rita Mastroianni and all of the support staff who have assisted in this project.

Financial support from Merck Serono is also gratefully acknowledged.

3. Abstract

High-throughput Bioanalytical Sample Preparation (HTBSP) is a method used in Research, Pre-Clinical and Clinical drug development to speed-up bioanalysis.

Bioanalysis is the quantitative determination of drugs and their metabolites in biological fluids. This technique is used very early in the drug development process to provide support to drug discovery programs on the metabolic fate and pharmacokinetics of chemicals in living cells and in animals. Its use continues through the preclinical and clinical drug development phases, into post-marketing support and may sometimes extend into clinical therapeutic drug monitoring[1].

Developing high-capacity sample preparation systems and strategies are of key importance in providing breakthrough in the time required to develop a drug by increasing the number/time of analyzed samples. This objective must be achieved without loosing quality within the obtained data and it is of paramount importance in Pharmacokinetic/dynamic studies.

The aim of this PhD project in Pharmaceutical, Biopharmaceutical and Toxicological Analysis at University of Milan developed in Merck Serono laboratories, is to automate the manual sample preparation processes for LC/MS analysis using high-throughput techniques, and to insert the developed processes in the frame of a Pharmaceutical Bioanalytical process managed by a LIMS system.

The main techniques examined are: Automatic Liquid Handling, Sample Dilution, Protein Precipitation Technique (PPT), Liquid-Liquid Extraction (LLE), Solid Phase Extraction (SPE) and other innovative sample preparation techniques such as Dried Blood Spot (DBS) Technique and Hybrid PPT-SPE.

The use of liquid handling technology, for example, to automate the protein precipitation procedures for collection microplates and filterplates, is an important step to improve the throughput of these universal sample preparation methods. All liquid handling steps can potentially be performed by a workstation: sample transfer, addition of internal standards and

1 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. 5, 1, (2003), 1.

precipitant solutions, transfer of supernatants to a clean microplates and preparation for injections via dilution or reconstitution in a mobile phase compatible solvents[2].

Additionally, automation can be utilized prior to the precipitation procedure in order to prepare the calibration standards and the quality control samples in a microplate format.

It is important, to apply laboratory automation, choosing the right projects and technology, developing a strategy and plan, finding the resources, managing the project and implementing, validating and preparing for long-term operation.

Therefore one of the first strategies developed during my project has been the implementation in the sample preparation process of a Hamilton Starlet Robot. This device should have then been interfaced with a LIMS system to further reduce manual routine operations like serial dilutions, tube-to-plate transfers and data management and storage.

Initially, I focused my attention on the technologies for LC/MS, LIMS, whose knowledge was a mandatory background to properly establish a liquid handling platform based on 96 well plate format for different LC/MS sample preparation techniques.

This strategy aimed to generate the following advantages:

- 96 samples purified at the same time;
- flexibility, i.e., enabling the scouting for the best approach by using different chemical strategies, such as PPT, LLE, SPE or others;
- purified samples available in less time than manual operations;
- precipitation and filtration in the same well;
- no laborious pipetting and/or centrifugation required and minimal manual liquid handling;
- use of LIMS interface to automatically generate and transfer the sample/plate maps to the liquid handling station.

It is also important to validate, in a GLP environment, the systems, materials and processes that are necessary to be applied to conduct one pre-clinical study. This means

2 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. 5, 7, (2003), 255.

that all the process parameters will be evaluated, optimized and validated for long-term process usage in a regulated environment and eventually transferred to other studies.

4. Pharmacokinetics and Bioanalytics

Pharmacokinetics, sometimes abbreviated as PK, (from Ancient Greek pharmakon "drug" and kinetikos "to do with motion") is a branch of pharmacology dedicated to the determination of the fate of substances administered externally to a living organism. In practice, this discipline is applied mainly to drug substances, though in principle it concerns itself with all manner of compounds ingested or otherwise delivered externally to an organism, such as nutrients, metabolites, hormones, toxins, etc.

Pharmacokinetics is often studied in conjunction with Pharmacodynamics. Pharmacodynamics explores what a drug does to the body, whereas Pharmacokinetics explores what the body does to the drug. Pharmacokinetics includes the study of the mechanisms of absorption and distribution of an administered drug, the rate at which a drug action begins and the duration of the effect, the chemical changes of the substance in the body (e.g. by enzymes) and the effects and routes of excretion of the metabolites of the drug[3].

As described before, Pharmacokinetics is divided into several areas which include the extent and rate of Absorption, Distribution, Metabolism and Excretion. This is commonly referred as to the ADME scheme. However recent understanding about the drug-body interactions brought about the inclusion of new term Liberation.

Now Pharmacokinetics can be better described as LADME.

Description in

Figure 1:

- Liberation is the process of release of drug from the formulation.
- Absorption is the process of a substance entering the body.
- Distribution is the dispersion or dissemination of substances throughout the fluids and tissues of the body.
- Metabolism is the irreversible transformation of parent compounds into daughter metabolites.

3 Pharmacokinetics (2006). In Mosby's Dictionary of Medicine, Nursing, & Health Professions. Philadelphia, PA: Elsevier Health Sciences. Retrieved December 11, 2008.

- Excretion is the elimination of the substances from the body. In rare cases, some drugs irreversibly accumulate in a tissue in the body.

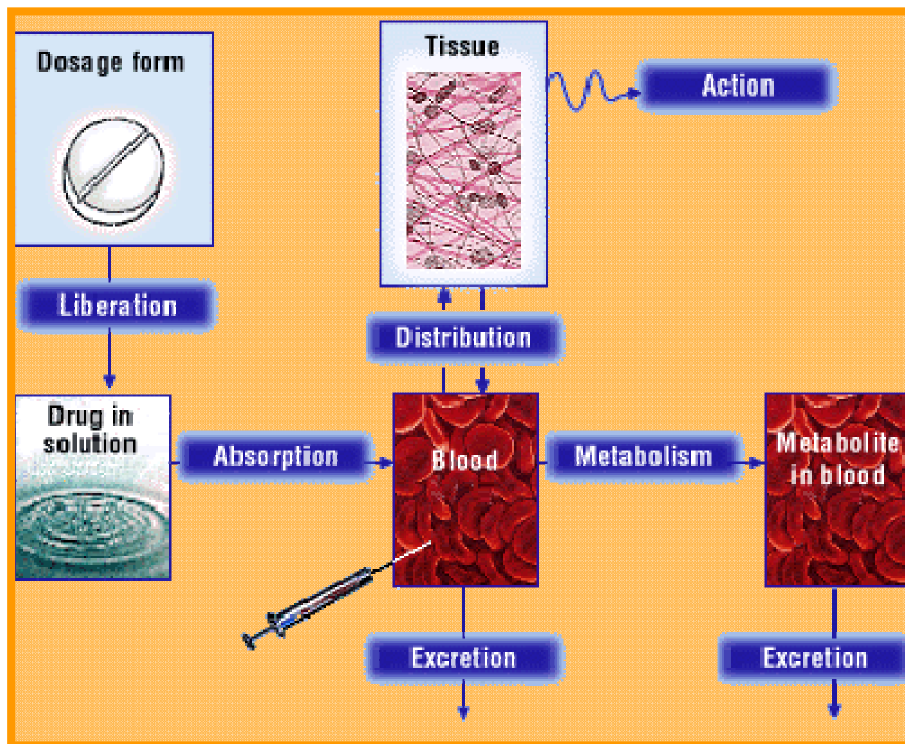

Figure 1: LADME scheme.

Pharmacokinetics describes how the body affects a specific drug after administration. Pharmacokinetic properties of drugs may be affected by elements such as the site of administration and the concentration in which the drug is administrated. These may affect the absorption rate[4].

Pharmacokinetic analysis is performed by noncompartmental (model independent) or compartmental methods. Noncompartmental methods estimate the exposure to a drug by estimating the area under the curve of a concentration-time graph. Compartmental methods estimate the concentration-time graph using kinetic models. Compartment-free methods are often more versatile in that they do not assume any specific compartmental model and procedure accurate results also acceptable for bioequivalence studies.

4 Kathleen Knights; Bronwen Bryant (2002). Pharmacology for Health Professionals. Amsterdam: Elsevier. ISBN 0-7295-3664-5.

4.1. Noncompartmental analysis

Noncompartmental PK analysis is highly dependent on estimation of total drug exposure. Total drug exposure is most often estimated by Area Under the Curve methods, with the trapezoidal rule (numerical differential equations) the most common area estimation method. Due to the dependence on the length of 'x' in the trapezoidal rule, the area estimation is highly dependent on the blood/plasma sampling schedule. That is, the closer your time points are, the closer the trapezoids are to the actual shape of the concentration-time curve.

4.2. Compartmental analysis

Compartmental PK analysis uses kinetic models to describe and predict the concentration-time curve. PK compartmental models are often similar to kinetic models used in other scientific disciplines such as chemical kinetics and thermodynamics. The advantage of compartmental over some no compartmental analyses is the ability to predict the concentration at any time. The disadvantage is the difficulty in developing and validating the proper model. Compartment-free modeling based on curve stripping does not suffer this limitation. The simplest PK compartmental model is the one-compartmental PK model with IV bolus administration and first-order elimination. The most complex PK models (called PBPK models) rely on the use of physiological information to ease development and validation.

4.3. PBPK model

Physiologically-based pharmacokinetic (PBPK) modeling is a mathematical modeling technique for predicting the absorption, distribution, metabolism and excretion (ADME) of a compound in humans and other animal species. PBPK modeling is used in pharmaceutical research and development, and in health risk assessment.

PBPK models strive to be mechanistic by mathematically transcribing anatomical, physiological, physical, and chemical descriptions of the phenomena involved in the complex ADME processes. Some degree of residual simplification and empiricism is still present in those models, but they have an extended domain of applicability compared to

that of classical, empirical function based, pharmacokinetic models. Given that property, PBPK models may have purely predictive uses, but other uses, such as statistical inference, have been made possible by the development of Bayesian statistical tools able to deal with complex models[5]. That is true for both toxicity risk assessment and therapeutic drug development.

PBPK models try to rely *a priori* on the anatomical and physiological structure of the body. These are usually also multi-compartment models, but the compartments correspond to predefined organs or tissues, for which the interconnections correspond to blood or lymph flows (more rarely to diffusions). A system of differential equations can still be written, but its parameters represent blood flows, pulmonary ventilation rate, organ volumes etc., for which information is available in scientific publications. Indeed the description of the body is simplified and a balance needs to be struck between complexity and simplicity. Besides the advantage of allowing the recruitment of *a priori* information about parameter values, these models also facilitate inter-species transpositions or extrapolation from one mode of administration to another (e.g., inhalation to oral).

It is interesting to note that the first pharmacokinetic model described in the scientific literature was in fact a PBPK model. It led, however, to computations intractable at that time. The focus shifted then to simpler models, for which analytical solutions could be obtained (such solutions were sums of exponential terms, which led to further simplifications). The availability of computers and numerical integration algorithms marked a renewed interest in physiological models in the early 1970s.

PBPK models are compartmental models like many others, but they have a few advantages over so-called "classical" pharmacokinetic models, which are less grounded in physiology.

An example of a 7-compartment PBTK model, suitable to describe the fate of many solvents in the mammalian body, is given in Figure 2.

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5 Gelman A., Bois F., Jiang J. (1996) Physiological pharmacokinetic analysis using population modeling and informative prior distributions Journal of the American Statistical Association, 91:1400-1412.

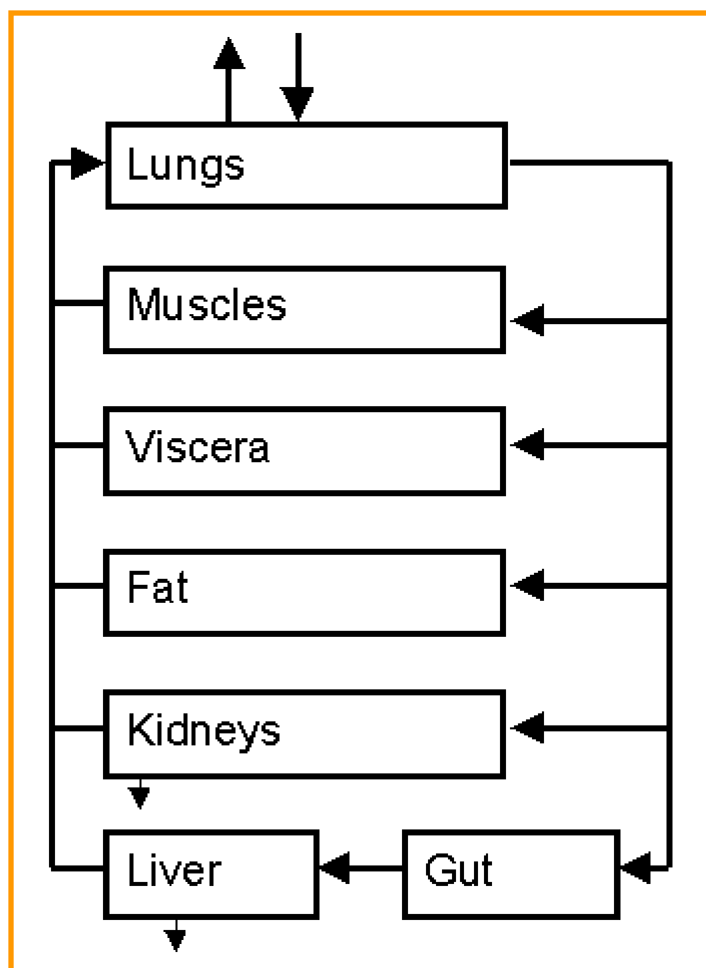

Figure 2: Example of a 7-compartment PBTK model.

PBPK models can first be used to abstract and eventually reconcile disparate data (from physico-chemical or biochemical experiments, *in vitro* or *in vivo* pharmacological or toxicological experiments, etc.). They give also access to internal body concentrations of chemicals or their metabolites, and in particular at the site of their effects, be it therapeutic or toxic. Finally they also help interpolation and extrapolation of knowledge between:

- Doses: e.g., from the high concentrations typically used in laboratory experiments to those found in the environment.
- Exposure duration: e.g., from continuous to discontinuous, or single to multiple exposures.
- Routes of administration: e.g., from inhalation exposures to ingestion.

- Species: e.g., transpositions from rodents to human, prior to giving a drug for the first time to subjects of a clinical trial, or when experiments on humans are deemed unethical, such as when the compound is toxic without therapeutic benefit.
- Individuals: e.g., from males to females, from adults to children, from non-pregnant women to pregnant.

Some of these extrapolations are "parametric": only changes in input or parameter values are needed to achieve the extrapolation (this is usually the case for dose and time extrapolations). Others are "nonparametric" in the sense that a change in the model structure itself is needed (e.g., when extrapolating to a pregnant female, equations for the fetus should be added).

4.4. Bioanalytical methods

Bioanalytical methods are necessary to construct a concentration-time profile. Chemical techniques are employed to measure the concentration of drugs in biological matrix, most often plasma or urine. Proper Bioanalytical methods should be selective and sensitive.

Pharmacokinetics is often studied using mass spectrometry because of the complex nature of the matrix and the need for high sensitivity to observe low dose and long time point data.

In order to measure the characteristics of individual molecules, a mass spectrometer converts them to ions so they can be moved about and manipulated by external electric and magnetic fields to be measured.

The three essential functions of a mass spectrometer, and the associated components, are:

- A small sample of compound is ionized, usually to cations by loss of an electron (the ion source).
- The ions are sorted and separated according to their mass and charge (the mass analyzer).
- The separated ions are then detected and tallied, and the results are displayed on a chart (the detector).

The most common instrumentation used in this application is LC-MS with a triple quadrupole mass spectrometer since multiple fragmentations can be induced a followed using MS/MS technique. Tandem mass spectrometry is in fact employed for adding specificity. The samples represent different time points as a pharmaceutical is administered and then metabolized or cleared from the body. Blank or t=0 samples taken before administration are important in determining background and insuring data integrity with such complex sample matrices. Standard curves and internal standards are used for quantitation of usually a single pharmaceutical in the samples. Much attention is paid to the linearity of the standard curve; however it is not uncommon to use curve fitting with more complex functions such as quadratics since the response of most mass spectrometers is less than linear across large concentration ranges[6,7,8].

There is currently considerable interest in the use of very high sensitivity mass spectrometry for micro dosing studies, which are seen as a promising alternative to animal experimentation[9].

4.4.1. HPLC-MS Technique

The HPLC technique is one of the most used techniques in environmental analytical chemistry, to separate mixtures of non volatile compounds.

Hyphenating HPLC with mass spectrometry (MS) permits the separation and characterization of a great number of compounds and it makes possible to collect information about molecular mass and structure of the species. Mass spectrometry detection increases the sensitivity and selectivity of the separation method.

The greatest problem of HPLC-MS technique is the interfacing, because:

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6 Hsieh Y, Korfmacher WA (June 2006). "Increasing speed and throughput when using HPLC-MS/MS systems for drug metabolism and pharmacokinetic screening". Current Drug Metabolism 7 (5): 479–89.

7 Covey TR, Lee ED, Henion JD (October 1986). "High-speed liquid chromatography/tandem mass spectrometry for the determination of drugs in biological samples". Anal. Chem. 58 (12): 2453–60.

8 Covey TR, JB Crowther, EA Dewey, JD Henion (February 1985). "Thermospray liquid chromatography/mass spectrometry determination of drugs and their metabolites in biological fluids". Anal. Chem. 57 (2): 474–81.

9 Committee for Medicinal Products for Human Use (CHMP) (2004-06-23). "Position Paper on Non-Clinical Studies to Support Clinical Trials with a Single Microdose" (in en) (PDF). CPMP/SWP/2599/02 Rev 1. European Medicines Agency, Evaluation of Medicines for Human Use.

- in HPLC samples are liquids and soluble in the mobile phase, while in MS they are gases;
- HPLC works at room temperature, while MS works at temperature between 100° and 350° C;
- in HPLC it is possible to use not volatile inorganic salt in the mobile phase, while in MS it is not possible.

API (Atmospheric Pressure Ionization) permits to interface liquid chromatography and mass spectrometry. The most common ionization systems are: Electro Spray Ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI) and Atmospheric Pressure Photochemical Ionization (APPI).

4.4.2. ESI

The electrospray ionization technique (ESI) (Figure 3) is a method to generate highly charged droplets from which ions are ejected by ion evaporation process. Ion Spray is an improved version of electrospray, in which droplets are created by both a high electric field and a pneumatic nebulisation which allows higher liquid flow rates to be employed and produces a more stable ion current.

The mechanism by which ions can be emitted from a liquid into the gas phase was first proposed in 1976[10] and the term Ion Evaporation was applied to the process. Experimental evidence combined with a theoretical model suggested that, as a charged droplet evaporates; a critical point may be reached at which it is kinetically and energetically possible for ions at the surface of the liquid to "evaporate".

The details of the process are independent on how the droplets are originally formed or how they are originally charged. A charged droplet contains the solvent plus both positive and negative ions, with ions of one polarity being dominant, the difference being the net charge.

The excess ions are those which were originally present in the liquid being sprayed. If there are enough excess ions, and the droplet evaporates far enough, a critical field is

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10 Iribarne, J.V. and Thomson, B.A. (1976). On the Evaporation of Small Ions from Charged Droplets. J. Chem. Phys. 64, 2287.

reached at which ions are emitted from the surface. The rate of ion emission depends on the solvation energies of the individual ions so that one type of ion may preferentially evaporate if it has lower solvation energy.

In both electrospray and ionspray, an electric field is generated at the tip of the sprayer by applying a high voltage directly to the sprayer. The sprayer itself is isolated by fused silica tubing connected to the pump. Ions of one polarity are preferentially drawn into the drops by the electric field as they are separated from the bulk liquid.

Electrospray and ionspray operate both with flow from 1 -5µl/min to 1 ml/min[11].

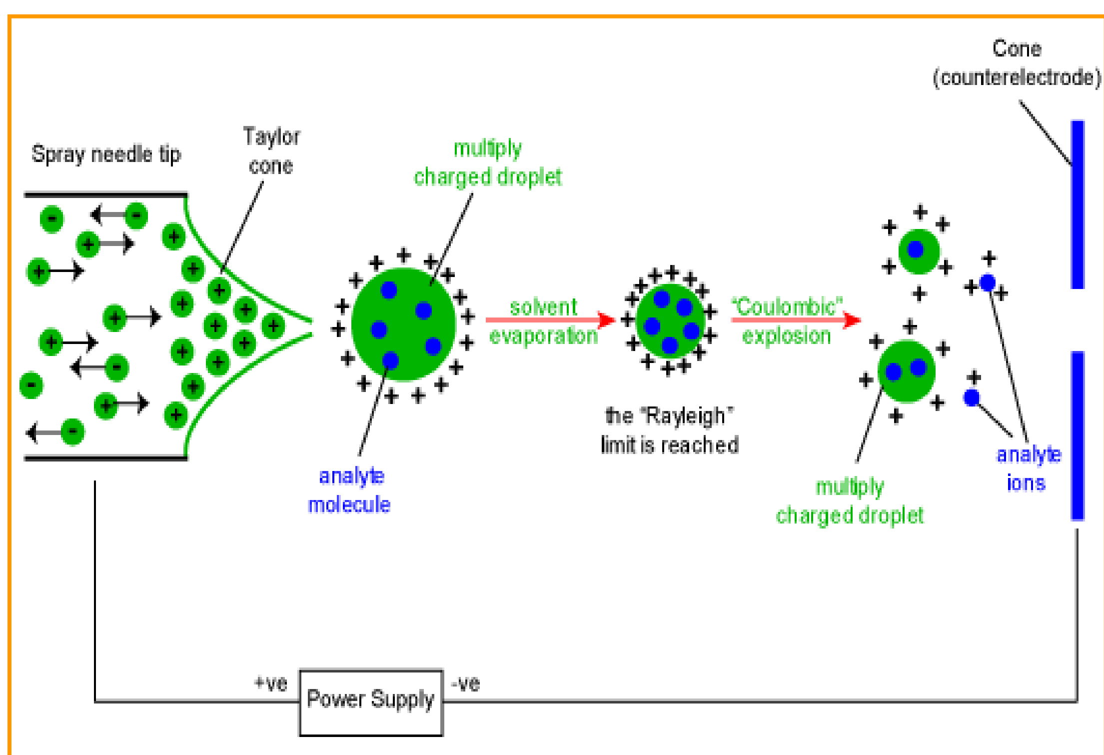

Figure 3

4.4.3. APCI

Atmospheric pressure chemical ionization (APCI) interface is mainly based on ion-molecule reactions in gaseous phase which take place between analyte molecules and a

11 The API Book, Perkin-Elmer Sciex Instruments. 1994.

reagent gas, generated by a series of reactions ion-molecule which, in turn, arise from a "crown discharge".

This technique allows the source to be operated at atmospheric pressure. In negative ion mode, electrons created by the crown discharge undergo collisions with neutral species and are captured by species such as O_2 to form O_2^- and O^-. The superoxide anion (O_2^-), and its hydrates ($O_2^-[H_2O]n$) and clusters ($O_2^-[O_2]n$), are the major reactant negative ions in an APCI source directly sampling ambient air. The neutral species formed in the corona discharge (NO, NO_2, CO, etc.) may react with O_2^- or O^- to form a series of minor reactant ions as NO_2^-, NO_3^- and CO_3^2.

The ions may react in several ways: charge transfer, where the positive or negative charge on the reactant ion is transferred to the analyte; and proton transfer, where a proton is transferred from a positive ion to the analyte or a proton is transferred from the analyte to a negative reactant ion. These processes are summarized in the following equations.

Charge transfer:

- $R^+ + A \longrightarrow A^+ + R$ **(1)**
- $R^- + A \longrightarrow A^- + R$ **(2)**

Proton transfer:

- $RH^+ + A \longrightarrow AH^+ + R$ **(3)**
- $R^- + AH \longrightarrow A^- + RH$ **(4)**

R represents the reactant and A the analyte.

The sensitivity of the APCI system is approximately three orders of magnitude less for charge transfer than for proton transfer. For example, benzene is detected as the M^+ ion with a detection limit in the 1µg L^{-1} range, whereas aniline is detected as the MH^+ in the 1ng l^{-1} range. To overcome this sensitivity problem in the charge transfer reactions, a suitable chemical ionization reagent may be added to the ambient air flow entering the source region[12].

Description in

Figure 4.

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12 The API Book, Perkin-Elmer Sciex Instruments. 1994.

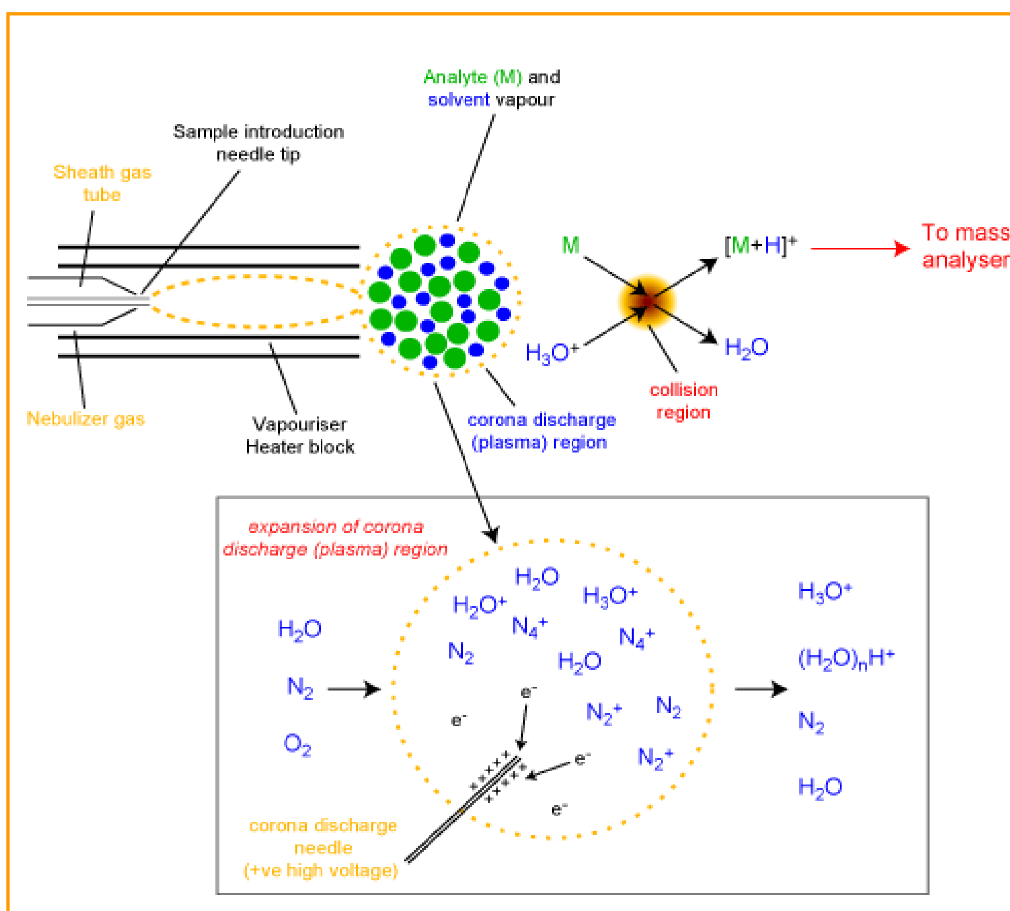

Figure 4

4.4.4. Mass analyzers

4.4.4.1. Quadrupole and triple quadrupole

The quadrupole is one type of mass analyzer. It is the component of the instrument responsible for filtering sample ions, based on their m/z ratio. A quadrupole mass analyzer is essentially a mass filter that is capable of transmitting only the ion of choice. The mass spectrum is obtained by scanning through the mass range of interest over time. The quadrupole consists of four parallel metal rods. Each opposing rod pair is connected electrically and a radio frequency (RF) voltage is applied between one pair of rods, and the other. A direct current (DC) voltage is then superimposed on the RF voltage. Ions travel down the quadrupole between the rods. Only ions of a certain m/z will reach the detector for a given ratio of voltages: other ions have unstable trajectories and will collide

with the rods. In liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry they serve as exceptionally high specificity detectors.

A linear series of three quadrupoles can be used; known as “triple quadrupole” mass spectrometer. The first (Q1) and third (Q3) quadrupoles act as mass filters, and the middle (Q2) quadrupole is employed as a collision cell.

This collision cell is an RF only quadrupole (non-mass filtering) using Ar or N_2 gas ($\sim 10^{-3}$torr) to induce collisional dissociation of selected precursor ions from Q1. Subsequent fragments are passed through to Q3 where they may be filtered or scanned.

This process allows for the study of fragments (product ions) which are crucial in structural identification. For example, the Q1 may be set to "filter" for a drug ion of a known mass, which is fragmented in Q2. The third quadrupole (Q3) can then be set to scan the entire m/z range, giving information on the sizes of the fragments made. Thus, the structure of the original ion can be deduced[13] from Figure 5.

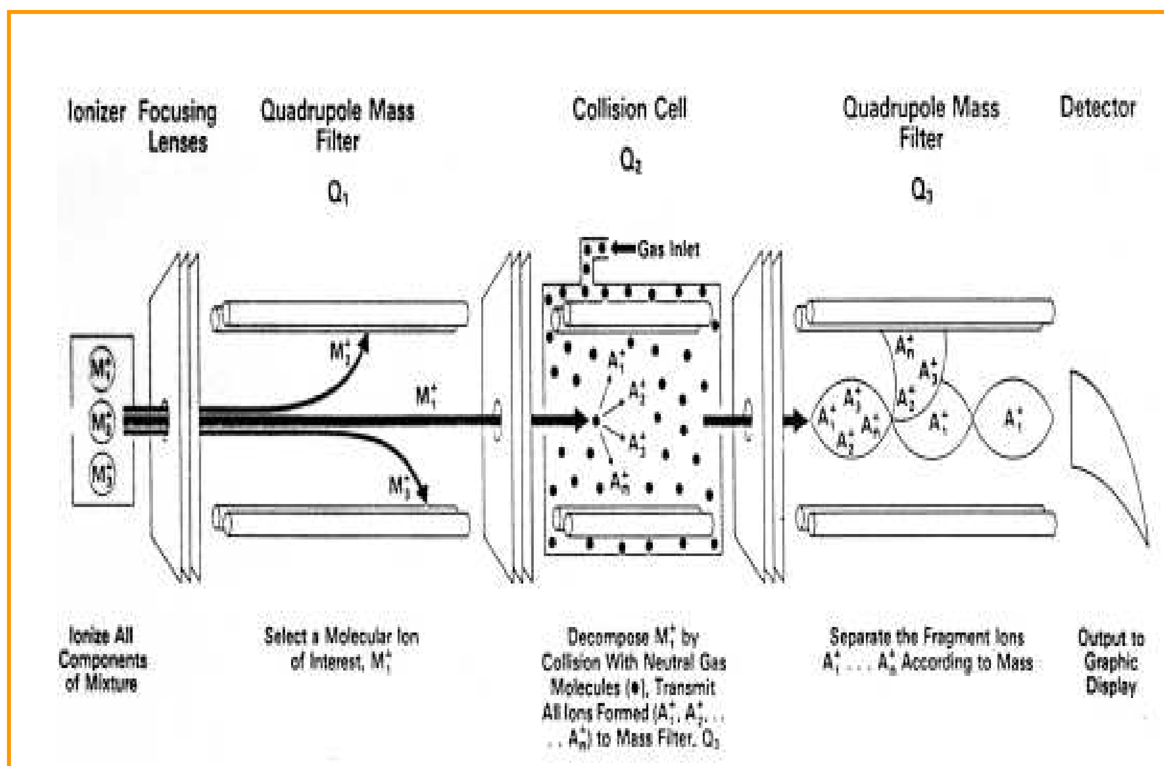

Figure 5

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13 M.Vincenti. MS/MS Triple Quadrupole Analyzers MSn Ion Trap Analyzers, Acquisitions Methods, Scuola Nazionale di Spettrometria di Massa, Parma.

Quadrupole instruments typically have unit mass resolution throughout the mass range.

In a triple quadrupole mass spectrometer, there are several types of experiment that can be performed. The figures show a schematic representation of four common types of MS/MS experiment:

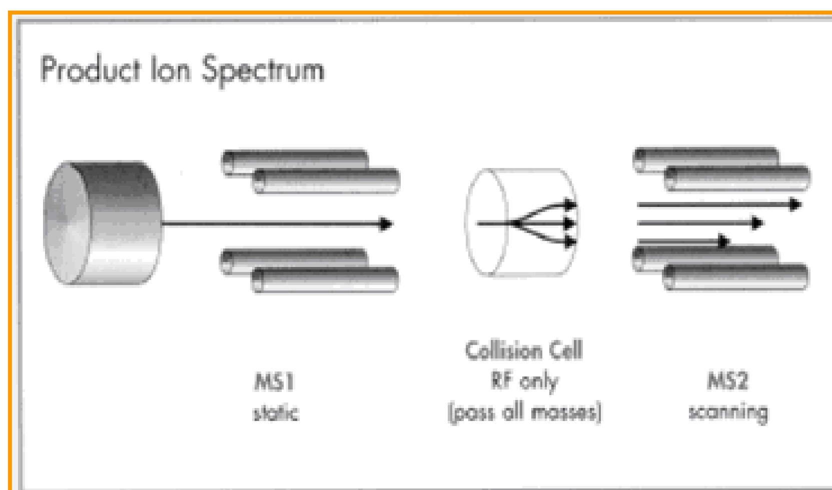

Figure 6: Product Ion Scan.

Product ion scan.

In this case, the precursor ion is focused in Q1 and transferred into Q2, the collision cell, where it interacts with a collision gas and fragments. The fragments are then measured by scanning Q3. This results in the typical MS/MS spectrum and is the method most commonly employed with ESI ionization and/or LC-MS, description in Figure 6.

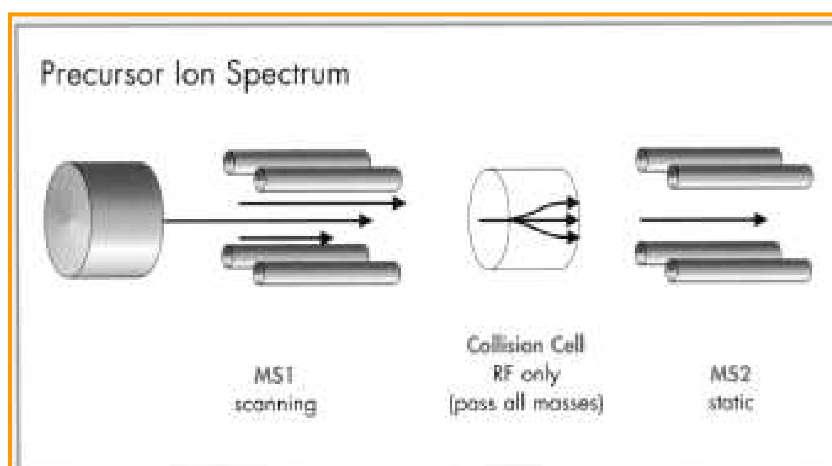

Figure 7: Precursor Ion Scan.

Precursor ion scan.

In this case Q3 is held to measure the occurrence of a particular fragment ion and Q1 is scanned. This results is a spectrum of precursor ions that result in that particular product ion, this is especially useful when used with EI or CI ionization and/or GC-MS, description in Figure 7

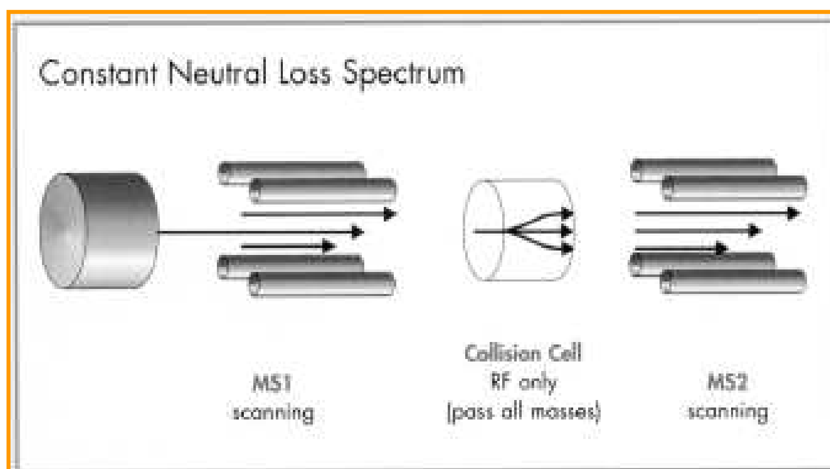

Figure 8: Neutral Loss Scan.

Neutral loss scan.

In this case Q1 is scanned as in precursor ion scan but this time Q3 is also scanned to produce a spectrum of precursor ions that undergo a particular neutral loss. Again this mode is especially useful for EI and CI ionization, description in Figure 8.

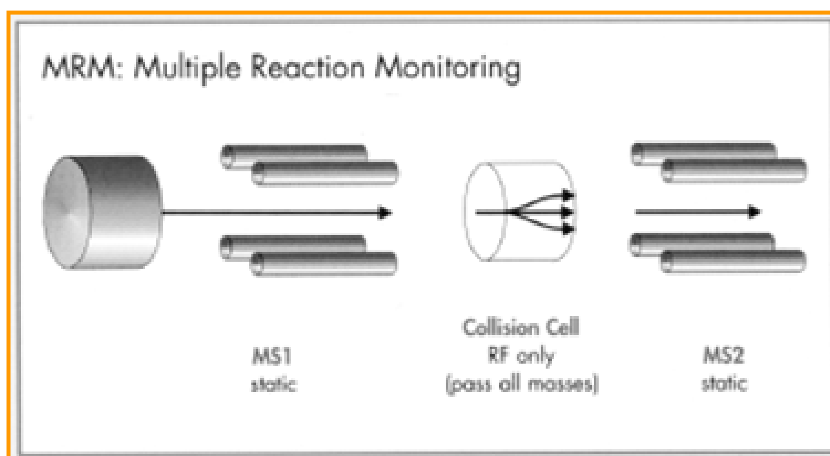

Figure 9: Multiple Reaction Monitoring.

Multiple Reaction Monitoring.

Also in this case, the precursor ion is focused in Q1 and transferred into Q2, the collision cell, where it interacts with a collision gas and fragments. Q3 is held to measure a particular fragment ion[14], description in Figure 9.

4.5. Population pharmacokinetics

Population pharmacokinetics is the study of the sources and correlates of variability in drug concentrations among individuals who are the target patient population receiving clinically relevant doses of a drug of interest[15,16]. Certain patient demographic, pathophysiological, and therapeutical features, such as body weight, excretory and metabolic functions, and the presence of other therapies, can regularly alter dose-concentration relationships. For example, steady-state concentrations of drugs eliminated mostly by the kidney are usually greater in patients suffering from renal failure than they are in patients with normal renal function receiving the same drug dosage. Population pharmacokinetics seeks to identify the measurable pathophysiologic factors that cause changes in the dose-concentration relationship and the extent of these changes so that, if such changes are associated with clinically significant shifts in the therapeutic index, dosage can be appropriately modified.

4.6. Pharmacokinetics in practice

The PK profile of a drug, in which the absorption, distribution, metabolism and excretion processes following *in vivo* administration are mathematically described, is derived from the plot of the systemic drug concentration versus time for that compound. Following either parenteral or oral administration, serial blood samples are collected as a

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14 M.Vincenti. MS/MS Triple Quadrupole Analyzers MSn Ion Trap Analyzers, Acquisitions Methods, Scuola Nazionale di Spettrometria di Massa, Parma.

15 Sheiner, L.B.; Rosenberg, B., Marathe, V.V. (1977). "Estimation of Population Characteristics of Pharmacokinetic Parameters from Routine Clinical Data". J. Pharmacokin. Biopharm. 5: 445–79.

16 Sheiner, L.B.; Beal, S.L., Rosenberg, B. Marathe, V.V. (1979). "Forecasting Individual Pharmacokinetics". Clin. Pharmacol. Ther. 26 (3): 294–305.

function of time and then analyzed for drug content. The complete process is summarized in Figure 10and can be divided into five main areas:

- the in vivo work, including the initial surgery required to prepare animals for dosing, actual dosing and collection of blood samples;
- the calibration of an analytical method;
- the preparation of samples prior to analysis;
- the actual quantitative analysis;
- data reduction and reporting.

Consideration of the *in vivo* aspects yields little opportunity for throughput improvements for single-compound studies as, by definition, the experiment must be conducted in real time. However, alternate experimental designs such as multicomponent analysis might provide new options. Clearly, the calibration, sample preparation and analysis steps have represented the major bottlenecks and it is these that have appropriated the most attention for the development of higher throughput approaches.

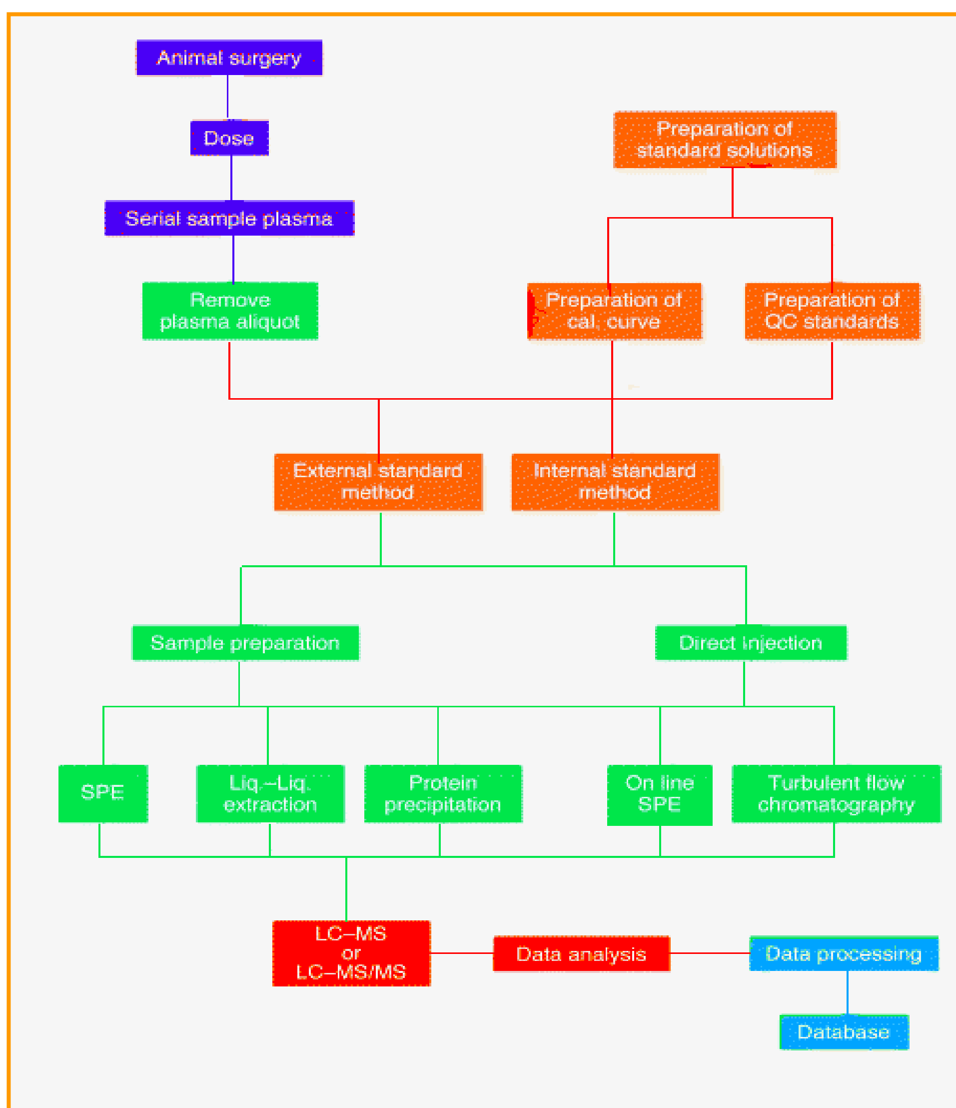

Figure 10: Flow diagram representing the processes required to implement a typical pre-clinical pharmacokinetic study. The various components required are colour coated as follows: in vivo work (purple), calibration (orange), sample preparation (green), sample analysis (red) and data analysis (blue).

4.7. Calibration

To ensure that measured concentrations truly reflect the actual sample concentrations, analytical methods are calibrated by adding varying known concentrations of drug into control matrix (e.g. plasma). These are then prepared and analyzed following a protocol identical to that used for the actual samples. Preparation of appropriate dilutions is time-

consuming and laborious, but must be performed to a high degree of accuracy as the integrity of the assay results depends on this. Similarly, quality controls are included to assess the performance of the assay over time as these are interspersed into the analytical run. These values are then compared with those obtained from the calibration standards.

4.8. Sample preparation methodologies

The aim of the sample preparation process is to provide a suitable specimen, usually for chromatographic analysis, which will not contaminate the instrumentation and where the concentration in the prepared sample is reflective of that found in the original. The selected method of sample preparation is generally dictated by the analytical techniques available and the physico-chemical characteristics of the analytes under investigation. The two main sample preparation methods are matrix cleanup or direct injection. In a matrix cleanup procedure, the aim is to remove as much endogenous material as possible from the drug sample, either by liquid- or solid-phase extraction (SPE) or by precipitation of the plasma proteins. In the direct injection, which is usually only amenable to relatively lipophilic compounds, the compound of interest interacts with a stationary phase, which is then eluted and analyzed, leaving behind the endogenous material.

Although it is imperative that all samples, standards and quality controls are treated identically, processing each sample sequentially is time-consuming and prone to error, even in the hands of a well-skilled analyst, as the operations are highly repetitive. This suggests that the introduction of new technologies and automation at the sample preparation stage should help improve throughput and reduce errors.

4.9. Analysis

In the early 1990s, there was a vast increase in the analysis of xenobiotics in biofluids with the introduction and widespread adoption of atmospheric pressure ionization (API)-MS and, in particular, MS/MS coupled to HPLC[17,18,19]. With non-selective analyses such

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17 Gilbert, J.D. et al. (1992) Determination of L-365260, a new cholecystokinin receptor (CCK-B) antagonist, in plasma by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. Biol. Mass Spectrom. 21, 63–68.

as HPLC–UV, which was the previous mainstay of bioanalysis, all sample components possessing a chromophore could be detected resulting in complex chromatograms in which the peak of interest was often difficult to distinguish from endogenous components. With API-LC–MS, however, detection is performed on the basis of the presence of protonated molecular ion of the parent compound such that only components with that mass (± 0.5Da) are detected. There is a further gain in specificity through using LC–MS/MS operating in a single-reaction monitoring (SRM) or multi-reaction monitoring (MRM) mode. Here, the transition from the mass-to-charge ratio of the precursor ion to a specific product ion produced within a collision cell in the mass spectrometer is determined by collisionally induced dissociation (CID). This essentially removes background interference, as it is likely that only the 'parent' ion will generate these precursor and product ions.

Improvements in signal-to-noise ratio result in higher simplified chromatograms with greater sensitivity and selectivity for the compound of interest. Additionally, the ability to rapidly scan different transitions enables the simultaneous analysis of several components without the requirement for chromatographic resolution, thereby reducing run times. Furthermore, this enables rapid generic gradients to be applied to all samples, therefore eliminating chromatographic method development time.

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18 Gilbert, J.D. et al. (1992) Determination of L-654066, a new 5areductase inhibitor in plasma by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. Biol. Mass Spectrom. 21, 341–346.

19 Gilbert, J.D. et al. (1996) High-performance liquid chromatography with atmospheric pressure ionization tandem mass spectrometry as a tool in quantitative bioanalytical chemistry. ACS Symp. Ser. 619,330–350.

5. High-throughput

The development of new therapeutic drugs is a long and costly process. With the introduction of combinatorial chemistry, high throughput screening, and other technologies, the speed of lead generation has increased[20]. However, prioritization of leads with regard to drug metabolism and pharmacokinetics (DMPK) in animals appears crucial in the selection of viable targets from millions of compounds.

With pressure on pharmaceutical companies to reduce time-to-market and improve the success rate of new drug candidates, higher-throughput pharmacokinetic support has become an integral part of many drug discovery programmers.

To deliver the promise of HTPK are fundamental the amalgamation of robotics, new sample preparation technologies and highly sensitive and selective mass spectrometric detection systems.

Discovery pharmacokinetics (PK) has traditionally been regarded as a low-throughput activity, a finding that is directly opposed to the need to address the high attrition rate of compounds in drug development caused by poor PK profiles[21].

Historically, the drug discovery cascade has been established as a sequential process with many drug candidates, which originate from a variety of sources, being initially screened for potency against a biological target. At best, limited PK and metabolism studies would be conducted prior to acceptance of a compound for safety evaluation with, as it transpires, the said high attrition rate.

A new paradigm in drug discovery has emerged in which the entire sample collection is rapidly screened using robotized high-throughput assays at the outset of a programme[22,23], often together with some selectivity testing against other targets. This frequently provides

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20 High-throughput pharmacokinetic method: Cassette dosing in mice associated with minuscule serial bleedings and LC/MS/MS analysis. Takaho Watanabe, Daniela Schulz, Christophe Morisseau and Bruce D. Hammock.

21 Broach, J.R. and Thorner, J. (1996). High-throughput screening for drug discovery. Nature 384, 14–16.

22 Burbaum, J.J. and Sigal, N.H. (1997). New technologies for highthroughput screening. Curr. Opin. Chem. Biol. 1, 72–78.

23 Gallop, M.A. et al. (1994). Applications of combinatorial technologies to drug discovery: Background and peptide combinatorial libraries. J. Med. Chem. 37, 1233–1251.

higher quality leads for optimization by the medicinal chemistry department, often employing new combinatorial synthetic technologies[24, 25].

Higher-throughput PK (HTPK) is being achieved through the introduction of new techniques, including automation for sample preparation and new experimental approaches for the evaluation of many substances in parallel.

Application of these technologies can now support multiple projects at a pace appropriate to the output of a synthetic chemistry team, thus providing timely and relevant information that will impact directly on drug design.

In this chapter, the PK process, the historical context of newer developments, current HTPK approaches (including upstream and downstream activities), and future prospects for further improvements are discussed.

5.1. Automation perspective

From the inception of modern chromatographic science, automation technologies have continuously developed, beginning with the introduction of autosamplers and microprocessor-controlled instrumentation in the late 1970s to early 1980s. This enabled the sequencing of samples, often requiring different analytical instrument characteristics, and this increased the number of samples that could be processed in a batch by enabling unattended, overnight operation.

In the mid-1980s, early adopters of robotic technologies were using the newly introduced Zymark Zymate II[26] to try to further improve throughput by programming a robot to reproduce manual operations from the sample preparation steps[27, 28, 29].

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24 Gallop, M.A. et al. (1994) Applications of combinatorial technologies to drug discovery: 1. Background and peptide combinatorial libraries. J. Med. Chem. 37, 1233–1251.

25 Gordon, E.M. et al. (1994) Applications of combinatorial technologies to drug discovery: 2. Combinatorial organic synthesis, library screening strategies, and future directions. J. Med. Chem. 37, 1385–1401.

26 Zymark Ltd, Runcorn, Cheshire, UK.

27 Fouda, H.G. and Schneider, R.P. (1987) Robotics for the bioanalytical laboratory: A flexible system for the analysis of drug in biological fluids. Trends Anal. Chem. 6, 139–147.

28 Fouda, H.G. and Schneider, R.P. (1988) Robotics for the bioanalytical laboratory: Analysis of antipyrine in human serum. Am. Clin. Prod. Rev. 7, 12–15.

Whilst some gains in productivity using this approach were evident, there were several problems with such systems:

- the use of robotics to purely reproduce a manual method is usually not the most efficient or cost-effective way of proceeding;
- many methods suffered from poor reliability and required frequent maintenance or recalibration;
- at best, most methods were semi-automated, often requiring substantial manual input, and therefore negating the advantages of employing the system;
- the timing of implementation, which was prior to the adoption of LC–MS/MS, was wrong as sample analysis was still the rate-determining step. Hence, automation of sample preparation did not bring substantial gains in productivity.

In fact, the initial slow adoption of systems such as the linear track robots in the late 1980s demonstrates the lack of commitment to automation at the time, as other areas of the infrastructure were not in place to allow this to be fully exploited nor justified from a cost perspective.

The traditional sample containers or collection devices used in bioanalysis have been individual test tubes and vials, available in many different dimensions and made from glass or plastic. Individual pipettors have been used with this tubes or vial to transfer sample, solvent and any other liquids as part of the overall sample preparation procedure.

Techniques such as protein precipitation (PPT) and liquid-liquid extraction (LLE) have also traditionally used single tubes for the extraction step; solid-phase extraction (SPE) has used individual cartridges.

Individual tubes or vials held a stronghold as the preferred sample container until the late 1990s. At this time, several advances occurred in parallel that brought about a change from tubes to microplates as the preferred sample and collection format[30].

With the introduction of the microtiter plate format as the standard platform on which to perform a multitude of assays, it was inevitable that the time gains seen in areas such as

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29 Fouda, H.G. et al. (1988) Liquid chromatographic analysis of doxazosin in human serum with manual and robotic sample preparation. J. Chromatogr. Sci. 26, 570–573.

30 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. 3, 1, (2003), 75-76.

biochemical screens through parallel sample processing would be adopted for bioanalytical assays.

Furthermore, the shift in formats from vials and tubes to microtiter plates has made it much more viable to apply robotic technologies. While 96-well microplates had been in use in other areas of pharmaceutical research they become more commonly available in larger well volumes. They also were molded in polypropylene, a plastic that has the required solvent resistance for use with common organic solvents. Filtration and solid-phase extraction were introduced in the 96-well plate format in flow-through process.

The microplate presents many efficiencies of operation such as easier labeling, sealing and manipulation.

When this format is combined with multiple probe workstations, dramatically faster pipetting throughput is achieved via parallel sample processing. The microplate architecture clearly offered the increased proficiency and productivity in sample preparation processes and analysis that were sought.

5.2. Current technologies

The current options available to increase the throughput of a PK assay can be divided into the two main areas of sample preparation and experimental design, although consideration should also be given to data handling and utilization, which will also be discussed.

5.2.1. Sample preparation

Simply grouping together the cartridges on one analysis plate, increases throughput, as it is easier to manually treat several samples simultaneously[31]. However, many groups are using this as the basis for more fully automated analysis[32,33,34] with typical sample

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31 Cheng, Y-F. et al. (1998) Straightforward solid-phase extraction method for the determination of verapamil and its metabolite in plasma in a 96-well extraction plate. J. Chromatogr. A 828, 273–281.

32 Allanson, J.P. et al. (1996) The use of automated solid phase extraction in the ‘96 well’ format for high throughput bioanalysis using liquid chromatography coupled to tandem mass spectrometry. Rapid Commun. Mass Spectrom. 10, 811-816.

preparation times of under two hours for 96 samples. A further extension of this approach is the use of SPE disk technologies[35,36]. Here, the thickness of the adsorbent bed is dramatically reduced, enabling elution in volumes approximating those used for chromatographic analysis.

Hence, the eluent from the SPE disk can be analyzed directly, therefore eliminating steps such as evaporation and reconstitution. Liquid–liquid extraction can also reliably work in a 96-well plate format[37]. Other techniques, such as trace enrichment[38] or online column switching[39], can improve throughput but are usually application specific.

5.3. Microtiter plate

A microtiter plate or microplate (Figure 11) is a flat plate with multiple "wells" used as small test tubes.

The microplate has become a standard tool in analytical research and clinical diagnostic testing laboratories. A very common usage is in the enzyme-linked immunosorbent assay (ELISA), the basis of most modern medical diagnostic testing in humans and animals.

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33 Harrison, A.C. and Walker, D.K. (1998) Automated 96-well solidphase extraction for the determination of doramectin in cattle plasma. J. Pharm. Biomed. Anal. 16, 777-783.

34 Pleasance, S. and Biddlecombe, R.A. (1998) Automating '96-well' format solid phase extraction for LC-MS-MS. Methodol. Surv. Bioanal. Drugs 25, 205-212.

35 Plumb, R.S. et al. (1997) Use of reduced sorbent bed and disk membrane solid-phase extraction for the analysis of pharmaceutical compounds in biological fluids, with applications in the 96-well format. J. Chromatogr. B Biomed. Sci. Appl. 694, 123-133.

36 Simpson, H. et al. (1998) High throughput liquid chromatography/mass spectrometry bioanalysis using 96-well disk

solid phase extraction plate for the sample preparation. Rapid Commun. Mass Spectrom. 12, 75-82.

37 Steinborner, S. and Henion, J. (1999) Liquid–liquid extraction in the 96 well plate format with SRM LC/MS quantitative determination of methotrexate and its major metabolite in human plasma. Anal. Chem. 71, 2340-2345.

38 Kronkvist, K. et al. (1998) Automated sample preparation for the determination of budesonide in plasma samples by liquid chromatography and tandem mass spectrometry. J. Chromatogr. A 823, 401-409.

39 Gao, V.C.X. et al. (1998) Column switching in high-performance liquid chromatography with tandem mass spectrometric detection for highthroughput preclinical pharmacokinetic studies. J. Chromatogr. A 828, 141-148.

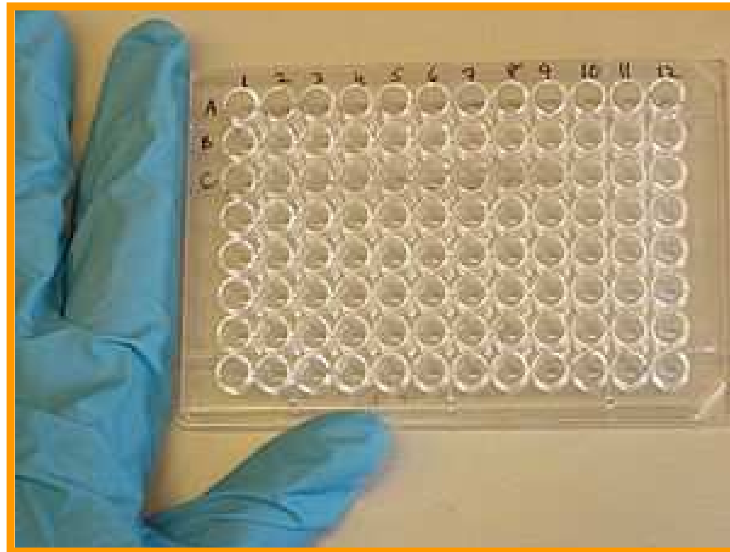

Figure 11: An example of 96-well microtiter plate.

High throughput applications are based on samples being reformatted from racks of test tubes or vials into 8-rows by 12-columns format. Once in this configuration, the samples are contained in a compact space and each well is uniquely identified by column number and row letter, e.g., A1, A2, A3, C5, F8 or H12. The tedious and time consuming task of individually labeling tubes or vials is now eliminated.

Other kinds of microplate could have 6, 24, 384 or even 1536 sample wells arranged in a 2/3 rectangular matrix. Some microplates have even been manufactured with 3456 or even 9600 wells, and an "array tape" product has been developed that provides a continuous strip of microplates embossed on a flexible plastic tape[40].

Each well of a microplate typically holds somewhere between tens of nanoliters to several milliliters of liquid. They can also be used to store dry powder or as racks to support glass tube inserts. Wells can be either circular or square, example in

Figure 12.

40 Elaine May (2007-06-15). "Array Tape for Miniaturized Genotyping". Genetic Engineering & Biotechnology News (Mary Ann Liebert, Inc.): p. 22. Retrieved 2008-07-06. "(subtitle) Processing hundreds of microplate equivalents without complex plate-handling equipment".

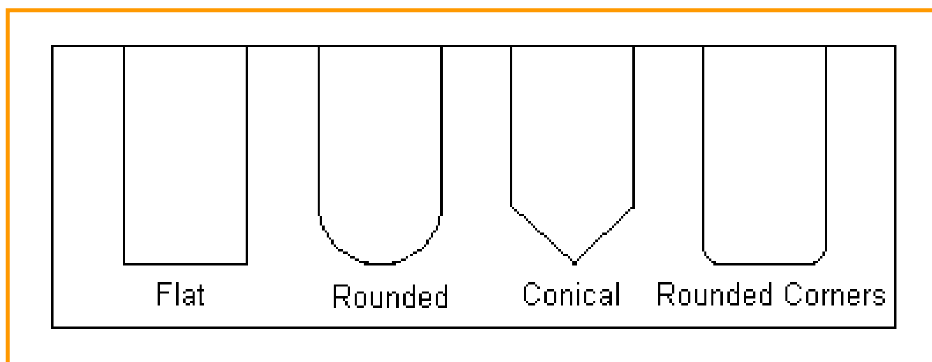

Figure 12: Different wells. Four well bottom geometries are commonly available in microplates: flat bottom (F-bottom), rounded bottom (U-bottom), conical (V-bottom) and rounded corners leading to flat bottom (C-bottom).

For compound storage applications, square wells with close fitting silicone cap-mats are preferred. Microplates can be stored at low temperatures for long periods, may be heated to increase the rate of solvent evaporation from their wells and can even be heat-sealed with foil or clear film. Microplates with an embedded layer of filter material were developed in the early 1990s by several companies, and in 1992, the world's first Solid Phase Extraction (SPE) microplate was launched by Porvair Sciences. This allowed simple column chromatography to be carried out in a microplate footprint for the first time. Today there are microplates for just about every application in life Science research which involves filtration, separation, optical detection, storage and reaction mixing or cell culture.

The enormous growth in studies of whole live cells has led to an entirely new range of microplate products which are "tissue culture treated" especially for this work. The surface of these products is modified, using a plasma discharge, to make it easier for adherent cells to grow on.

A number of companies have developed robots to specifically handle SBS microplates. These robots may be liquid handlers which aspirate or dispense liquid samples from and to these plates, or "plate movers" which transport them between instruments, plate stackers which store microplates during these processes, plate hotels for longer term storage or microplate incubators to ensure constant temperature during testing.

Instrument companies have designed plate readers which can detect specific biological, chemical or physical events in samples stored in these plates.

5.3.1. Manufacture and composition

Microplates are manufactured in a variety of materials. The most common is polystyrene, used for most optical detection microplates. It can be colored white by the addition of titanium dioxide for optical absorbance or luminescence detection or black by the addition of carbon for fluorescent biological assays. Polypropylene is used for the construction of plates subject to wide changes in temperature, such as storage at -80°C and thermal cycling. It has excellent properties for the long-term storage of novel chemical compounds. Polycarbonate is cheap and easy to mould and has been used for disposable microplates for the polymerase chain reaction (PCR) method of DNA amplification. Cyclo-olefins are now being used to provide microplates which transmit ultraviolet light for use in newly developed assays.

The most common manufacturing process is injection moulding, used for polystyrene, polypropylene and cyclo-olefin. Vacuum forming can be used with softer plastics such as polycarbonate. Composite microplates, such as filter plates and SPE plates and even some advanced PCR plate designs use multiple components which are moulded separately and later assembled into a finished product. ELISA plates may now be assembled from twelve separate strips of eight wells, making it easier to only partially use a plate. This saves cost for the scientist.

5.3.2. History

The earliest microplate was created in 1951 by a Hungarian, Dr. G. Takatsky, who machined 6 rows of 12 "wells" in Lucite. However, common usage of the microplate began in the late 1950s when John Liner in USA had introduced a molded version. By 1990 there were more than 15 companies producing a wide range of microplates with different features. It was estimated that 125 million microplates were used in 2000 alone. The word "Microtiter" is a trademark registered by the Dynatech Company; it is now more usual to use the generic term "microplate".

In 1996, the Society for Biomolecular Screening (SBS) began an initiative to create a standard definition of a microtiter plate. A series of standards was proposed in 2003 and

published by the American National Standards Institute (ANSI) on behalf of the SBS. The standards govern various characteristics of a microplate including well dimensions (e.g. diameter, spacing and depth) as well as plate properties (e.g. dimensions and rigidity).

As early as the first meeting of the Society for Biomolecular Screening (SBS) in 1995, a need for clearly defined dimensional standards of a microplate was identified. At the time, the microplate was already becoming an essential tool used in drug discovery research. At the time, the concept of a microplate was similar among various manufacturers, but the dimensions of microplates produced by different vendors, and even within a single vendors catalog line varied. This often caused numerous problems when microplates were to be used in automated laboratory instrumentation.

In late 1995, members of the SBS began working on defining dimensional standards for the standard 96 well microplate. The first written proposal was released in December 1995 and presented at numerous scientific conferences and journals throughout 1996. This initial proposed standard was officially presented to the membership of SBS for approval at the annual meeting in October 1996 in Basel, Switzerland. Between then and late 1998, various versions of the proposed standards for 96 and 384 well microplates were circulated to the membership of the society. In early 1999, efforts to begin formalizing the proposed standards in preparation for submission to a recognized standards organization were begun. The email ListServ was started in March, and the first regular quarterly meeting of the working committee met in August of that year. To date, the ListServ contains members representing over 100 corporations, educational institutions, and government organizations from over 15 nations.

This standard was processed and approved for submittal to ANSI by the Microplate Standards Development Committee of the Society for Biomolecular Screening.

5.3.3. Fundamental microplate specification as proposed by The Society for Biomolecular Screening (SBS)[41]

- The wells in a 96-well microplate should be arranged as 8 rows by 12 columns.
- Center-to-center well spacing should be 9.0 mm (0.3543 in).
- The outside dimension of the base footprint, measured at any point along the side, shall be: Length 127.76 mm (5.0299 in), Width 85.48 mm (3.3654 in).
- The footprint must be continuous and uninterrupted around the base of the plate.
- The overall plate height (shallow well design) should be 14.35 mm (0.5650 in).
- The maximum allowable projection above the top stacking surface is 0.76 mm (0.0299 in).
- The four outside corners of the plate's bottom flange shall have a corner radius to the outside of 3.18 mm (0.1252 in).

41 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. 3, 1, (2003), 81.

6. Automation

Bioanalysis is used very early in the drug development process to provide support to drug discovery programs on the metabolic fate of chemicals in animals and living cells. Its use continues throughout the preclinical and clinical drug development phases.

The detection techniques of choice, liquid chromatography interfaced with tandem mass spectrometry (LC-MS/MS), allows more samples to be analyzed per unit time than ever before.

As a result of this capability for rapid analysis, and the greater number of compounds able to be synthesized by combinatorial chemistry techniques, faster turnaround became expected for both Pharmacological and Bioanalytical evaluations.

The industry demanded faster sample analysis as the race to market became more aggressive.

Sample preparation was targeted as a rate limiting step in the overall procedure for bioanalysis.

The automation in sample preparation is an important goal for laboratories to meet the high throughput demands required in pharmaceutical research and development. The choices for automation differ in complexity according to the required tasks.

Processes can be introduced to make a manual procedure less tedious, to replace most or all steps in a manual procedure, to perform a specific application comprised of multiple linked steps or to perform a procedure around the clock.

The size of automated instruments can vary as well, from a small benchtop instrument to a large instrument placed on a tabletop to a room-sized throughput ultra high configuration composed of individual modules linked together.

Faster off-line approaches were developed, more productive formats (96-well plate) and more efficient automation solutions for the off-line sample preparation methods. The term "off-line" refers to performing the sample preparation procedure independently of the LC-MS/MS analysis.

The throughput of sample preparation techniques for drug bioanalysis has increased dramatically in many approaches to keep peace with the speed of detection.

Improving overall throughput and laboratory productivity are important goals for introducing automated processes into bioanalytical sample preparation methodologies, but there are other motivating factors.

Automation can reduce the hands-on time required by an analyst and allows the scientist to perform other tasks in the laboratory. Unattended operation of a process may permit multiple or overnight runs, further increasing throughput. Removing an individual from hazardous or mundane tasks can maintain worker health and safety.

The automation of most processes has been shown to bring a degree of reproducibility and quality to the results that cannot be realized among different workers each performing the method manually. In addition, compliance with GLP is considerably improved as a result of the remarkable process consistency that automation provides.

Automation can often facilitate troubleshooting by removing the manual component processes. It can introduce process documentation into a method and also allow for less skilled workers to perform a complex task. An important overall goal for implementing automation into a laboratory workflow is greater employee job satisfaction[42].

6.1. Strategies for Selecting Automation

6.2. Defining how Automation will be used and supported

A laboratory must properly assess its needs for automation and define how it will be used and supported in the workspace.

The focus of my PhD project is on the off-line processes for sample preparation.

How: it will be used for performing the off-line techniques for protein precipitation, liquid-liquid extraction, solid phase extraction and other techniques in microplates.

Which: the flexibility of the system is the main objective and it could be reach with the adaptability of the instrument to perform multiple tasks which is an important criterion in the decision making process. Also upgradeability is very important, one configuration can be purchased and over time its capabilities can be upgraded.

Where: multiple instruments can be scattered among individual laboratories or one instrument can be located in a common area and shared by a large user group.

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42 D.A.Wells and T.L.Lloyd, In.: J.Pawliszyn, Ed., Sampling and Sample preparation for Field and laboratory: fundamentals and New Directions in Sample Preparation, Elsevier, Amsterdam (2002).

What type of access: when a dry down step is required off-line the automation should remain available. When the sample has been dry down the user returns to the instrument and performs the reconstitution step.

How flexible should be the software: it can be a decision factor in selecting among automation choices. Software with great flexibility and many options can be daunting to some analyst taking weeks to master. Only experienced users or custodians are able to program them.

How much support: Standard Operation Procedure (SOP) must be developed and followed to ensure accuracy and precision of pipetting with proper attention to maintenance and service issues.

How much space: if the dimensions permit the best way to accomplish vapor removal is to place the unit inside a custom built enclosure.

6.3. General considerations

Aspects to be considered are the number of samples processed per hour in the automation productivity and the hands-on time required by the analyst. For example a semi-automated method that requires 15 min hands-on time may be preferable to a fully automated system that might cost more but perform at similar productivity.

Usually the available founds strongly influence the capability that automation can provide and the rate at which it can be implemented. Having a 8-tip and a 96-tip sample preparation workstation is ideal one for reformation and one for extraction. When it is not possible the necessity to find other solutions grows.

Continuous training and education in automation has a big priority even if training in the principles and operation of mass spectrometers is supported. Only individuals that have been properly trained can use a validated system, therefore training is an important requirement.

Conferences in automation are a great source of continuing education. Vendor courses are also available at their site or in house for in-depth training on specific automation platforms.

As described before in Merck Serono Ivrea Automation Laboratory the main instrument is a Microlab Starlet Hamilton Robotics, a flexible pipetting workstation with 8 probes that display variable spacing.

Unique to the STAR is a patented CO-RE technology (Compressed induced O-Ring Expansion). CO-RE represents advancement in tip coupling that allows positive grasping, sealing and releasing between the channel and the tip. Compatibility is demonstrated with both disposable tips and steel probes.

Dual mode (capacitive and pressure based) liquid level detection provides sensing for both conductive and non-conductive liquids. An optional internal gripper called iSWAP is available to manipulate plates on the instrument deck or transfer the to adjacent microplate compatible instrument.

A vacuum system can be placed to the deck of the STAR. A requirement of the vacuum manifold is that it must be self-sealing when a plate is placed on top, so the gasket material must perform every time the vacuum is applied. Also, in order to provide flexibility, a manifold has to be able to accommodate varieties in heights of flow-through microplates and collection plates. Another useful option is a temperature controlled microplate carrier that can maintain temperatures on the instrument deck from -22°C to +60°C[43].

Automation is a crucial resource in today's bioanalytical pre clinical and clinical laboratory environment, helping to offset a number of healthcare industry trends—specifically, laboratory errors caused by manual mishandling of samples, a worsening labor shortage in laboratories.

An automated solution provides faster, more-efficient, and more-accurate results.

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43 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. 5, (2003).

7. Hamilton Robotics

The aim of this PhD project in Pharmaceutical, Biopharmaceutical and Toxicological Analysis is to automate assays successfully and within budget such as the manual sample preparation processes for LC/MS analysis.

Hamilton's workstations and software serve as a high precision and flexible base upon which to provide automated solutions with air displacement pipetting and monitoring technology as well as the software controlling systems.

7.1. Liquid Handling Workstations

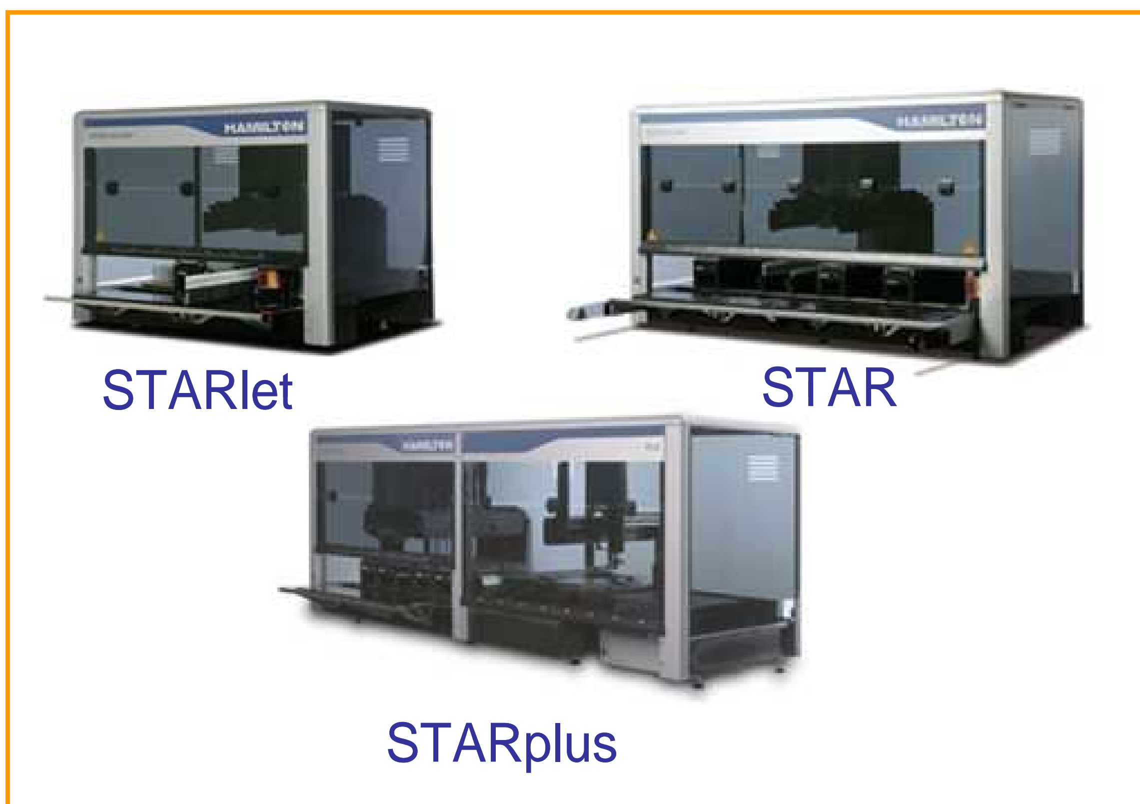

Figure 13: Hamilton robotic workstation STAR Line.

Figure 14: Automation Lab.

In

Figure 13 and Figure 14 are shown the three MicroLab Star lines. The STAR line workstations are based on superior air displacement pipetting technology. This increases accuracy and repeatability while providing chain of custody with pipette condition monitoring and recording. Each workstation can be configured with multiple arms and each arm can be configured with multiple pipetting and labware gripping devices. Pipetting channels and labware grippers move independently of each other, supporting the use of a wide range of labware. The autoload option provides barcode tracking of samples, labware, racks and carriers. All workstation functions and integrated third-party devices are controlled by the Vector 4.1.1. Software.

There are four different levels of authorization for the Vector Software

- "Lab Operator", routine user: operators may run any method.
- "Lab Operato 2", routine user: operators 2 may run any method and move elements on deck.
- "Lab Method Programmer", method programmer laboratory manager: method programmers may modify method and labware definitions.
- "Lab Service", service technician laboratory manager: service technician for Hamilton's laboratory software,

This software offers an intuitive, flexible and powerful programming control and all the tools to allow simple to complex programming, without limiting imagination or compromising requirements.

Data can be tracked and processed within the application as well as interfaces to internal and external databases, including LIMS. The STAR can serve as a simple pipettor for serial dilutions or act as the center of a large system with multiple workstations and third party devices such incubators, cell counters, centrifuges, etc.

7.1.1. CORE-Technology: Compressed O-Ring Expansion

Bioanalytical applications require precision tip attachment and positioning. To meet this demand, the workstation uses quality engineered components and the CO-RE tip attachment technology. The CO-RE system (Figure 15) attaches disposable tips or washable steel tips to the pipetting channels with a stable lock-and-key fit and a precision of ±0.1 mm in all axes. The Hamilton STAR is therefore ideal for tasks where the highest precision is required such as MALDI target spotting, 1536 well pipetting and, in my case, low volume dispensing.

The Hamilton STAR requires no vertical force for tip attachment or tip ejection, thus eliminating mechanical stress and improving the overall system reliability along with pipetting speed and dexterity. Added benefits of CO-RE technology include:

- Use of both disposable and washable tips within the same run.
- Ability to pick up CO-RE grippers and other tools, Figure 15.
- Elimination of aerosol production upon tip ejection.

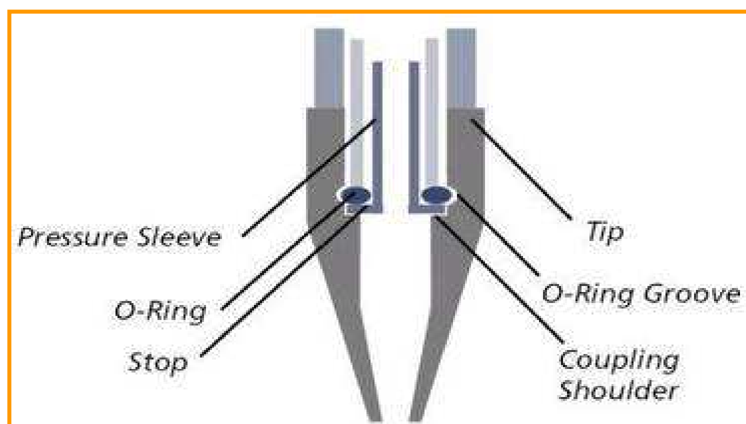

Figure 15: CORE Technology.

7.1.2. Total Aspirate and Dispense Monitoring: TADM

During crucial sample transfers, the STAR can monitor aspiration and dispense steps in real time. TADM verifies with a traceable digital audit trail that a sample has been transferred.

High and low pressure limit bands are first predetermined from multiple test sample runs. During routine pipetting, errors are time reported in real-time when the measured pressure exceeds these bands. The system can then simply record the errors or react to them, Figure 16.

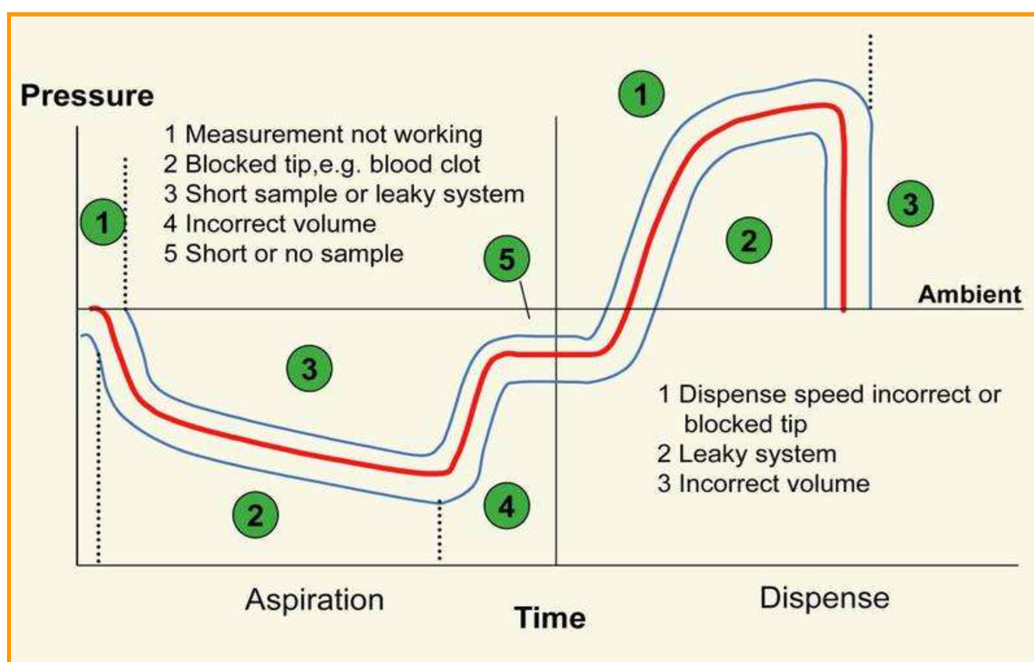

Figure 16: TADM

7.1.2.1. Error event handling

Pressure data is continuously recorded during pipetting for Chain of Custody.

When the pressure goes outside the pressure limits prescribed for different times during the aspiration or dispense cycles, an error event is registered and communicated to the script.

Vector scripts can be written in a variety of ways to handle these events. It can ignore them, stop the method, ask for user intervention or intelligently attempt to deal with the

error without user intervention. For example, in the event of a clog or too low pressure during aspiration, the script could evacuate the tip or needle and try to aspirate again.

It could even move the tip slightly to avoid a swab in a tube and perhaps be programmed to make several aspiration attempts before requesting user intervention or flag the sample as an error and automatically move on to the next sample.

Profile is described in

Figure 17.

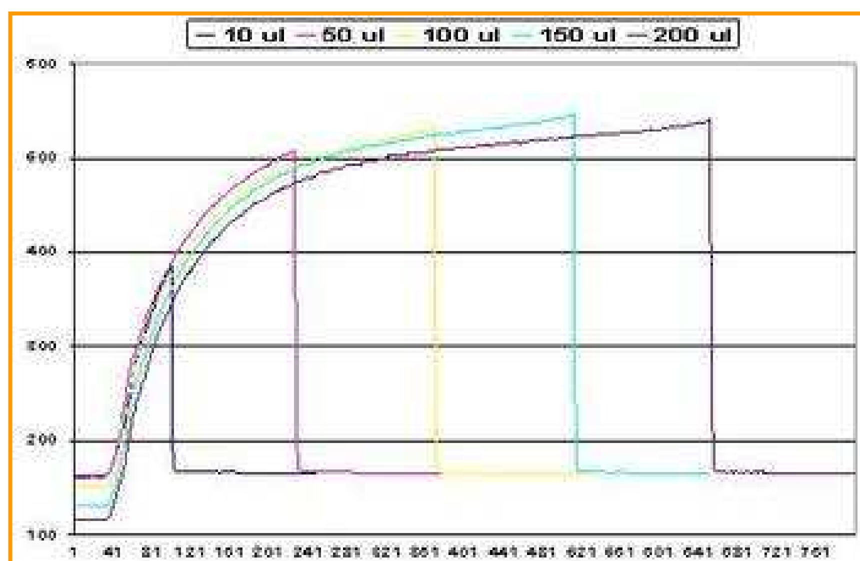

Figure 17: typical curves.

7.1.2.2. Aspiration Error Detection

Tip blockage (2) is detected when the pressure drops below the minimum set values.

The minimum set values vary with aspiration/dispense time and are predetermined by the results of test runs. Limits can be widened or tightened to minimize false errors.

Insufficient sample (3) or incorrect aspiration is detected when the pressure rises above the maximum limit during aspiration.

7.1.2.3. Dispense Error Detection

Blocked tip (1) is detected when dispense pressure rises above the maximum pressure limit during dispense.

Leaking seals (2) are detected when the pressure declines below the minimum pressure

limit during dispense.

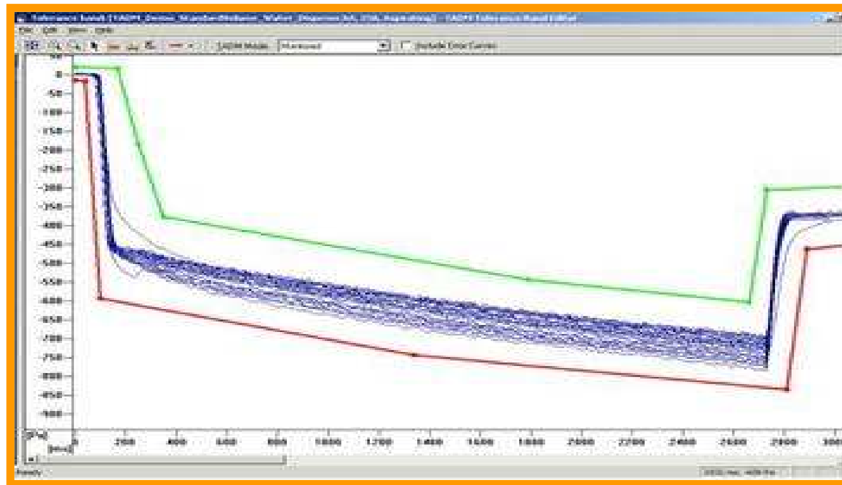

Figure 18: Typical curves - 50 successful runs

TADM curves (mbar vs. msec) from aspirating 50 samples (Figure 18) of the same volume with no errors. Maximum and minimum error bands are set just outside this range of data to allow for variation within observed successful dispenses. Band settings are stored in the software by liquid class. A unique liquid class can be created for each liquid type and pipetting volume range.

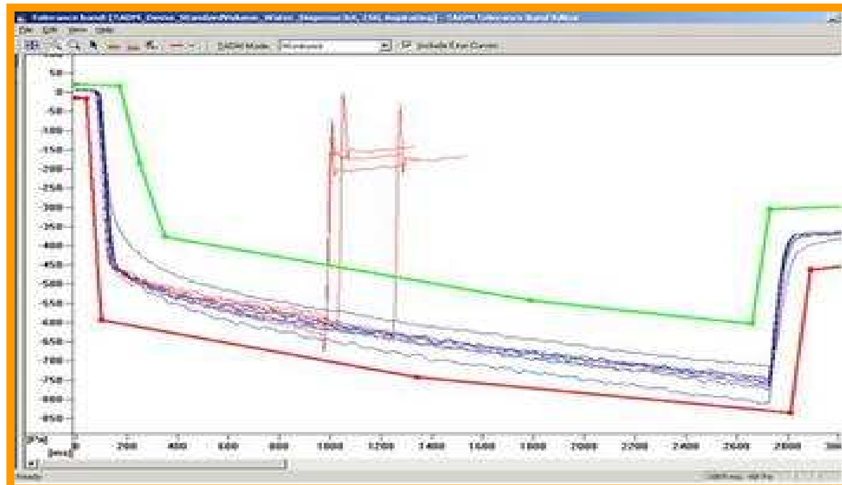

Figure 19: Typical curves - Insufficient samples

Insufficient sample aspiration detected in three samples when the pressure abruptly increased above the maximum TADM error band. Figure 19.

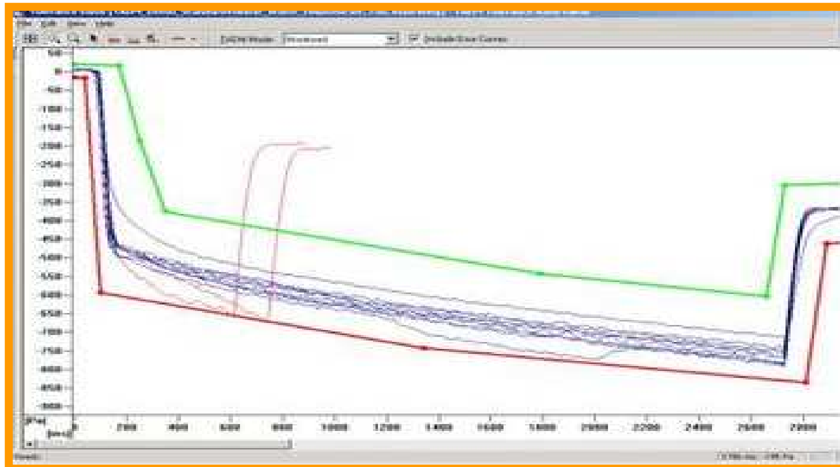

Figure 20: TADM - clots

Clots detected and aspiration aborted in two samples when the pressure gradually decreased below the minimum TADM error band. Figure 20.

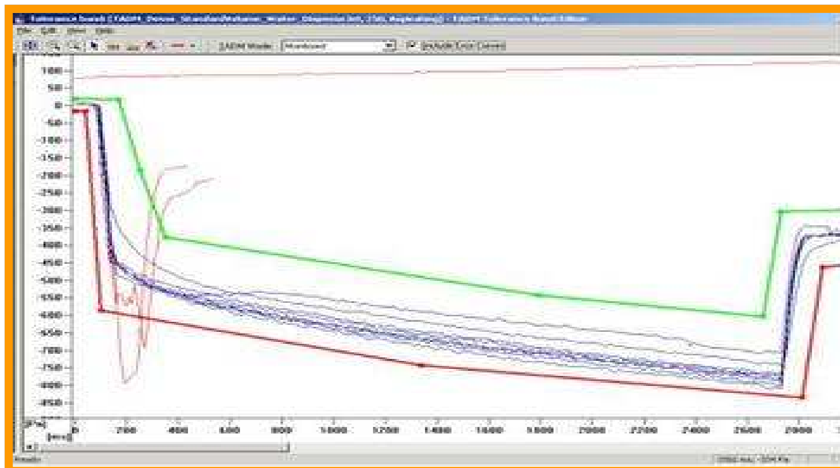

Figure 21: TADM - foam

Foam detected and aspiration aborted in two samples when the pressure abruptly dropped below the minimum. Figure 21.

7.1.3. Flexible and Precise tip Positioning

The STAR's Dynamic Positioning System allows each pipetting channel to move independently in both the Y and Z axes. Each channel uses its own high-precision motors and electronics to reach any position on the deck. In applications such as hit-picking,

where samples need to be transferred in an irregular pattern, this flexibility improves throughput. Since it is possible to have up to 16 channels on the same arm, multiple microplates may be processed simultaneously, drastically increasing throughput, Figure 22.

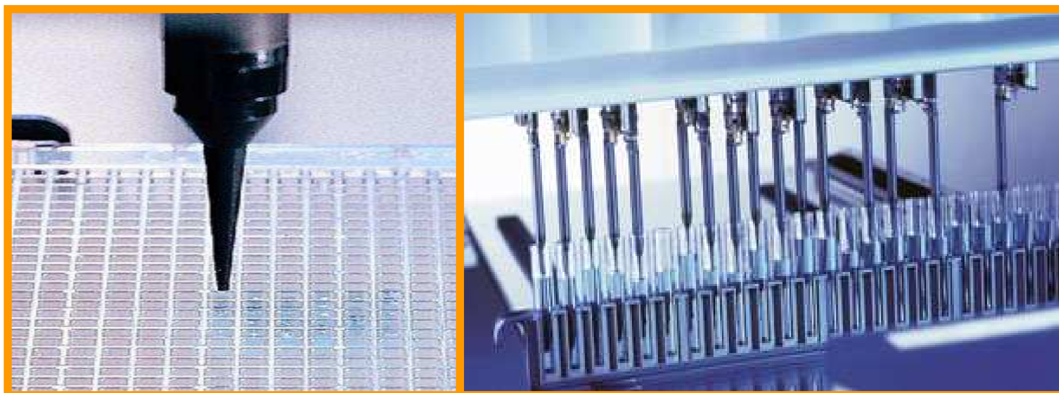

Figure 22

7.2. General liquid handling

7.2.1. Serial Dilution

Hamilton Microlab STAR automated liquid handling workstations are ideal for carrying out the laborious task of serial dilutions. Sample liquid can be transferred to and from tubes, vials, and microtiter plates.

Using the program DDM (Dynamic Dilution Module) included in ML_STAR Software System. It includes tip sequence (tip size and tip re-use), tip ejection after each dilution step, sample liquid pick-up sequence (1 to 8 wells), dilution liquid pick-up sequence, dispense sequence, dilution level, and sample volume. The template automatically calculates the transfer volumes, liquid volumes, pre- and post volumes and mix volumes. The serial dilution method is then run by simply clicking the play button.

7.2.2. Plate Replication

Super Simple Method allows the user to rapidly copy plates. After selecting this pipette method, the user is asked to select one of the variations of the method from a list. The user

then enters the number of plates to process, pipetting volume(s) and range. Pictorial views are then sequentially displayed to show which carrier and labware to load on the deck. The user then clicks the okay button. A pipetting script is automatically generated and run to completion.

7.2.3. Reagent Addition

Super Simple Method allows the user to quickly add reagents to plates. After selecting this pipetting method, the user is asked to select one of the variations of the method from a list. The user then enters the number of plates to process, pipetting volume(s) and range. Pictorial views are then sequentially displayed to show which carrier and labware to load on the deck. The user then clicks the okay button. A pipetting script is automatically generated and run to completion.

7.2.4. Combining or Merging Plates

Super Simple Method allows the user to conveniently merge plates. After selecting this pipetting method, the user is asked to select one of the variations of the method from a list. The user then enters the desired pipetting volume(s) and range. Pictorial views are then sequentially displayed to show which carrier and labware to load on the deck. The user then clicks the okay button. A pipetting script is automatically generated and run to completion.

7.3. Chain of Custody

The STAR liquid handling workstation supports the chain of custody in every aspect of pipetting, labware handling and sample tracking.

7.3.1. Barcode Tracking

When the Autoload option is used, the barcodes of all tips, labware, and carriers can be read as they are loaded. This confirms the correct items are loaded in the correct spot. A record of all loaded items can be stored and used to control the pipetting process. Barcode data sets can be compared to worklists from LIMS, exported as data files, or printed.

7.3.2. Total Aspiration and Dispense Monitoring (TADM)

Each channel has its own pressure sensor to monitor pressure in the pipette. This data is monitored and stored in real-time. This provides independent evidence that the pipetting operated within standard conditions or not. Under pressure or over pressure conditions experienced during specific times of the pipetting cycle can reveal if a clog, foam, insufficient sample or improper dispense occurred.

7.3.3. Tip Sensing

Each tip size is physically different at the tip connection site so that the tip channel can sense which tips is attached. The workstation automatically checks type with the tip size called out in the application script. A record of this data can be stored.

7.3.4. Labware Grip Sensing

Hysteresis in both the CO-RE grippers and iSWAP articulating labware gripper motors are monitored and recorded during their use. This data is used to confirm if a labware was pick-up or not during labware transfers.

7.3.5. Labware Sensing

The STAR can monitor and record the hysteresis on the z-drive (up/down) of the pipetting channels. This information can be used to determine if labware is present or not prior to pipetting further confirming that the method executed correctly.

7.3.6. Liquid Level Sensing

The STAR pipetting channels are equipment with capacitive and pressure level sensing and recording functions. These techniques can be used to check or confirm the liquid level in an individual cell prior to or after pipetting.

7.3.7. Liquid Level Detection

Hamilton offers both the traditional capacitive liquid level detection as well as pressure liquid level detection. The software can use these functions to drive the pipette tip to just above the surface to do a jet dispense, or touch the surface for a wet dispense, or dive below sufficiently to aspirate liquid without drawing in any air.

7.3.7.1. Capacitive Liquid Level Detection

A weak electrical potential is created between the pipetting channel and the labware carrier. When using conductive tips (black) and an ionic liquid the capacitance of the circuit is measured. From this data the software can determine the height of the liquid surface and take appropriate action.

7.3.7.2. Pressure Liquid Level Detection

A pressure transducer inside the air displacement channel measures the pressure inside the barrel during pipetting. The data from this sensor changes as the tip approaches the liquid surface, touches the surface and drives below,

Figure 23. This data can be used to control pipetting in real-time. Pressure level sensing is the only way to determine the level of non-ionic liquids.

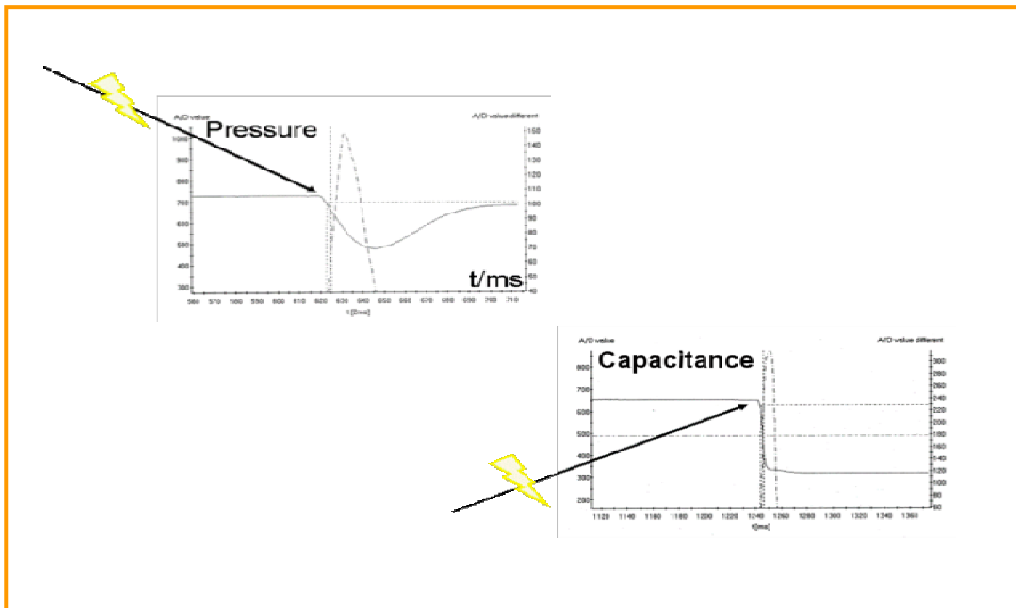

Figure 23: pressure Liquid Level Detection, capacitive Liquid Level Detection.

7.3.8. Monitored Air Displacement: MAD

Monitoring air displacement allows for real time detection of pipetting errors during pipetting steps. This includes detecting:

- if an insufficient amount of liquid has been aspirated for a single, multiple or partial dispense;
- aspiration of air, because the level of the liquid in a reservoir/tube was too low;
- blocked tips due to clotting.

Errors can then be corrected by user-defined, software-guided error handling. The STAR's Monitored Air Displacement eliminates uncertainty in automated assays by providing reliable, consistent, walk away automation.

7.3.9. Anti Droplet Control (ADC)

The combination of air-tight tip seals and air displacement pipetting with pressure monitoring allows Hamilton to offer anti droplet control (ADC). It can be pipetted liquids with extremely low viscosity and high vapor pressure like Acetone and Methanol with ease and confidence knowing that there will be no loss of liquid and no contamination of other samples because of dripping tips.

- Enables pipetting of highly volatile liquids;
- Increases process safety;
- Allows safe automation of Assays that require pipetting of substances like Acetone and Methanol.

ADC,

Figure 24, works by continuously monitoring the pressure in the channel.

The STAR channels sense the minute increase in the vapor pressure inside the barrel. When the pressure reaches a point where a drip is likely to occur, the plunger is withdrawn a step or two to keep the liquid inside the tip and prevent dripping. Volatile liquids such as Ether and Acetonitrile can now be safely pipetted from one side of the deck to the other.

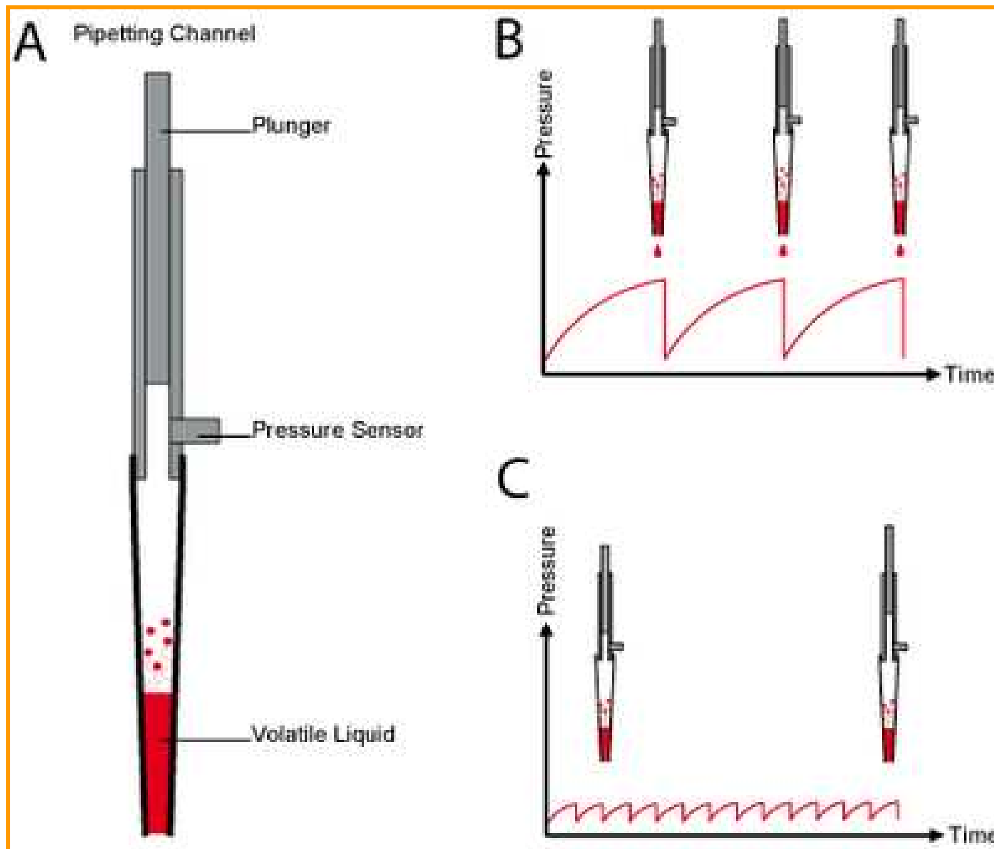

Figure 24: Anti Droplet Control. A: schematic drawing showing a pipetting channel with its pressure sensor. The volatile liquid contained in the tip evaporates into the air space. B: without ADC, as the pressure in the tip increases, a droplet forms at the end of the tip, reducing the pressure in the tip when it falls off. C: with ADC, pressure differences are detected by the pressure sensor and compensated for in real time by piston movements: droplet formation is prevented.

7.3.10. Action Editor

The Action Editor offers you a very intuitive user interface which includes access to all possible actions (pipetting, transport, incubation, etc.) in a toolbox for simple drag & drop programming. With the Action Editor you can quickly carry out throughput calculations and easily customize the actions by inserting action details like pipetting volume, pipetting source and target.

The new 3D deck and the rich graphics make deck creation easy:

- select carriers by category or just type some keywords;
- drag the photo of the carrier on the deck;
- add labware - find your plates by name, keyword and photo;

- switch to the Action Editor and drag a pipetting step into the method;
- choose from simple pipetting steps, aliquoting or pooling;
- answer a few steps about source, target, volume and start pipetting;
- the Action Editor supports drag and drop of target and source plates.

7.4. Pipetting Options

7.4.1. 1000µl Channels

The 1000µl Independent Channels are based on air displacement pipetting technology similar to the best hand-held pipettes. The pipette volumes range from 0.5 to 1000µl. The channels support pipetting with disposable tips. Disposable tips are available in four different sizes: 10, 50, 300, 1000µL CO-RE tips, Table 1.

Movements in the Y (spreading) and Z (up and down) directions are independent. This provides the greatest flexibility to adapt to asymmetric labware positions and pipetting sequences.

Pressure and Capacitive Liquid Level Sensing Each channel is equipped with its own pressure transducer and capacitance measuring circuitry. This allows it to measure liquid level either by pressure or capacitance or both. Pressure sensing is vital for measuring the liquid level of non-ionic liquids, supporting TADM and chain of custody.

Tip Type Sensing Each channel senses which tip type is being attached. If the tip type does not support the pipetting conditions set in the software script, the user is alerted. This ensures that liquid never accidentally enters the channel.

Straight Alignment Each channel uses Hamilton's CO-RE technology for tip attachment. An internal o-ring is expanded to assure a tight seal and straight alignment. Hamilton tips also are individually imaged during manufacturing for straightness.

Together with the CO-RE tip attachment design; this makes sure the tip of each tip on channel is in the right position even for 1536 plate pipetting.

The Hamilton MICROLAB® STAR expands the high throughput of multichannel liquid handling with a96 channel pipetting head.

The highly configurable and flexible Hamilton MICROLAB® STAR supports multiple arms, each with a multichannel pipetting head. The throughput and flexibility can be

achieved with both the 384 and 96 channel head on a single workstation with the highest on-deck plate density in the industry.

Table 1: Pipetting specifications for disposable tips

Tip size	Volume	Precision	Trueness
10µl	0,5µl	6,0%	10,0%
10µl	10µl	1,0%	1,5%
50µl	1µl	4,0%	5,0%
50µl	50µl	0,75%	2,0%
300µl	300µl	0,75%	1,0%
1000µl	1000 µl	0,75%	1,0%

7.4.2. Disposable Tips

The Hamilton Company designed the CO-RE tips for its MICROLAB pipetting workstations in order to solve the problem of sealing and alignment plagued by press-fit tip attachment techniques. Each CO-RE disposable tip has a circular groove inside the attachment orifice. An o-ring on the pipetting channel is expanded to fit this groove precisely providing an air-tight seal and straight alignment between tip and pipetting channel.

Tip Type Signature Each CO-RE tip type has a unique set of ridges molded into it so that the pipette channel can detect the tip type and the software can compare tip type with pipetting settings to make sure they are compatible.

Tip Quality Hamilton goes beyond on relying just on statistical analysis of tip quality to assure the highest product quality. Instead it has invested in an automated computer-based visualization system to inspect every tip before it is packed in its rack. Each tip is checked for straightness, concentricity of its orifice, and absence of flash from the molding process. This way you can be assured that Hamilton is shipping 100% performing product for accurate pipetting with each tip.

Optimal Tip Design Hamilton tips are designed for optimal liquid handling. Each dimension of the tip has been analyze and optimized using numerical grid simulations and empirical testing to produce accurate and repeatable results in the widest range of pipetting modes.

7.4.3. Tip Types

- Conductive (black) / Non-conductive (clear)
- Filtered / non-filtered
- Individual racks (blue frame) / stacked (black frame)

Stacked Tips In order increase tip capacity on the workstation deck for longer walk-away times and to increase tip packaging density, Hamilton designed a new stackable tip frame for the CO-RE 10µl, 50µl and 300µl unfiltered tips, Figure 25. These tips come in a package containing a disposable frame, five stacks with 4 tip racks, each rack with 96 tips. With these packs a user can load 1,920 tips on the deck in one step. And, all these tips take up only 6 tracks on the deck. The frames are able to be manipulated by the iSWAP, CO-RE grippers and SWAP. Tips can be picked up by the independent channels or 96 channel mulitprobe head directly from the stack eliminating the need to move the frames to a tip pick-up position. This saves not only processing time but also frees up an extra position on the deck. Used tips and frames can be ejected into the standard waste bag or front/side mounted tip waste chute.

Stacked tip racks can be loaded in bulk on the deck using a single low profile standard carrier, or individual frames or single stack of frames can be loaded on multiflex modules.

Figure 25: CO-RE Disposable Tips and Washable Needles

7.5. Labware Manipulation Tools

7.5.1. Microplate Gripping: CO-RE Grip

Thanks to the CO-RE tip attachment technology, the STAR features an innovative plate transfer option. In addition to tips, the pipetting channels can pick up the compact CO-RE gripping tool during a run. With this gripping tool the pipetting channels act as a gripper, picking plates from a stack, moving plates around the deck, removing lids or placing a filter plate vacuum manifold. The CO-RE gripper is an inexpensive option than moves labware without time consuming positional teaching.

Advantages:

- Small budget plate handler;
- Gripper paddles picked up by two channels;
- Default position for labware;
- Reaches all deck space;
- Landscape and portrait plate gripping;
- Facilitates stacking.

7.5.2. Plate Gripping Tools / Plate handling

Two different types of plate gripping tools are available on the STAR line of liquid handling robots, and an external robotic arm provides a third option for plate handling when building integrated systems that require labware handling away from the STAR deck.

Two pipetting channels can pick up the CO-RE Gripper tool, providing a flexible, low-cost solution for moving labware on the STAR deck, Figure 26.

Figure 26: CO-RE Gripper

A robotic gripper with a full range of motion, the iSWAP can also reach off the STAR deck to access 3rd party equipment or custom labware positions, Figure 27.

Figure 27: iSWAP

The MICROLAB eSWAP is designed for seamless and simple integration of 3rd party peripheral devices with Hamilton liquid handling robots to create completely automated workstations, Figure 28.

Figure 28: eSWAP External Gripper

7.6. Carriers & Accessories

7.6.1. STAR Multiflex Modules

The latest addition to the STAR accessories line is based on a modular concept. On a base plate, a variety of different modules can be mounted. There are modules available for plate or tip rack stacking, cooling, heating, tilting, shaking and many more. Compared to standard carriers, Multiflex allows the free combination of various modules on one base plate according to your needs. It's customization based on standard parts. The number of modules is steadily increasing, so check back for new products frequently.

7.6.2. Volume Verification Kit

The MICROLAB STAR verifies pipetting volumes via gravimetric testing. This is much more reliable than traditional verification testing using dyes and plate absorbance readers.

7.6.3. Autoload and Barcode Reading

The Autoload option on the MICROLAB STAR automatically loads carriers as well as reads barcodes. With this option a loading shelf is supplied which attaches to the front of the workstation. This acts as a staging area for carriers to be inserted or removed from the workstation for either loading/unloading labware or changing carriers for another application. The track numbers are imprinted on deck to help the user position the carriers in the correct position. In addition backlit track numbers are mounted on the front safety panel and can light up green to indicate the correct tracks to insert a particular carrier at a particular time. Plastic torpedoes guide the carriers into position. Then the workstation grabs the carrier and pulls it in front of the barcode reader.

The barcode reader can read the barcodes on carriers to make sure the correct carrier is inserted in the correct track. It can read the barcodes on tip racks and plates to make sure the correct items are loaded on the correct carrier. This information can be compared to a work list either created by the user or downloaded from a LIMS. The barcode reader can also read the barcodes on tubes as well. These functions are paramount for IVD and chain of custody compliance. Also, with this design barcodes are read as the carrier is being loaded. If the barcode check fails, the user is alerted immediately before the carrier is pulled all the way on the deck thus saving the user time to get the correct carrier in the right position with the correct labware and consumables.

7.6.4. Basic Vacuum System

The MICROLAB STAR Basic Vacuum System (BVS) (Figure 29) is designed to automate solid phase extraction assays using either commercially available kits or custom-made assays.

The option consists of a dedicated carrier, vacuum pump and controller. The vacuum is completely controlled by the Venus software. The carrier consists of a vacuum chamber for the destination plate, a manifold with seals to place the source elution filter, a park position for the manifold and two empty MTP positions. iSWAP or CO-RE grippers can manipulate the manifold and access the target plate.

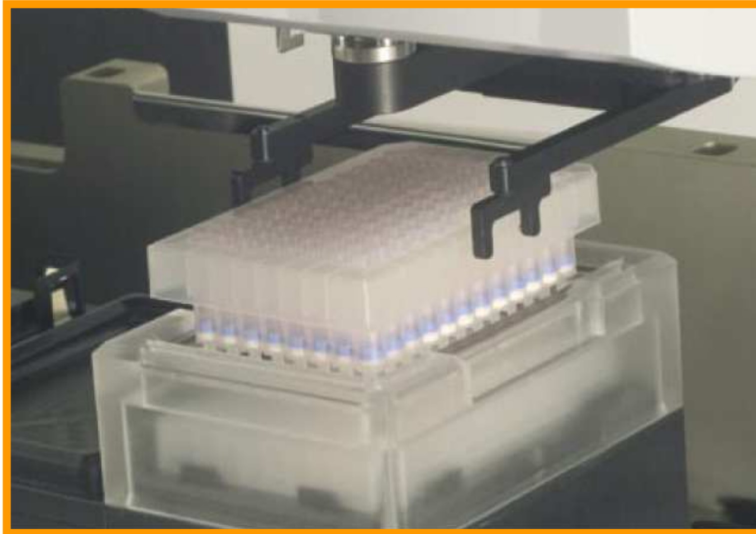

Figure 29: Basic vacuum System.

8. Sample Preparation Techniques

The importance of accurate sample preparation techniques cannot be overstated, meticulous sample preparation is essential. Often overlooked, it is the midway point where the analytes from the sample matrix are transformed so they are suitable for analysis.

In pharmaceutical bioanalysis, researchers develop and run various assays to quantitate drugs, pharmaceutical candidates, and their metabolites in biological fluids such as serum and plasma. The data resulting from these assays are used to help determine the pharmacodynamic and pharmacokinetic properties as well as the toxic and therapeutic concentrations of existing and emerging pharmaceutical compounds in living cells, tissues, and animals. Although advances in Liquid Chromatography-Mass Spectrometry (LC-MS) technology have reaped overwhelming benefits in terms of increased throughput and sensitivity, good sample preparation continues to be a critical component of bioanalysis.

A sample matrix for bioanalysis almost always contains some amount of protein, along with other endogenous macromolecules, small molecules, metabolic byproducts, salts and possibly co administered drugs. These components must be removed from the sample before analysis in order to attain a selective technique for the desired analyte.

It is important to remove the protein from a biological sample because that protein, when injected into a chromatographic system, will precipitate upon contact with the organic solvents and buffer salts commonly used in mobile phases[44].

The three most common sample preparation techniques used in Bioanalytical Sample Preparation are Protein Precipitation (PPT), Liquid-Liquid Extraction (LLE), and Solid Phase Extraction (SPE). Each technique offers unique advantages and disadvantages that are considered during the method development process. For example, protein precipitation methods are simple (2-3 steps), fast, and often require minimal method development. However, the technique offers minimal selectivity as it only removes gross levels of protein from a sample prior to analysis. In contrast, SPE offers significant benefits in terms of selectivity/sample cleanup, but the technique often requires moderate to extensive levels of expertise and time for adequate method development. In addition, SPE often

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44 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. 6, 199, (2003).

requires multiple steps (5-8), resulting in increased assay time. Modern analytical laboratories are full of significant technological investment. Many are surprised to learn that the highest cost in a laboratory is still not the equipment but the analyst, and more specifically, analyst time. With analysts spending as much as 60% of their time on sample preparation, it is no wonder companies are turning to automation to recapture their investment in human resources. Only through a careful analysis of what tests, or portions of tests, are automated will one be able to assess the return on investment from automation. In the worst cases, an analyst could be replaced with an equipment repair technician, or a step that is not a critical path in the laboratory could be automated. In the best case, significant additional testing capacity and reduced cycle times can be created.

Both routine and intensive analyst tasks are being automated because of favorable financial return. However, for laboratories in regulated industries, using this automation is not as effortless as simply turning on the switch. A significant amount of planning, along with additional investment in validation and documentation, must be invested prior to relying upon automation to generate a return.

8.1. IQ/OQ/PQ

Details for a successful instrument qualification, operational qualification, and performance qualification, known collectively in the pharmaceutical industry as IQ/OQ/PQ, must be established under GxP, Table 2, lists the general definitions of the qualifications, with the added definitions for equipment qualification (EQ) and design qualification (DQ). (EQ and DQ are newer interpretations of equipment qualification and not as widespread in application.) Why all the fuss about instrument qualification? GxPs mandate that analysts establish and document procedures to indicate that the instrumentation has been fully controlled. The industry adage “if the scientist didn't write it down, you didn't do it” applies not only to data but to instrumentation documentation as well. For now, let it suffice to say that all measurement equipment in a GxP laboratory must be qualified. Automated equipment not only must be qualified to demonstrate its

utility, but also to address concerns from directing much of an analysis away from human intervention and control[45].

Table 2

	Qualification definitions
Installation Qualification (IQ)	IQ establishes that the instrument is received as designed and specified. IQ also verifies that the instrument environment is suitable for the operation and use of the instrument and that the instrument is properly installed.
Operational Qualification (OQ)	OQ is the demonstration that an instrument will function according to its specifications in the selected environment.
Performance Qualification (PQ)	PQ is the demonstration that the instrument performs according to a specification appropriate for its routine use.

8.1.1. Validation vs. Qualification

8.1.1.1. Validation

Validation is the process of evaluating the performance of a specific measuring procedure and checking that the performance meets certain preset criteria. Validation establishes and provides documented evidence that the measuring procedure is fit for a particular purpose. Qualification is the verification that an instrument is performing under predetermined specifications. It should also be noted that “calibration” is also unique; it is the verification that an instrument is standardized against a nationally accepted standard (or a secondary standard that has been calibrated against such a standard). The terms “validation” and “qualification” are often mistakenly interchanged.

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8.1.1.2. Qualification

Regulated industries look for equipment to undergo a series of qualifications: Equipment Qualification (EQ), Design Qualification (DQ), Installation Qualification (IQ), Performance Qualification (PQ), and Operational Qualification (OQ). Specifications for these qualifications are often predefined by the manufacturer and negotiated within the company.

Automated methods can be introduced to the GxP laboratory by validating the automated method directly or establishing a successful analytical method transfer from an existing method to the automated procedure[46].

8.1.2. Method Validation

The level to which a method is validated for GxP is normally based on where the methodology is being applied. In early drug development, there is less information about the chemical characteristics of the drug and a high likelihood it will not succeed and become a marketed product. It is not economically prudent to allocate significant resources to the drug, and the level of method validation is to a level consistent with the state of knowledge regarding the manufacturing processes and the concern for safety of patients taking the drug in a clinical setting. Reference standards are seldom available at this stage, leaving the validation of the method focuses on precision and selectivity of the drug from its matrix. Linearity of response is needed for all stages of development; however, sensitivity only needs to be established to the reporting limit. If the GxP study involves human testing, the level of validation increases to a point where confidence in the designed indication going into the patient is established. Finally, as the drug progresses to market, full analytical characterization is established.

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8.1.3. Analytical Validation Principles

Validation is establishing documented evidence that the analytical test method performs to established specifications. These specifications are generally defined in a study protocol, in company standard operating procedure (SOP), or in a compendial reference. If the method is being used for stability investigations, the validation must establish its suitability as a stability-indicating method for the analysis of the intended analyte.

To some degree, all analytical method validations under GxP will test for specificity, precision (repeatability and intermediate precision), linearity, range, accuracy, robustness, and quantitation limit of the intended analyte.

Sample preparation has been and continues to be the critical step for fast, accurate, and reliable LC-MS/MS assays.

Conventional biological matrix extraction methods such as solid phase extraction, liquid-liquid extraction, and protein precipitation (PPT) are both time-consuming and labor-intensive. Due to its simplicity and universality, protein precipitation continues to be the main methodology employed for biological sample preparation, especially in the drug discovery arena. However, all three major sample preparation methods remain tedious processes and, consequently, bottlenecks limiting the throughput of current fast LC-MS/MS analysis. Current trends in sample preparation have focused on the use of 96-well technology as well as automated sample preparation via 96-well parallel processing.[1]While the introduction of 96-well liquid handlers, such as the Tomtec Quadra, has helped overcome some of these bottlenecks, a completely automated sample preparation solution remains elusive.Current 96-well automation is composed solely of the liquid-transfer steps of sample preparation, but not the preparation of standards and quality control samples or sample dilutions. In addition, current automation is incapable of interfacing with either laboratory information management systems (LIMS) such as Watson or mass spectrometric data acquisition software. This report demonstrates the utility of a commercially available advanced robotic workstation, the Hamilton MicroLab Star, and the resultant fully automated 96-well sample preparation methodology for biological sample preparation.

To achieve the automated Hamilton programming, we then considered whether bioanalytical sample preparation procedures could be standardized. Bioanalytical sample preparation basically consists of two main liquid transfer steps. The first step is sample

transfer, which includes transferring different types of samples, i.e., standards (STD), quality controls (QC), double blanks (BLKs) (blank matrix without internal standard [IS]), control blanks (blank matrix with IS), and study samples, from different source vials or containers to their destination wells on a 96-well sample collection plate. Then, IS is added to all wells except for the BLK. The second step is extraction and reconstitution. The most commonly used extraction methods are SPE, protein precipitation (PPT), and liquid-liquid extraction (LLE). The general procedures are shown below in Table 3.

Table 3

PPT	LLE	SPE
Addition of precipitant solution	Addition of organic solvent to the sample plate	SPE 96-well plate conditioning
Supernatant transferring	Organic layer transferring	Loading sample to SPE 96-well plate
Dilution of supernatant, or evaporation and reconstitution	Evaporation and reconstitution	Interference washing
		Analyte eluting Evaporation and reconstitution

The first step is quite complicated and prone to error if the manual programming is used because:

1. Different types of samples are in different types of vials or containers, and thus they are on different carriers. Although it is possible to put all types of samples on the same carrier by manually transferring different types of samples into the same type of vials before sample transfer, the advantage of using a liquid handler is minimized.
2. For high-dose study samples, dilution with a dilution factor (DF) up to 1,000-fold is sometimes needed to bring the concentration of those samples to the standard curve range.
3. IS is only added to the part of wells on the 96-well sample collection plate, i.e., no IS will be added to blank samples.

8.1.4. Liquid Classes

The concept of Liquid Class is important. A liquid class is a set of parameters determining the most appropriate aspiration and dispense performances of the pipette for any given liquid, tip type and dispense mode. For all aspiration and dispense steps I must select the most appropriate liquid class.

The accurate transfer of liquids depends on their specific properties and other physical parameters. For example, the volume of liquids is affected by the temperature and the ambient pressure, and organic solvents frequently tend to evaporate due to their often high vapor pressure. Density, viscosity and the surface tension of liquids and solutions are additional characteristics which strongly influence the precision and accuracy of liquid handling. The capillary action of a liquid will be significantly determined by its surface tension. The dimensions, shape and material of tips, needles and wells affect formation of drops, meniscus and bubbles.

Liquids which are used in life science research include pure water, media, buffer solutions, DMSO, other organic solvents, protein and nucleic acid solutions, and many other liquids. All must be transferred with high accuracy and precision for satisfactory automation of applications.

Allowing the liquid handling automation to accomplish all pipetting tasks can eliminate the introductions of human pipetting errors. The accuracy of pipetting is much higher when performed by a liquid handler compared with manual operation, especially when small volumes are aliquotted.

The accuracy and precision of the automation with regard to pipetting performance are determined at regular intervals. When maintained and used properly automation performance is reliable. Many varieties of automation allow the user to adjust default settings for pipetting in order to allow for individual variation in the liquid type. A specific set of pipetting defaults can be used for plasma samples, organic solvents, aqueous solutions and 50/50 mixtures of organic/aqueous solutions. These settings can usually be overridden for individual steps when necessary.

The highly flexible Hamilton robotics system is capable of handling multiple liquid class types within a given method. This feature allows accurate pipetting of a variety of solvents or matrixes by assigning a specific liquid class to an individual solvent throughout the method. However, in order to ensure accurate pipetting, each liquid class must be independently calibrated, typically on a quarterly basis. This is accomplished using the eight-channel head and a balance. Several liquid classes required calibration and validation, including a plasma liquid class for analysis of biological matrix, an organic liquid class for transfer of stock solutions. The validation process for each liquid class was conducted by measuring the weight of eight replicates, one replicate per channel on the

unit across a pipetting range of 5- 300µl. Volumes were calculated using the density of each liquid class.

For quantitative bioanalysis, a variety of solvents, including methanol, water, mixed solvents, and plasma, are typically used to prepare standards and unknown samples. Density information is usually unknown for the mixed solvents, and the density of plasma can vary from species to species. For volumes of 5, 100, and 300 µl, the volume measurements were calculated from the weight values divided by the density of the given liquid class[47].

I could find several standard liquid classes in the software, but they are not enough to fulfill all the needs present in my methods, since sometimes there were particular liquids or liquid mixtures not covered by any ready-made liquid classes.

In the section "Liquid Details" I can set the parameters I need such as the flow rate that correspond to plunger speed for aspirating, dispensing and mixing, the air transport volume, the over-aspirate volume which is a kind of pre-wetting volume, etc.

In the section "Correction Curve" I can introduce the corrected values calculated with gravimetric tests. The "corrected volume" is the volume that actually needs to be moved by the plunger for this purpose. In aspiration or dispense steps, the "target volume" which will be actually dispensed into the vessel must be entered. For example a corrected volume of 107.2 µl for a target volume of 100µl does not mean that 107.2µl of liquid will be dispensed. The high flexibility of the liquid classes allows to pipette any liquid with high accuracy. The liquid classes I have calculated for the methods I utilized since now (MeOH-ACN 50-50; MeOH-H_2O 50-50; ACN-MeOH 75-20; ACN-Formic Ac. 15-85; etc.) have good characteristics of accuracy and precision in dispensation more than the one requested[48].

In Table 4 are described the main liquid classes created in Automation Laboratory.

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47 Flexible Automated Approach for Quantitative Liquid Handling of Complex Biological Samples Joe Palandra, David Weller, Gary Hudson, Jeff Li, Sarah Osgood, Emily Hudson, Min Zhong,‡ Lisa Buchholz, and Lucinda H. Cohen Bioanalytical Research, Department of Pharmacokinetics, Dynamics.

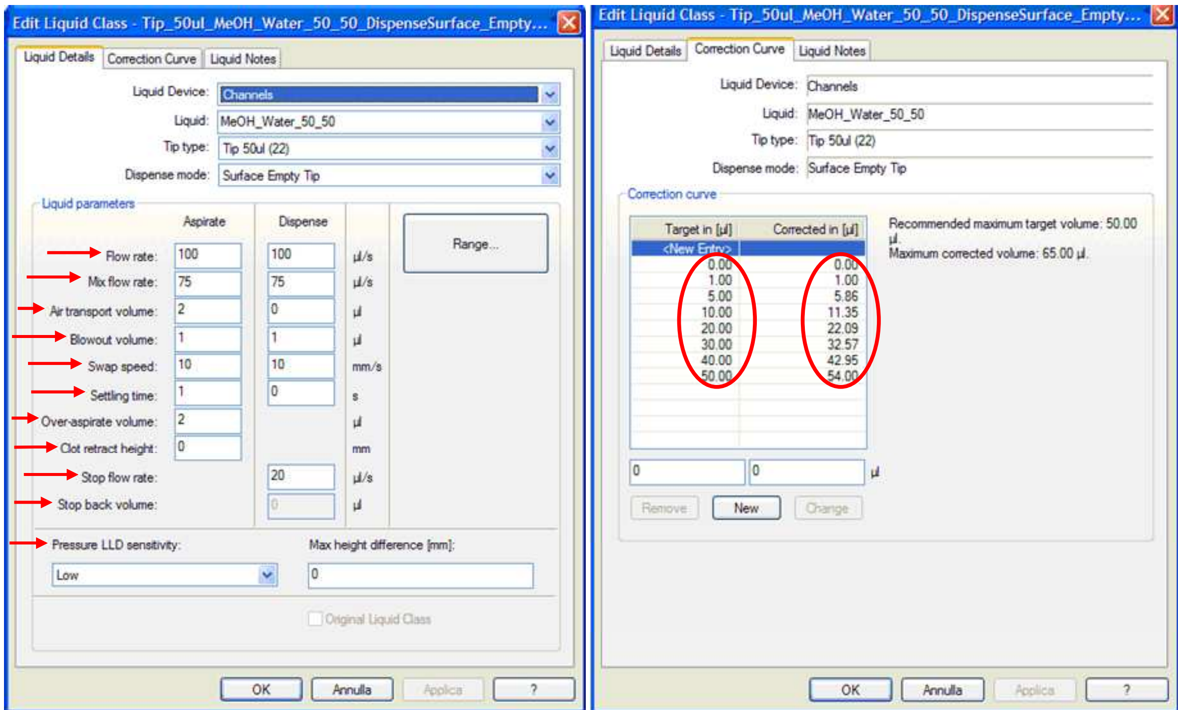
48 Microlab Star Let Operator Manual, Hamilton Robotics (13-20)

Table 4

Liquid Classes	
Tips 50ul and tips 300ul	**%**
CH_3CN	100
CH_3CN	90
CH_3CN-H_2O	50-50
CH_3CN-MeOH	75-25
H_2O 0.1%HCOOH-CH_3OH 0.1% HCOOH	95-5
H_2O-CH_3OH	50-50
CH_3OH	100
CH_3CN-HCOOH	75-15
Rat Plasma	100
Human Plasma	100
Dog Plasma	100
Urine	100

Statistical calculations are performed to determine values for the mean, standard deviation and CV% (coefficient of variation). Repeated failure signifies a mechanical adjustment issue or other problem; perhaps a correction needs to be made in the default settings for pipetting and system variables, Figure 30.

Accuracy in solvent delivery is a function of the reagent type (biological matrix, aqueous, organic or aqueous/organic mixture) the speed of pipetting, and the physical dimensions of the delivery hardware (tips or probes). A gravimetric determination using specific gravity calculations is an accepted way to generate accuracy data.



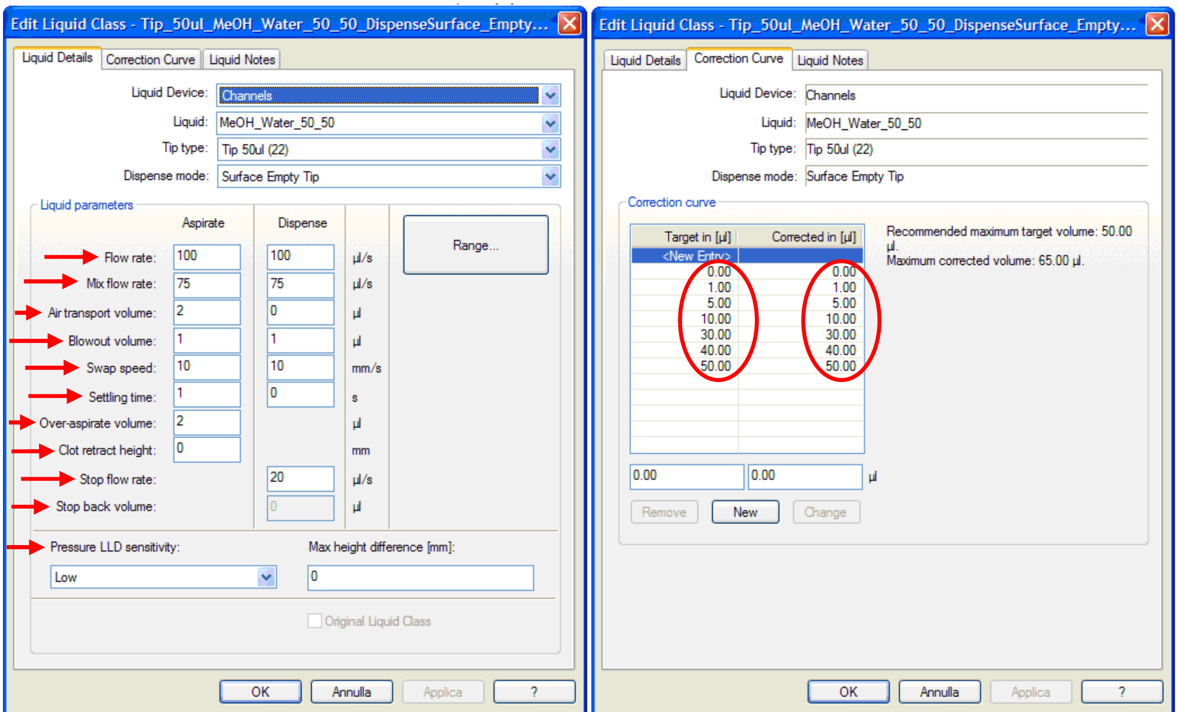


Figure 30: Mechanical adjustments and parameters to evaluate in the creation of a liquid class.

9. Protein Precipitation Technique (PPT)

9.1. Introduction

PPT is a technique used to remove proteins from a biological sample matrix prior to analysis. It is not selective as other sample preparation methods but the simplicity and universality of this approach have broad appeal for many applications, particularly in support of drug discovery.

High-throughput procedures for removing proteins from the matrix are important in fact these matrixes for bioanalysis always contain some amount of protein, other endogenous macromolecules, small molecules, metabolic byproducts, salt and possibly coadministrated drugs. These components must be removed from the sample before analysis in order to attain a selective technique for the desired analyte.

Proteins play an important role in the transport and storage of drug substances. Most biological matrices contain protein to varying extents. Protein binding phenomena are known to influence drug-drug interactions in the clinical setting. Among the various plasma proteins serum albumin is the most widely studied and is regarded as the most important carrier for drugs. The presence of these materials for bioanalysis, however, is problematic.

It is important to remove the protein from a biological sample because that protein, when injected into a chromatographic system, will precipitate upon contact with the organic solvents and buffer salts commonly used in mobile phases. The precipitated mass of protein builds up within the column inlet. The result is reduction of column lifetime and an increase in system backpressure. When protein is carried through the analytical system it may reach the mass spectrometer and foul the interface, requiring cleaning.

A common approach for removing protein from the injected sample, amenable to high-throughput applications in microplates, is precipitation using organic solvents, ionic salt and/or inorganic acids. The precipitated mass can be separated by either centrifugation or filtration; analysis of the supernatant or filtrate, respectively, is then performed.

9.1.1. Precipitating agents

9.1.1.1. Acids

Proteins are known to precipitate from solutions when subjected to strong acids, organic solvents and certain salts of heavy metal cations.

TCA 10% w/v, perchloric acid 6% w/v and metaphosphoric acid. 5% w/v, for example, protonate basic sites on the protein to change its conformation, subsequently forming insoluble salts at pH below their isoelectric point.

The isoelectric point is the pH at which a protein molecule has a neutral charge, at a pH higher than its isoelectric point, the protein will acts as a base, and at pH lower it will acts as an acid. A protein has its minimum solubility at its isoelectric point and can be coagulated more easily at this value.

These acids are very effective protein precipitants but they are generally regarded as too harsh for many labile analytes when injected directly into LC-MS/MS system.

9.1.1.2. Organic solvents

Organic solvents are known to lower the dielectric constant of protein solutions and increase the protein-protein interactions. Solvents such as methanol, ethanol, acetonitrile (ACN) and acetone are reported to be slightly less effective than acids for their degree of protein precipitation but are preferred in bioanalysis because the conditions are very mild and analyte degradation is avoided. These organic solvents are also more compatible with the LC mobile phases commonly used and sometimes can be injected directly.

Among choice for organic solvents used as precipitating agents, acetonitrile and methanol are most often utilized for bioanalytical sample preparation, each has its advantages and drawbacks in certain situations. For example, using ACN can result in poor recovery and also late eluting peaks. Methanol is not as efficient as ACN when used in the same ratio but it does tend to produce precipitate that facilitates mixing; the supernatant appears clear rather than the dark one produced by ACN.

The ratio of precipitating agent to sample matrix is important for efficient removal of proteins. Acids achieve efficient removal of proteins at very low ratios (>98%). However the commonly used organic solvents require greater volumes relative to plasma.

The best results for precipitating proteins from plasma are obtained using a ratio of 1 part plasma to 3 parts ACN or 1 part plasma to 4 parts methanol.

9.1.2. Advantages

PPT is one of the four main drug sample preparation methodologies for several reasons:

- It is a simple and universal procedure for small drug molecules in plasma.
- There is usually no pH modifications involved as with other techniques so the exact nature of the analyte (ionized or unionized) is not as important to success of the method.
- The low speed of the procedure.
- Little method development time invested before proceeding to method validation.
- High recovery of analyte (>95%) can be obtained even when the extent of protein binding exceed 99%.
- Very small sample volumes (e.g., 20-50µl plasma) can be efficiently eliminated of proteins using small volume microplate wells since this precipitation technique has minimal sample transfer and isolation steps.

9.1.3. Disadvantages

Satisfactory analyses have been demonstrated with this rapid sample preparation approach, but it has several disadvantages:

- PPT typically dilutes the sample by a factor of three or more, so it is a useful technique only when analyte concentrations are relatively high (1-10ng/ml) and the detection limits allows an adequate quantitation. However the supernatant can be evaporated using nitrogen and heat to reverse this dilution effect.
- The evaporation step requires an additional transfer step (with possible transfer loss) and time for the dry-down and reconstitution procedures.
- The analyte volatility for newly synthesized compounds is often unknown, and lower recovery may result by introducing a dry-down and reconstitution procedures.

- A lower recovery upon dry-down may also be due to the potential contributions of analyte absorption to the sample container and inefficiency in resolubilizing the dried extract.
- Gradual accumulation of small amount of protein or particulate may occur and be noticeable after injection of a large number of samples.
- The use of an additional wash step in this manner adds time to the overall procedure and dilutes the sample further.
- Lipid components are also undesirable. Various types of lipids can potentially accumulate in the LC column and elute slowly upon each injection, deteriorating column efficiency over time

The case of variable ionization suppression, discussed in the next chapter, is even more of a problem than the accumulation of matrix components and the percentage of organic in the injected sample.

9.1.3.1. Matrix effects

The major precaution when using protein precipitation as a sample preparation technique is that matrix components are not efficiently removed and will be contained in the isolated supernatant or filtrate.

In MS/MS detection systems, matrix contaminants have been shown to reduce the efficiency of the ionization process using API techniques and in ESI ion suppression is more severe. The observation seen is a loss in response and this phenomenon is referred to as ionization suppression.

This effect can lead to decreased reproducibility and accuracy for an assay and failure to reach the desired limit of quantitation.

Attempts have been made to minimize matrix effects from protein precipitation extractions. The best approach is a more selective extraction technique.

When the technique is decided a recommended approach is to use a stable isotope of analyte as internal standard (IS) so that both compounds coelute and are affected in the same manner by ESI suppression.

9.1.4. High-throughput PPT techniques using collection microplates

The first approach to automation was the development of a fully automated protein precipitation technique for biological sample preparation and it has been developed for the quantitation of drugs in various biological matrixes.

All liquid handling during sample preparation was automated using a Hamilton MicroLab Star Robotic workstation, which included the preparation of standards and controls, shaking of 96-well plates, vacuum application and an interface with a Watson 7.X LIMS to manage a work list.

An overview of the process workflow is discussed, including the software development. Validation data are also provided, including specific liquid class data as well as comparative data of automated vs. manual preparation using both quality controls and actual sample data. The efficiencies gained from this automated approach are described.

PPT traditionally has been performed in test tubes, but this approach is labor intensive with its required tube labeling and frequent manipulations.

The manual approach does not meet the high-throughput needs required by the emphasis on rapid pharmaceutical drug development. Since the microplate format is commonly used in autosamplers for injection into the chromatographic system, it is desirable to retain that format and perform the sample preparation procedure in a microplate.

Two general approaches are common for performing protein precipitation in the high-throughput microplate format:

- Use a collection plate or microtube rack, pellet the precipitated protein at the bottom of wells by centrifugation and collect the supernatant for analysis.

- Use a filtration microplate to trap the precipitated protein on top of the filter and collect the filtrate for analysis.

9.1.5. Filter Plates

Figure 31: Sirocco filter plate

Sirocco filter plate Sirocco (Figure 31)

The Sirocco 96-well filtration plate is an update in the automation and throughput of protein precipitation in drug metabolism/ADME/toxicology labs by providing a simplified means of sample preparation.

The plate combines 96-well plate tip design features with proprietary membranes that result in rapid "in-well" sample preparation methods. This plate contains a unique filter system, a sealing cap mat and a patented valve technology designed specifically to allow “in-well” processing which prevents clogged wells, cross-talk or leaking during use.

Is possible applying a vented cap mat, using a cap roller to ensure secure and uniform sealing. This will prevent leakage and cross talk during mixing. With Sirocco filter plates there is no need to remove the vented cup mats or valve tips from the plate before filtration because the valves and vented cap mat are designed to open under vacuum to allow controlled flow during filtration.

Mix time 0.5-1 min at medium setting, filtration 3 min, 10” Hg.

Excessive mixing could cause cross talk and under mixing this will cause clogging or a cloudy filtrate.

Whatman Fast Flow

The plate is made with 2ml, 96-well, rigid glass filled polypropylene which make the plate both robust and chemically resistant. The plate contains specially formulated dual membranes with two distinct layers. The top layer acts as a prefilter to remove coarse

particulates. The bottom layer is oleophobic for retaining the well contents without dripping. This provides a final filter for removing fine particulate matter when vacuum is applied.

9.1.6. Automation of PPT in filter plates using a robotic workstation

The use of filtration plates offers the advantage of eliminating the manual off-line steps of mixing and centrifuging, as used with a collection microplate.

The precipitation occurs immediately and protein is trapped on the surface of the filter in each well. Vacuum is used to process liquid through the filter plate.

The deck layout we created for PPT is shown in and Figure 33.

- Position 1 is the location for the filter plate and at the same time is the mixing position.
- Position 2 is the vacuum manifold containing the collection plate, in the same position will be located the filter plate after mixing.
- Position 3 contains the precipitant solution, white plasma for the calibration curve.
- Position 4 contains the standard solutions, the QCs and the IS.
- Position 5 contains the sample plate which was prepared off line by mixing and, if occurs, sonication.
- Position 6 contains different size tips as better shown in Figure 33.
- Position 7 is the waste position.

The liquid handling workstation first aspirate an aliquot of precipitation solvent, followed by an air gap, and than aspirates plasma from the sample plate. The plasma and solvent are forcibly dispensed into wells of the filtration plates, thus avoiding any manual intervention. The plate is mixed in the same position and than transferred by co-re gripper in vacuum manifold position. Vacuum is applied to collect the filtrate. Proteins remain on the top of the filter and are discarded with the plate. The underlying collection microplate containing filtrate is removed, dried and reconstituted by the liquid handler for injection.

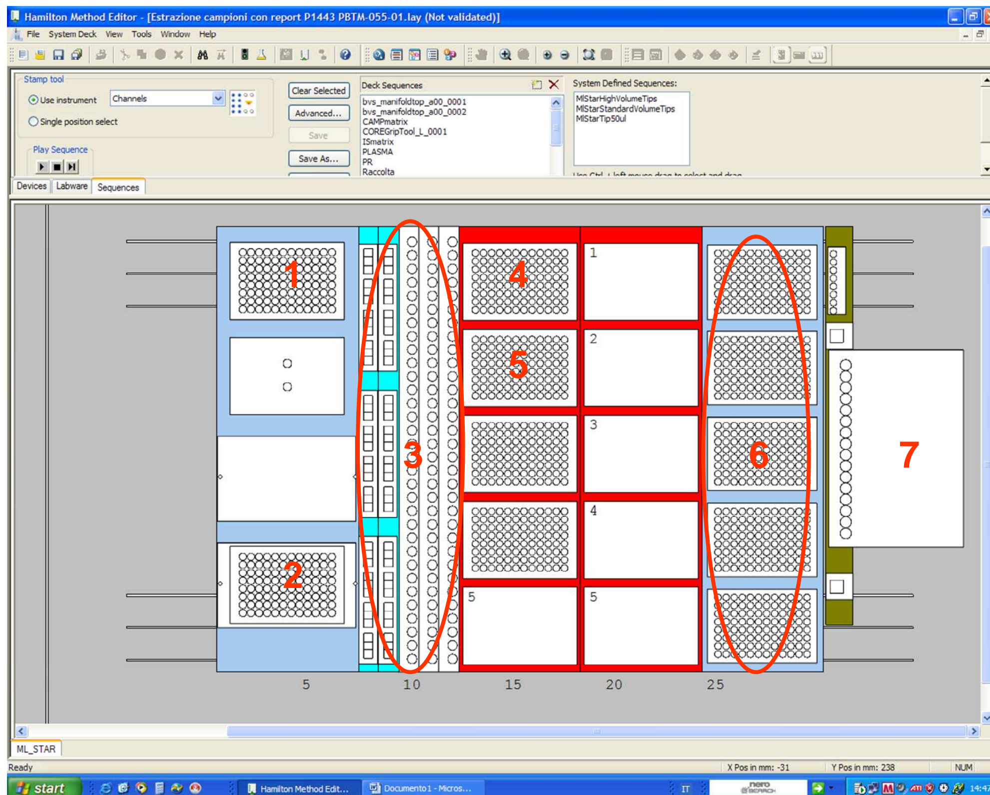


Figure 32: deck layout

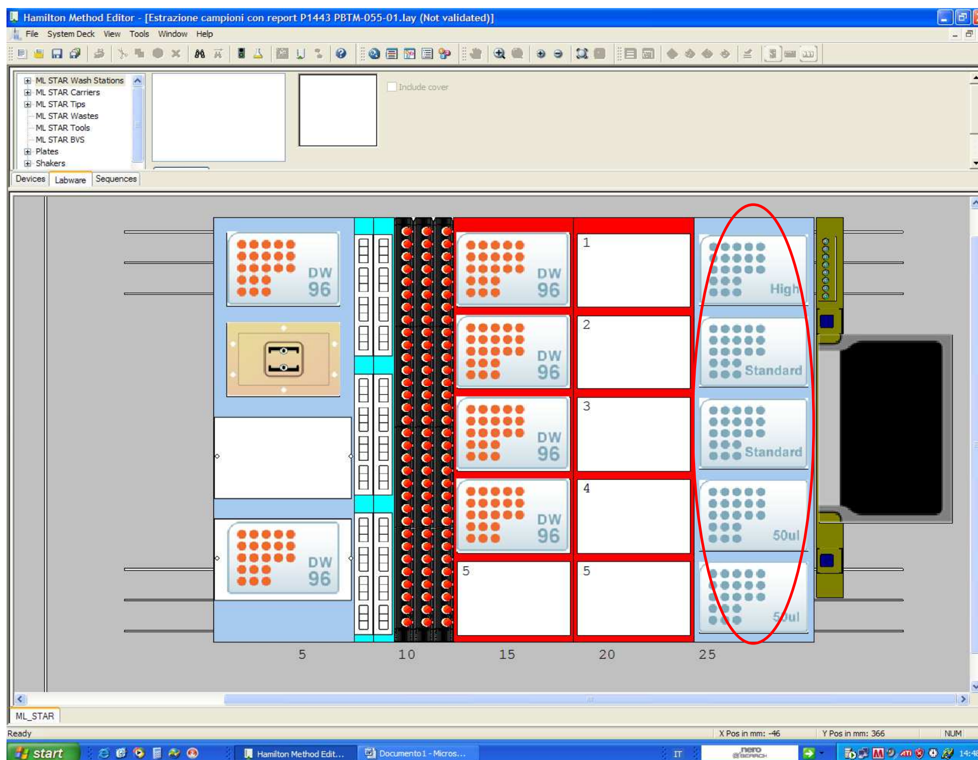


Figure 33: deck layout

9.2. Experimental

Then I translated the protocol method in several steps to be performed by the robotic workstation.

I had to choose the best parameters to reach the best performance.

One of the most important parameters is the way to dispense. At the beginning I used a “jet” dispensation but in this way I could not obtain the expected results. So, step by step, changing every time all the parameters that gave me the wrong results, I reached the right configuration on labware and layout, enabling to perform for (the) first (time) correct calibration curves.

Each method remaining constant in his performance could be different in solvents and volumes.

9.2.1. Instrumentation

9.2.1.1. Reagents and chemicals

Analysis is performed with the following reagents and chemicals:

Acetonitrile:	HPLC grade
Deionized water:	Milli-Q quality
Methanol:	HPLC grade
Dimetil Sulfoxide (DMSO):	Analytical reagent grade
Diluent Solvent: Methanol/Water :	50/50
Reconstitution Solvent (RS):	Acetonitrile/Water : 50/50

9.2.1.2. Equipment

Analytical balance:	Sartorius Research R200D or equivalent
Centrifuge:	ALC 4239R
Vacuum system:	Speed-Vac plus SC210A System, Savant
Ultrapure waters system:	MilliQ Plus Millipore
Protein precipitation system:	Waters Sirocco filter plates, Whatman Fast Flow filter plates

Hamilton Workstation:	MICROLAB STARLet

9.2.1.2.1. HPLC/MS System

Autosampler:	CTC HTS PAL or Gerstel MP3
HPLC pump:	Agilent HP 1100 binary pump
Guard column:	Frit 2.1 mm for narrow bore column
Column:	Waters SunFire, 3,5 μm 50 x 2.1 mm
Switching valve:	Valco 2 position
Mass spectrometer:	PE-SCIEX API 4000 triple quadrupole equipped with a turbo ion spray source (TIS)
Data system:	Analyst ver. 1.4.1

9.3. Preliminary tests

A well programmed test method is first performed with water, then with the final liquid classes accurately tested and prepared.

The software is a Windows based, menu driven interface allowing the user to define deck layouts and methods.

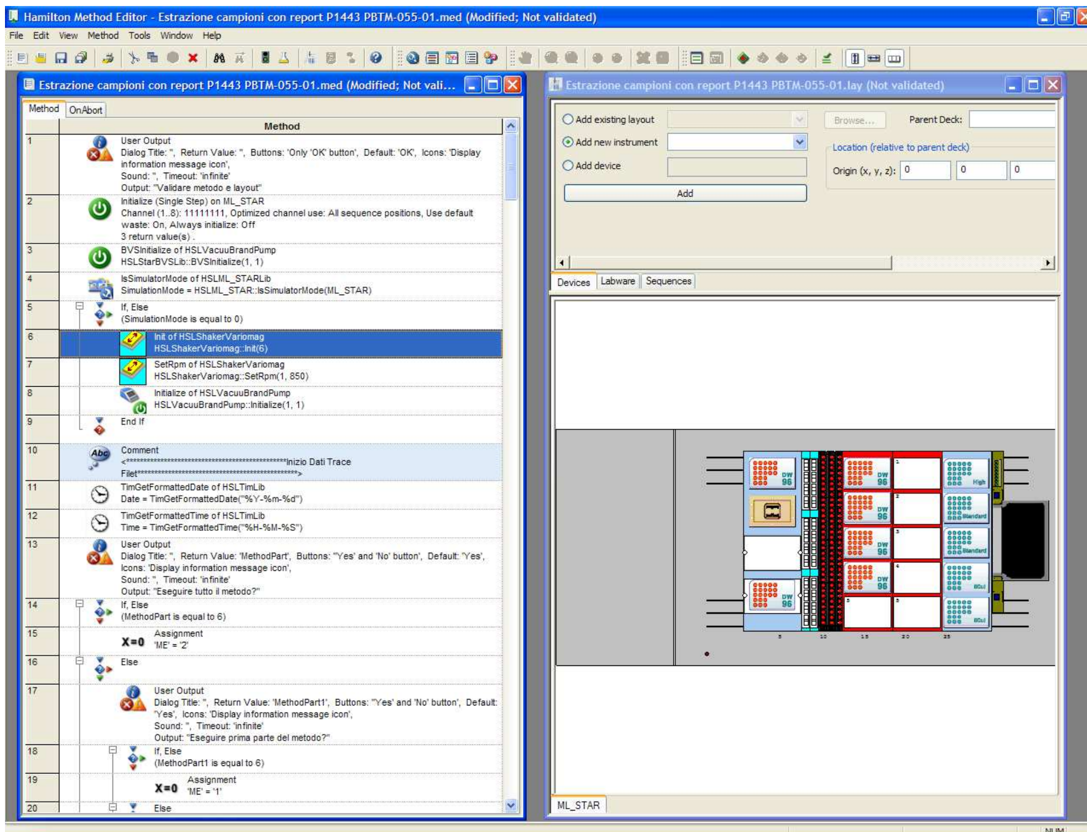
In the method tasks there is the possibility to introduce all the information required about positions (by the creation of the deck layout), liquid classes, way of dispensation, error detections etc.

First the goal was to obtain an automated calibration curve.

The first calibration curves obtained did not reach the linearity required.

Optimization tests for the right settings were performed on rpm settings, dispensation settings, antidroplet activation etc.

A screen view of a method is presented in Figure 34



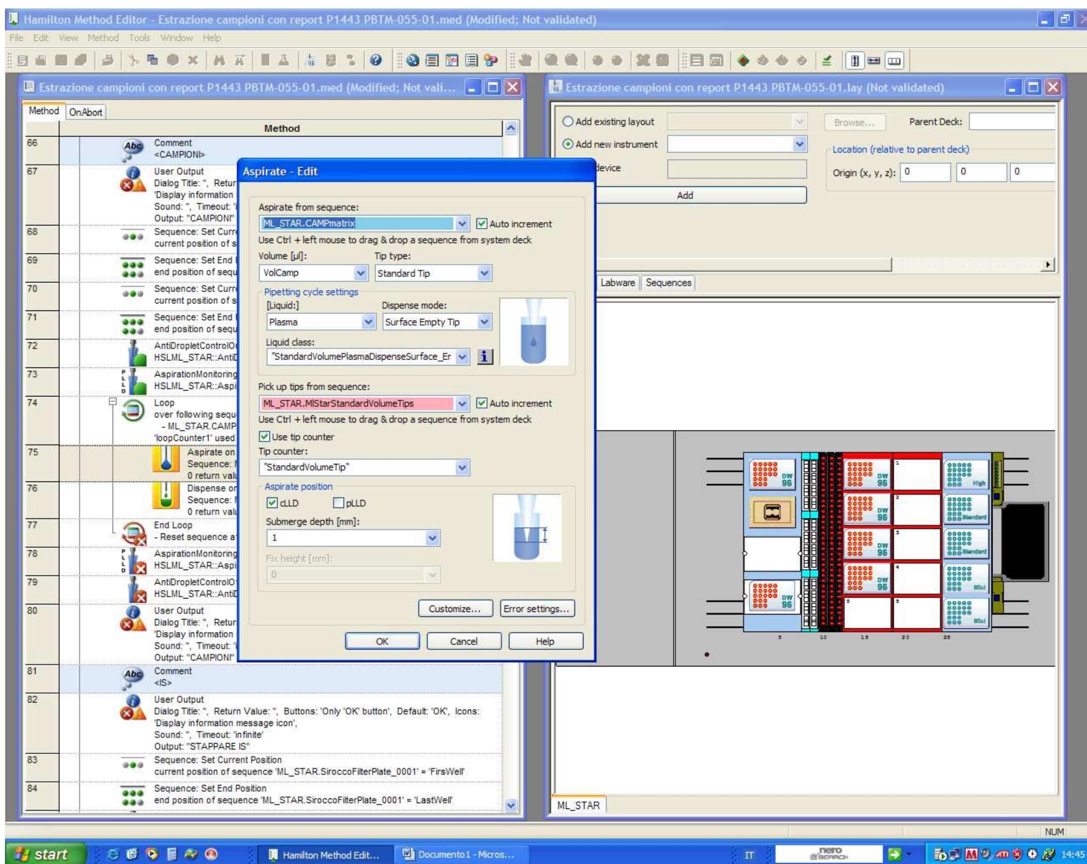


Figure 34: Hamilton method editor

One of the first correlations between manual and automated results is described in Table 5 and Figure 35.

Table 5

	Conc, ng/ml	Analyte 1 Peak Area	Analyte 1 - blank Peak Area	IS, Peak Area	Area Ratio	Area Ratio Blank Subtracted
STD.1	0.2	8173.9	6478.85	218634.2	0.03738619	0.029633287
STD.2	0.5	11301.2	9606.15	157178.7	0.07190033	0.061116105
STD.3	1	19851.7	18156.65	164576.1	0.12062323	0.110323735
STD.4	5	95444.6	93749.55	164415	0.58051029	0.570200712
STD.5	10	160284	158588.95	147294	1.08819097	1.076683029
STD.6	25	432467.6	430772.55	163285.4	2.64853808	2.638157178
STD.7	50	919286.5	917591.45	158967.1	5.78287268	5.772209784
STD.8	100	1823729.8	1822034.75	160665.5	11.3510978	11.3405476
QCL.p1.1	0.75	14376.8	12681.75	162686.9	0.08837098	0.077951882
QCL.p1.2	0.75	13914	12218.95	164018.4	0.08483195	0.074497434
QCL.p2.1	0.75	13499.8	11804.75	169830.8	0.0794897	0.069508888
QCL.p2.2	0.75	13013.8	11318.75	167334.3	0.07777126	0.067641542
QCM.p1.1	7.5	114149	112453.95	160212.9	0.7124832	0.701903218
QCM.p1.2	7.5	108063.5	106368.45	159996.8	0.67541038	0.664816109
QCM.p2.1	7.5	115849.1	114154.05	168143	0.68899151	0.678910511
QCM.p2.2	7.5	113474.1	111779.05	163498.3	0.69403841	0.683671023
QCH.p1.1	75	1035114.8	1033419.75	153332.8	6.75077218	6.739717464
QCH.p1.2	75	1079335.9	1077640.85	162036.1	6.66108293	6.650621991
QCH.p2.1	75	1177580.7	1175885.65	171239.3	6.87681332	6.866914604
QCH.p2.2	75	1060440.1	1058745.05	162998.8	6.50581538	6.495416224

Linear Reg w 1/X2						Linear Reg			
Rec conc	Acc%	Rec conc-blank	Acc%			Rec conc	Acc%	Rec conc-blank	Acc%
0.6616906	**88.2254133**	0.645262	**86.03496**	0.7405	98.7333333	0.9156337	**122.084493**	0.913	**121.73328**
0.6296289	**83.95052**	0.613686	**81.82481333**	0.7081509	94.42012	0.8845237	**117.936493**	0.882631	**117.684147**
0.5812309	**77.4974533**	0.568087	**75.74497333**	0.6593189	87.9091867	0.8375623	**111.674973**	0.838776	**111.836827**
0.5656627	**75.4216933**	0.551018	**73.46912**	0.6436112	85.8148267	0.8224562	**109.660827**	0.82236	**109.648027**
6.315822	**84.21096**	6.348618	**84.64824**	6.445326	85.93768	6.401941	**85.3592133**	6.398231	**85.3097467**
5.979961	**79.7328133**	6.009615	**80.1282**	6.106454	81.4193867	6.07605	**81.014**	6.072194	**80.9625867**
6.102999	**81.37332**	6.138448	**81.84597333**	6.230595	83.0746	6.195436	**82.6058133**	6.196099	**82.6146533**
6.148721	**81.9829467**	6.181962	**82.42616**	6.276727	83.6896933	6.239801	**83.1973467**	6.237949	**83.1726533**
61.01957	**81.3594267**	61.5385	**82.05133333**	61.63954	82.1860533	59.48199	**79.30932**	59.47738	**79.3031733**
60.20703	**80.27604**	60.7241	**80.96546667**	60.81973	81.0929733	58.69357	**78.2580933**	58.69413	**78.25884**
62.16143	**82.8819067**	62.70118	**83.60157333**	62.79165	83.7222	60.58997	**80.7866267**	60.59559	**80.79412**
58.80039	**78.40052**	59.30541	**79.07388**	59.40047	79.2006267	57.32868	**76.43824**	57.3297	**76.4396**

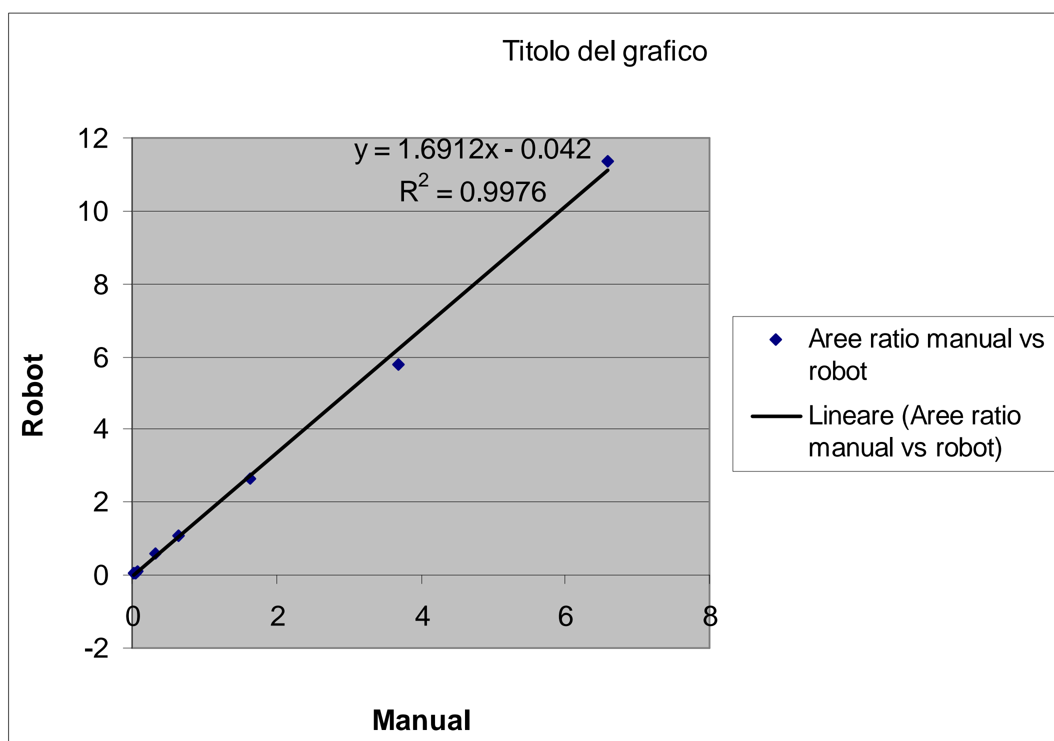


Figure 35

9.4. Validation of the System: method description

After the optimization of the calibration curve tests, the liquid classes creations, the Hamilton StarLet Validation making use of an internal validation protocol was performed. A parallel validation of the method using the two different filer plates (Sirocco and Whatman) described above was made.

The aim of the present study is to perform, at the RBM Preclinical Bioanalysis Lab., the validation of a LC-MS/MS method, for the quantitation of a new chemical active entity in Na-heparinized dog and rat plasma samples. The Full Validation will be performed to determine this new chemical active entity in Na-heparinized dog and rat plasma samples.

Detailed description of LC-MS/MS procedure is described in the Test Method PBTM. Spiked plasma samples will be prepared and frozen at –20 ± 5°C before starting each validation program, in sufficient aliquots to complete the study.

Spiked samples (Low, Medium and High SSs) will be prepared in the same way as QC samples as described in the test method and used for the following test.

The validation was conducted by the use of two different kind of filter plates, Sirocco and Whatman.

9.4.1. Features and Benefits of automation in the method validation development

The manual procedure is very difficult and time consuming; the manipulation of volatile solvent is not easy and has safety issues. Good and reproducible mixing of immiscible solvents is not easy to achieve. The manipulation of organic solvents, as told before, is very difficult to do manually.

Using a workstation there are challenging aspects to consider. The non conductivity and the high volatile characteristics of the organic solvents can be overcome by the technological advantages that the workstation offers. A pressure transducer inside the air displacement channel measures the pressure inside the barrel during pipetting.

Another aspect to observe is the dripping that may occur while pipetting organic solvents. In this case with the Anti Droplet Control technology pressure differences are detected by the pressure sensor and compensated for in real time by piston movements: droplet formation is so prevented.

Other advantages are:

- Automation friendly procedure improves productivity – no off-line steps required.
- Efficient extraction process maximizes analyte recovery.
- Only mechanical method development using the same solvent systems as LLE allowing direct transfer of methods.
- Fixed well plate format is compatible with all common liquid handling systems.

9.4.2. Method validation Key Performance characteristics[49]

Validation is defined as "process of providing documented evidence that the method does what it is intended to do" it identifies the following parameters as important key performance parameter in method validation: accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Measurements for each analyte in the biological matrix

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should be validated. In addition, the stability of the analyte in spiked samples should be determined.

Typical method development and establishment for a bioanalytical method include determination of (1) selectivity, (2) accuracy, precision, recovery, (3) calibration curve, and (4) stability of analyte in spiked samples.

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ).

Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics.

If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

The *accuracy* of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte.

Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

The *precision* of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended.

The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability,

which assesses precision during a single analytical run, and between-run, interbatch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.

The *recovery* of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

Calibration/Standard Curve

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ

1. Lower Limit of Quantification (LLOQ)

The lowest standard on the calibration curve should be accepted as the limit of quantification.

- The analyte response at the LLOQ should be at least 5 times the response compared to blank response.
- Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%.

2. Calibration Curve/Standard Curve/Concentration-Response

The simplest regression model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The following conditions should be met in developing a calibration curve:

- 20% deviation of the LLOQ from nominal concentration.
- 15% deviation of standards other than LLOQ from nominal concentration.

At least four out of six non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution.

All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

1. Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle should be repeated two more times, and then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -80◦C during the three freeze and thaw cycles.

2. Short-Term Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

3. Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high QC concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

4. Stock Solution Stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

5. Post-Preparative Stability

The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be

assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

Although the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits for evaluation of analyte stability in a biological matrix can be used. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.

9.4.3. Application of validate method to routine drug analysis

Assays of all samples of an analyte in a biological matrix should be completed within the time period for which stability data are available. In general, biological samples can be analyzed with a single determination without duplicate or replicate analysis if the assay method has acceptable variability as defined by validation data. This is true for procedures where precision and accuracy variabilities routinely fall within acceptable tolerance limits. For a difficult procedure with a labile analyte where high precision and accuracy specifications may be difficult to achieve, duplicate or even triplicate analyses can be performed for a better estimate of analyte.

A calibration curve should be generated for each analyte to assay samples in each analytical run and should be used to calculate the concentration of the analyte in the unknown samples in the run. The spiked samples can contain more than one analyte. An analytical run can consist of QC samples, calibration standards, and either (1) all the processed samples to be analyzed as one batch or (2) a batch composed of processed unknown samples of one or more volunteers in a study. The calibration (standard) curve should cover the expected unknown sample concentration range in addition to a calibrator sample at LLOQ. Estimation of concentration in unknown samples by extrapolation of standard curves below LLOQ or above the highest standard is not recommended. Instead, the standard curve should be redefined or samples with higher concentration should be diluted and reassayed. It is preferable to analyze all study samples from a subject in a single run.

Once the analytical method has been validated for routine use, its accuracy and precision should be monitored regularly to ensure that the method continues to perform satisfactorily. To achieve this objective, a number of QC samples prepared separately

should be analyzed with processed test samples at intervals based on the total number of samples. The QC samples in duplicate at three concentrations (one near the LLOQ (i.e., LLOQ), one in midrange, and one close to the high end of the range) should be incorporated in each assay run. The number of QC samples (in multiples of three) will depend on the total number of samples in the run. The results of the QC samples provide the basis of accepting or rejecting the run. At least four of every six QC samples should be within 15% of their respective nominal value. Two of the six QC samples may be outside the 15% of their respective nominal value, but not both at the same concentration.

The following recommendations should be noted in applying a bioanalytical method to routine drug analysis:

- A matrix-based standard curve should consist of a minimum of six standard points, excluding blanks (either single or replicate), covering the entire range.
- Response Function: Typically, the same curve fitting, weighting, and goodness of fit determined during prestudy validation should be used for the standard curve within the study. Response function is determined by appropriate statistical tests based on the actual standard points during each run in the validation. Changes in the response function relationship between prestudy validation and routine run validation indicate potential problems.
- The QC samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.
- Any required sample dilutions should use like matrix (e.g., human to human) obviating the need to incorporate actual within-study dilution matrix QC samples.
- Repeat Analysis: It is important to establish an SOP or guideline for repeat analysis and acceptance criteria. This SOP or guideline should explain the reasons for repeating sample analysis. Reasons for repeat analyses could include repeat analysis of clinical or preclinical samples for regulatory purposes, inconsistent replicate analysis, samples outside of the assay range, sample processing errors, equipment failure, poor chromatography, and inconsistent pharmacokinetic data. Reassays should be done in triplicate if sample volume allows. The rationale for the repeat analysis and the reporting of the repeat analysis should be clearly documented.

- Sample Data Reintegration: An SOP or guideline for sample data reintegration should be established. This SOP or guideline should explain the reasons for reintegration and how the reintegration is to be performed. The rationale for the reintegration should be clearly described and documented. Original and reintegration data should be reported.

9.4.3.1. Acceptance Criteria for the Run

The following acceptance criteria should be considered for accepting the analytical run:

- Standards and QC samples can be prepared from the same spiking stock solution, provided the solution stability and accuracy have been verified. A single source of matrix may also be used, provided selectivity has been verified.
- Standard curve samples, blanks, QCs, and study samples can be arranged as considered appropriate within the run.
- Placement of standards and QC samples within a run should be designed to detect assay drift over the run.
- Matrix-based standard calibration samples: 75%, or a minimum of six standards, when back-calculated (including ULOQ) should fall within ±15%, except for LLOQ, when it should be ±20% of the nominal value. Values falling outside these limits can be discarded, provided they do not change the established model.
- Quality Control Samples: Quality control samples replicated (at least once) at a minimum of three concentrations (one within 3x of the LLOQ (low QC), one in the midrange (middle QC), and one approaching the high end of the range (high QC)) should be incorporated into each run. The results of the QC samples provide the basis of accepting or rejecting the run. At least 67% (four out of six) of the QC samples should be within 15% of their respective nominal (theoretical) values; 33% of the QC samples (not all replicates at the same concentration) can be outside the ±15% of the nominal value. A confidence interval approach yielding comparable accuracy and precision is an appropriate alternative. The minimum number of samples (in multiples of three) should be at least 5% of the number of unknown samples or six total QCs, whichever is greater.

- Samples involving multiple analytes should not be rejected based on the data from one analyte failing the acceptance criteria.
- The data from rejected runs need not be documented, but the fact that a run was rejected and the reason for failure should be recorded.

9.5. Sample Preparation and Automation

9.5.1. Validation Results

The results obtained during the validation of the assay fulfilled all requirements and recommendations regarding linearity, accuracy, and precision generally accepted for bioanalytical studies.

A fully automated high-throughput Protein Precipitation Validation of an existing analytical method was developed to demonstrate the effectiveness of the Robotic System.

9.5.1.1. Linearity

The linearity of the method will be assessed by analyzing calibration samples at various concentrations between the lower (LLOQ) and upper limit of quantification (ULOQ). One set of calibration standards at 9 different non-zero concentration levels including the LLOQ and ULOQ will be analysed in one validation batch.

Table 6:Linearity

	Slope	**Mean**	**%Diff Slope ± 25.0**
Run02	0.0144		0.70
Run03	0.0138	0.0143	-3.50
Run04	0.0148		3.50

Run02	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
	1.00	2.50	5.00	10.00	25.00	50.00	100.00	250.00	500
Back calculated	1.00	2.50	4.90	9.80	24.90	51.50	98.40	255.60	504.60
Acc%	100.00	100.00	98.00	98.00	99.60	103.00	98.40	102.24	100.92

Run03	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
	1.00	2.50	5.00	10.00	25.00	50.00	100.00	250.00	500
Back calculated	1.00	2.50	4.60	9.80	20.80	48.80	102.70	254.90	527.70
Acc%	100.00	100.00	92.00	98.00	83.2*	97.60	102.70	101.96	105.54

Run04	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
	1.00	2.50	5.00	10.00	25.00	50.00	100.00	250.00	500
Back calculated	1.00	2.30	4.70	9.90	25.40	49.80	106.70	246.50	512.40
Acc%	100.00	92.00	94.00	99.00	101.60	99.60	106.70	98.60	102.48

9.5.1.2. Accuracy and Precision

The concentration of low QC is 3.00ng/ml, near the lower limit of quantitation but no more than 3 times the LLOQ concentration; the medium QC concentration is 40.00ng/ml about in the middle of the calibration range it has to be near the geometric mean of the low and high QC concentrations; the high QC is 200.00ng/ml and is the upper end of the calibration curve, within the upper quartile of the calibration range.

The analyses will be performed in 3 validation runs (on 3 different days) in order to assess the overall accuracy and precision.

From the results is possible to notice that the mean intra-batch as well as the overall accuracy is within 85-115% at QC levels higher than LLOQ.

The intra-batch and overall precision, expressed as CV%, do not exceed 15% at QC levels higher than LLOQ, as suggests from the guidance results validation lines.

In both Table 7 and Table 8 are shown the results of intra and inter- run accuracy and precision.

Table 7: Intra run accuracy and precision

Run02	True Conc. ng/ml	Estimated Conc. ng/ml	Bias%	Mean Bias% each level ≤20.0	ACC%	CV% ACC% ≤15.0%	Mean Bias% each day ≤15.0	CV% ACC% each day ≤15.0
	SS-Low	2.8	-6.67		93.33			
	3.00	2.8	-6.67	**-8.89**	93.33	**4.23**		
		2.6	-13.33		86.67			
	SS-Medium	41.2	3.00		103.00			
	40.00	38.4	-4.00	**-4.33**	96.00	**7.85**	**-7.52**	**5.45**
		35.2	-12.00		88.00			
	SS-High	182.1	-8.95		91.05			
	200.00	175.5	-12.25	**-9.35**	87.75	**3.00**		
		186.3	-6.85		93.15			
Run03	**True Conc. ng/ml**	**Estimated Conc. ng/mL**	**Bias%**	**Mean Bias% each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low	3.2	6.67		106.67			
	3.00	2.7	-10.00	**-1.67**	90.00	**11.99**		
		N.A.						
	SS-Medium	39.50	-1.25		98.75			
	40.00	43.10	7.75	**4.33**	107.75	**4.68**	**-0.39**	**9.21**
		42.60	6.50		106.50			
	SS-High	207	3.50		103.50			
	200.00	203.5	1.75	**-4.27**	101.75	**12.50**		
		163.9	-18.05		81.95			
Run04	**True Conc. ng/ml**	**Estimated Conc. ng/mL**	**Bias%**	**Mean Bias% each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low	2.80	-6.67		93.33			
	3.00	2.70	-10.00	**-5.56**	90.00	**5.39**		
		3.00	0.00		100.00			
	SS-Medium	36.00	-10.00		90.00			
	40.00	35.90	-10.25	**-12.08**	89.75	**3.86**	**-11.36**	**7.34**
		33.60	-16.00		84.00			
	SS-High	159.00	-20.50		79.50			
	200.00	182.00	-9.00	**-16.45**	91.00	**7.73**		
		160.30	-19.85		80.15			

Mean ACC% 80.0%-120.0%	SD ACC%	Mean ACC% each day	SD ACC% each day
91.11	3.85		
95.67	7.51	92.48	5.04
90.65	2.72		
Mean ACC%	**SD ACC%**	**Mean ACC% each day**	**SD ACC% each day**
98.34	11.79		
104.33	4.88	99.61	9.17
95.73	11.97		
Mean ACC%	**SD ACC%**	**Mean ACC% each day**	**SD ACC% each day**
94.44	5.09		
87.92	3.39	88.64	6.51
83.55	6.46		

Table 8: Inter run Accuracy and Precision

Overall Mean Bias% ≤ 10.0	Overall CV% ACC% ≤15.0	Overall Mean ACC%	SD ACC%
-7.08	8.68	93.58	8.12

9.5.1.3. Selectivity

Selectivity of the method towards possible impurities or degradation products of the IS and of the analyte was so established by analyzing blank samples spiked with the molecules of interest, the good results obtained are shown in Table 9

Table 9: Selectivity

Run 01	Replicate	Peak Matrix Area	Peak LLOQ Area	SELECT% ≤20.0%
	1.00	794.20	4771.80	16.64
	2.00	843.10	5444.10	15.49
	3.00	0.00	4176.80	0.00
	4.00	596.30	5202.80	11.46
	5.00	0.00	4375.40	0.00
	6.00	0.00	4269.50	0.00

9.5.1.4. Carryover

Table 10

Run1	Peak Area Std 1	DBK after ULOQ	Criteria: ≤50.0%
	6503.70	1921.00	29.54

Run1	Average Area IS	DBK after ULOQ	Criteria: ≤20.0%
	278303.82	1888.10	0.68

	270850.20
	300629.40
	280800.60
	280304.00
	270467.10
	276414.10
	280515.90
	300312.70
	244440.40
Mean IS	278303.82

9.5.1.5. Dilution Test

Table 11

Run03	True Conc. ng/ml	Estimated Conc. (ng/ml)	BIAS%	Mean BIAS% ≤20.0%	CV% ACC% ≤15.0%	ACC% 80.0%-120.0%	Mean ACC%	S.D
	250.0	268.3	7.3			107.3		
		267.1	6.8			106.8		
		274.3	9.7	**265.9**	**1.9**	109.7	106.3	**2.06**
		261.8	4.7			104.7		
		263.3	5.3			105.3		
		260.3	4.1			104.1		

9.5.1.6. Autosampler Stability

Table 12

Run02	True Conc. (ng/ml)	Estimated Conc. t = 0 h ng/ml	Estimated Conc. t = 24 h ng/ml	STAB % ≥ 80.0 - ≤ 120.0	Mean STAB%
	SS-Low	3.80	3.60	94.74	
	3.00	3.10	3.20	103.23	**100.40**
		3.10	3.20	103.23	
	SS-High	404.40	416.80	103.07	
	400.00	409.90	434.00	105.88	**104.13**
		405.30	419.30	103.45	

9.5.1.7. Short Term Stability

Table 13

Run02	True Conc. (ng/ml)	Estimated Conc. t = 0 h ng/ml	Mean	Estimated Conc. t = 4 h ng/ml	Mean	STAB % ≥ 80.0 - ≤ 120.0
	SS-Low	3.80		3.60		
	3.00	3.10	**3.33**	3.80	**3.53**	**106.01**
		3.10		3.20		
	SS-High	404.40		425.30		
	400.00	409.90	**406.53**	486.20	**463.57**	**114.03**
		405.30		479.20		

9.5.1.8. Freeze&thaw stability

Table 14

Run02	True Conc. (ng/ml)	Estimated Conc. t = 0 h ng/ml	Mean	Estimated Conc. 3 Cycles ng/ml	Mean	CYCLES % ≥ 80.0 - ≤ 120.0
	SS-Low	3.80		3.60		
	3.00	3.10	**3.33**	3.30	**3.43**	**103.00**
		3.10		3.40		
	SS-High	404.40		413.00		
	400.00	409.90	**406.53**	446.70	**437.67**	**107.66**
		405.30		453.30		

9.5.1.9. Long Term Stability

Table 15

Run02	True Conc. (ng/ml)	Estimated Conc. t = 0 h (ng/ml)	Mean
	SS-Low	3.80	
	3.0	3.10	**3.33**
		3.10	
	SS-High	404.40	
	400.0	409.90	**406.5**
		405.30	

Run05	Estimated Conc. 1 month (ng/ml)	Mean	STAB % ≥ 80.0 - ≤ 120.0
	3.50		
	3.40	**3.30**	**99.10**
	3.00		
	333.50		
	394.00	**380.30**	**93.55**
	413.40		

All the results were in accordance with the range required and all the tests passed. Even if the results in Run terms were in behalf of Whatman plates I decided to present and consider Sirocco's results. For the future experiments of pharmacokinetic and toxicokinetic I'll use Sirocco filter plates because in term of recovery demonstrated a better performance.

Even if linearity of the calibration curve (over a concentration range of 1-250 ng/ml), as measured by r^2, was comparable for both plates and precision of calibration curve parameters, filtration results, mixing results, inter-day precision etc were comparable for both the PPT filter plate, the relative recovery of analyte and Internal Standard using Whatman plates was lower of 16%.

After having optimized and validate the system, I begun to perform the real samples' extraction comparing them with manual extraction in real time.

9.5.1.10. Real samples automated vs. manual extraction

Table 16

subj A	**1 manual**	**2 manual**	**1 robot**	**2 robot**	**CV% manual**	**CV% robot**	**1 ACC%**	**2 ACC%**	**3 ACC%**	**4 ACC%**	
day 85 4h	111.30	109.50	109.90	107.00	1.15	1.89	98.74	100.37	96.14	97.72	
day 85 6h	54.20		50.30				92.80				
day 85 24h	1.00	1.10	0.80	0.90	6.73	8.32	80.00	72.73	90.00	81.82	
day 89 4h	91.30	84.90	88.80	86.10	5.14	2.18	97.26	104.59	94.30	101.41	
day 89 6h	59.70		56.30	55.50		1.01	94.30		92.96		
day 89 24h	1.40	1.50	1.20	1.20	4.88	0.00	85.71	80.00	85.71	80.00	
					CV% mean	**CV% mean**	**ACC% 1 mean**	**ACC% 2 mean**	**ACC% 3 mean**	**ACC% 4 mean**	**ACC% total mean**
					4.48	2.68	91.47	89.42	91.82	90.24	**90.74**

Table 17

subj B	1 manual	1 robot	2 robot	CV% robot	1 ACC%	2 ACC%	
day 1 4h	198.900	198.700	198.100	0.214	100.101	100.404	
day 1 6h	89.400	85.300	85.400	0.083	104.807	104.684	
day 1 24h	2.100	2.000	2.100	3.449	105.000	100.000	
day 5 4h	212.200	205.600	206.200	0.206	103.210	102.910	
day 5 6h	116.600	114.000	113.200	0.498	102.281	103.004	
day 5 24h	7.400	8.200	8.200	0.000	90.244	90.244	
day 50 4h	138.000	138.900	133.100	3.016	99.352	103.681	
day 50 6h	56.700	56.700	59.600	3.526	100.000	95.134	
day 50 24h	1.900	2.200	2.200	0.000	86.364	86.364	
day 54 4h	76.400	76.400	78.500	1.917	100.000	97.325	
day 54 6h	39.300	38.500	38.900	0.731	102.078	101.028	
day 54 24h	1.000	1.200	1.100	6.149	83.333	90.909	
				CV% mean	**ACC%1 mean**	**ACC%2 mean**	**ACC%1 ACC%2 total mean**
				1.649	98.064	97.974	**98.019**

In Table 16 and Table 17 are presented the results obtained by a parallel extraction manual vs. automated of real plasma samples. The level of accuracy and CV% reached is high.

9.5.1.11. Dilutions using Dynamic Dilution Module

Table 18

C 1 to 5	1 manual	1 robot	2 robot	CV%	1 ACC%	2 ACC%	
subj 72	5.300	5.100	5.300	2.720	96.226	100.000	
subj 73	294.600	255.600	249.000	1.850	86.762	84.521	
subj 74	1859.700	1719.200	1744.700	1.041	92.445	93.816	
subj 75	229.7	221.100	224.300	1.016	96.256	97.649	
				CV% mean	**ACC% 1 mean**	**ACC% 2 mean**	**ACC% total mean**
				1.657	92.922	93.997	**93.459**

Table 19

D 1 to 10	1 manual	1 robot	2 robot	CV%	1 ACC%	2 ACC%	
subj 48	177.000	185.200	179.600	2.171	104.633	101.469	
subj 49	223.300	220.200	243.600	7.135	98.612	109.091	
subj 50	172.500	181.800	181.000	0.312	105.391	104.928	
subj 51	156.200	145.800	147.300	0.724	93.342	94.302	
				CV% mean	**ACC% 1 mean**	**ACC% 2 mean**	**ACC% total mean**
				2.585	100.494	102.447	**101.471**

Table 20

E 1 to 10	1 manual	1 robot	2 robot	3 robot	CV%	1 ACC%	2 ACC%	3 ACC%	
day 1 30min	474.000	428.000	414.000	434.000	2.413	90.295	87.342	91.561	
day 5 30min	187.000	198.900	195.700	206.700	2.823	106.364	104.652	110.535	
day 50 30min	746.000	840.800	835.800	823.700	1.055	112.708	112.038	110.416	
day 54 30min	200.300	216.000	216.000	206.900	2.467	107.838	107.838	103.295	
					CV% mean	**ACC% 1 mean**	**ACC% 2 mean**		**ACC% total mean**
					2.189	104.301	102.967	103.952	**103.740**

Using DDM it is possible to interface directly the software to a macro containing the dilution factors indicated in the LIMS. The results obtained, described in Table 18, Table 19 and Table 20, for both 1:5 and 1:10 dilution factor has accuracy high level.

9.6. Conclusions

Through use the Hamilton MicroLab Star liquid handling workstation, protein precipitation sample preparation has been successfully automated and implemented for several biological matrix samples. This in turn has greatly reduced the intensive labor as well as the possibilities of systematic error associated with the manual volumetric transfers. Significant advantages in terms of efficiency and throughput have now been realized by this automated process while maintaining the integrity of the precision and accuracy.

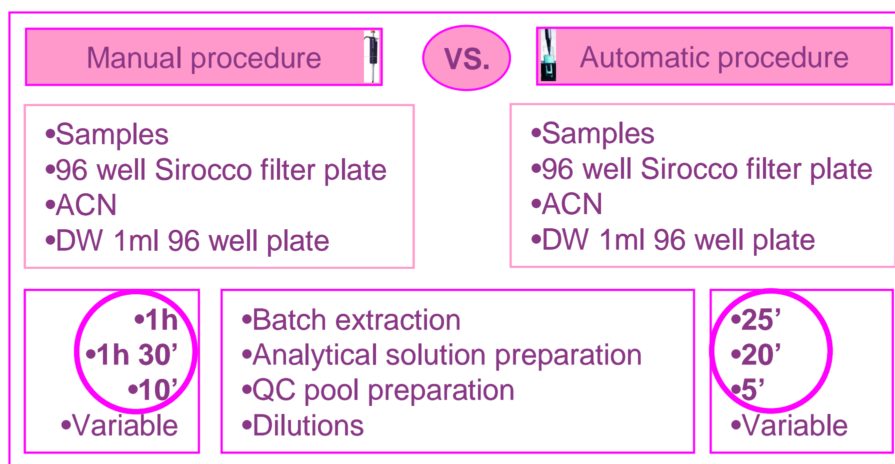

Figure 36

Processing time is less than 30 s per sample or 25 min per 96-well plate (Figure 36), which is then immediately ready for injection onto an LC-MS/MS system or dried and reconstituted..

Over 12 studies and 5000 samples have been prepared in the Automation Laboratory since implementation in the first six months. The original goal to automate the sample workload using this approach was met.

Table 21

Project	Study	Quality	Run	Number of Samples
P964/P1074		QMS	Validation Run 1 2 3 Whatman Run 1 2 3 4 5 Sirocco	274
P1016	RE5900	GLP	Extraction Run on Parallel	231
P1016	RE6620	GLP	Extraction Run on Parallel	58
P1417	RE5820	GLP	Run 1 2 3 Reassay + QC Pool Preparation	1165
P1417	RE9030		WS QC IS Preparation	
P1417	RE9680	QMS	Run 1 2 6	482
P1417	RE9840	QMS	Run 1 2	673
P1417	RF0150	GLP	Run 1 2 3 4 5 + Incurred Samples	1032
0326-2008		GLP	Run 1 2 3 4 7	1068
P1443	RE7730	GLP	Extraction Run on Parallel	40
P1443	RE7930	GCLP	Run 30 31	48
P1443	RE7930		Extraction Run on Parallel	54
P1443	RE8500	GLP	Run 1 2 3 4	445
P1443	RE8510		QC Pool Preparation	
P1443	RE8550	QMS	Validation	40
P1492			WS QC IS Preparation	
			Tot	5610

Furthermore, the open configurability of both the Hamilton hardware and software allows for the continual refinement, enhancements, or additions to our program, which in turn enables us to keep pace with the ever-changing landscape and external demands that drive and shape our future.

These findings were presented in poster sessions at the following meetings:

"R&D Science Day", Thursday, September 30th 2010 NCD Day, Merck Serono Headquarters. 9, Chemin des Mines CH-1202 Geneva.

"Automation in Regulated Bioanalytics: High-throughput via Robotic Liquid Handling"

Maria Chiara Zorzoli[2], Luca Barbero[2], Luigi Colombo[1]

1 Global Bioanalytics, 2 Automation Laboratory.

10. Liquid-Liquid Extraction (LLE)

10.1. Introduction

Liquid-Liquid Extraction, also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubility in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid phase into another liquid phase. LLE can be labor-intensive and difficult to automate since it often requires off-line processing such as mixing or centrifuging.

High-throughput LLE is performed when a collection microplate is used in place of multiple test tubes. An alternative approach to LLE is the use of solid support diatomaceous earth particles contained in tubes or cartridges.

Due to advances in manufacturing, supported liquid extraction can now be done in high throughput 96-well plates compatible with most automated systems, requiring no off-line steps.

10.1.1. Fundamental Principles

LLE is a technique used to separate analytes from interferences in the sample matrix by partitioning the analytes between two immiscible liquids.

A given volume of aqueous sample solution, as plasma or urine, containing analytes is mixed with an internal standard in solution. When necessary a volume of buffer at a known pH is then added to maintain the analytes in their unionized state.

The resulting solution is then vigorously mixed with several ratio volumes of water immiscible organic solvent or mixture of two or more solvents such as hexane, diethyl ether, methyl tert-butyl ether (MTBE) or ethyl acetate (EA).

Analytes distribute between the two liquid phases (aqueous and organic) and partition preferentially into the organic phase when the analytes are unionized and demonstrate solubility in that organic solvent.

Agitation increases the available surface area for this interaction to occur and aids in the mass transfer process.

When LLE procedure is optimized, the hydrophilic compounds in the sample matrix will prefer to remain in the aqueous phase and the more hydrophobic compounds (ideally analytes to the exclusion of interfering substances) will migrate into the organic phase, description in Figure 37.

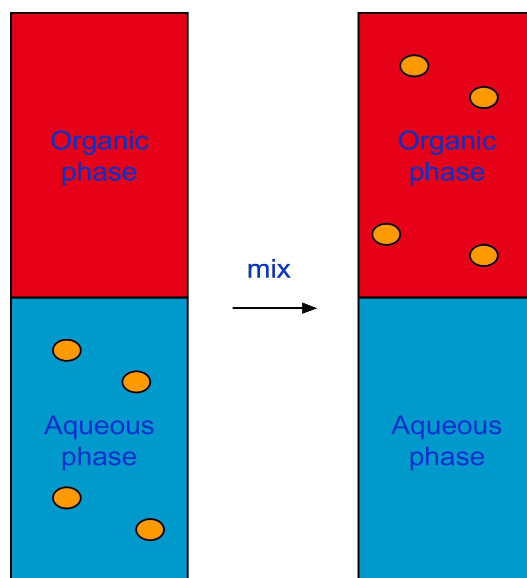

Figure 37

The organic phase is isolate and, since it is typically not compatible with solvents used in liquid chromatography (LC), is concentrated by evaporation. Reconstitution in a mobile phase compatible solvent is then performer prior to analysis.

10.1.1.1. Advantages

A major benefit is that LLE is of wide general applicability for many drug compounds and this technique presents many advantages:

- Very clean extracts can be obtained with good selectivity for the target analyte using proper selection of organic solvent and adjustment of sample pH.
- Inorganic salts are insoluble in the solvents commonly used for LLE and remain behind in the aqueous phase along with proteins and water soluble endogenous components.
- Interferences are excluded are excluded from the chromatographic system and a cleaner sample and a cleaner sample is prepared for analysis.

The removal of these unwanted matrix materials provides potential benefits of extending LC column lifetime and minimizing the downtime of the mass spectrometer caused by interface fouling.

10.1.1.2. Disadvantages

Several disadvantages exist with the Liquid-Liquid Extraction:

- It is a very labour intensive procedure because of multiple transfer steps and the need to frequently cap and uncap tubes.
- It requires a large volume of organic solvents which can be expensive to purchase and presents added costs for disposal as hazardous waste.
- Exposure of these solvents to personnel can present health hazards.
- The procedure has been difficult to fully automate using traditional liquid handling instruments.
- Evaporative losses may sometimes occur upon dry-down with volatile or oxygen labile reactive analyte.
- Emulsion formation is a potential problem.

10.2. Experimental

A small molecule currently under clinical development for multiple sclerosis (MS) was used to develop a fully automated extraction method.

The nucleoside analog 2-chlorodeoxyadenosine (Cladribine, CdA) is used in the treatment of patients with several hematological malignancies. After administration of CdA, the major catabolite measured in plasma and urine is 2-chloroadenine (CAde)[50].

Oral Cladribine is a proprietary oral tablet formulation of Cladribine that is being studied in an effort to demonstrate possible benefits as a treatment for patients with relapsing forms of MS. Cladribine is a purine nucleoside analogue that interferes with the behavior and the proliferation of certain white blood cells, particularly lymphocytes,

50 Cytotoxicity and pharmacokinetics of cladribine metabolite, 2-chloroadenine in patients with leukemia Synno¨ve Lindemalm, Jan Liliemark, Gunnar Juliusson, Rolf Larssonc,Freidoun Albertioni, 8 March 2004 Cancer Letters 210 (2004) 171–177.

which are involved in the pathological process of MS. Through its differentiated mechanism of action, Cladribine tablets may offer an effective new option to patients with MS.

The aim of this part of the project was to find the correct way to automate a validation method for the LLE of the molecule.

The use of a different extraction solvent from the more common end more manually manageable MTBE required the automation of the procedure.

The solvent used was ethyl acetate.

The advantage of MTBE in LLE is that it allows faster and cleaner recovery and is well suited for manual extraction because the aqueous solution free from the molecule of interest frozen in liquid nitrogen and it is easy to tip out from the tube in a new vial or in a 96 well plate the solvent containing the molecule. In this case the best solvent to perform the extraction was ethyl acetate. It presents different chemical physical characteristics and it is impossible to separate the phases by freezing only one of them.

The best mechanical way was evaluated in order to create the best full automated method.

Hamilton offers both the traditional capacitive liquid level detection as well as pressure liquid level detection. By the use of organic solvents the capacitive level detection is not possible. Using the pressure liquid level detection it is permitted to drive the pipette tip to just above the surface to do a jet dispense, or touch the surface for a wet dispense, or dive below sufficiently to aspirate liquid without drawing in any air.

The developed method was created for a pre study method validation. During pre-study validation, method precision and accuracy are determined through the analysis of QCs (validation samples) prepared in a biological matrix equivalent to that anticipated for study samples. Because of the endogenous nature of some biopharmaceuticals, it may be necessary to deplete the matrix of the analyte or employ a “surrogate” matrix to evaluate method accuracy and precision and molecule stability.

10.2.1. Instrumentation

10.2.1.1. Reagents and chemicals

The LLE extracts of the biological samples were analyzed by high-performance liquid chromatography with APCI (Atmospheric Pressure Chemical Ionization/Heated Nebulizer)) tandem mass spectrometry (LC-MS/MS).

Analysis is performed with the following reagents and chemicals:

Ammonia solution 2.5%:	Analytical reagent grade
Deionized water:	Milli-Q quality
Methanol:	HPLC grade
Formic Acid 98%:	Analytical reagent grade
Ethyl Acetate:	HPLC grade
Dimetil Sulfoxide (DMSO):	Analytical reagent grade
Dilution solvent:	Water/Methanol 95/5 with 0.1% Formic Acid
Reconstitution Solvent (RS):	Water/Methanol 95/5 with 0.1% Formic Acid.

10.2.1.2. Equipment

Analytical balance:	Sartorius Research R200D.
Centrifuge:	ALC 4239R.
Vacuum system:	Speed-Vac plus SC210A System, Savant.
Ultrapure waters system:	MilliQ Plus Millipore.
Hamilton Workstation:	MICROLAB STARLet.

10.2.1.2.1. UPLC/MS System (Figure 38)

Autosampler:	Waters Acquity UPLC Sample Organizer/Sample Manager
UPLC pump:	Waters Acquity UPLC Binary Solvent Manager
Column:	Waters Acquity UPLC BEH C18 1.7µm 2,1x50 mm
Mass spectrometer:	Applied Biosystems API4000 triple quadrupole e quipped with APCI (Heated Nebulizer)
Data system:	Analyst version. 1.4.2.

Figure 38: Ultra Performance Liquid Chromatography.

Ultra Performance Liquid Chromatography (UPLC, Figure 38) is a relatively new technique giving new possibilities in liquid chromatography, especially concerning decrease of time and solvent consumption. UPLC advantages are clearly obvious. The separation mechanisms is still the same, chromatographic principles are maintained while speed, sensitivity and resolution is improved.

10.2.2. Sample Preparation and Automation

Automated LLE was carried out on the 8-channel Hamilton Robotic Workstation using EA as the extraction solvent. Each individual channel for every different kind of tips was calibrated during the creation of the relative liquid class for accuracy and precision. EA is a volatile organic solvent difficult to handle.

The standard manual method indications suggest to thaw and centrifuge samples for 1 min at 10000 rpm if necessary, than to transfer a small volume of each sample in a tube.

Than is add a very small volume of working solution of internal standard and the solvent of choice to plasma.

These operations have always been conducted manually in small tubes requiring a very labour and time consuming work.

Method:

- Thaw samples and centrifuge for 1 min at 10000 RPM;
- Transfer 100µl into an Eppendorf tube (1.5 ml);
- Add working solution of internal standards and 0.5 ml ethylacetate to plasma;

- Close tubes, vortex thoroughly, centrifuge (5 min/10000 RPM) and put samples into -80°C freezer for app. 15 min;
- Transfer organic phase into a 96 deep well plate;
- Evaporate to dryness under a stream of nitrogen at 50°C;
- Add 100µl of methanol 10% in water;
- Close 96 deep well plate and shake well (5 min at 1000 RPM);
- Centrifuge 96 deep well plate for 2 min;
- Put 96 deep well plate in autosampler and inject 25µl of each sample.

In the automated extraction all the operations are conducted in 96-wellplates, not only the final transfer but also the extraction procedure. During the vortex robotic step care must be exercised to prevent potential contamination across the wells, the plate is cover with a dimpled sealing mat.

Automated procedure:

- Plasma samples aliquot are added with IS with 8 channel-Head;
- Samples are vortex mixed to equilibrate IS with plasma;
- EA is added with 8 channel-Head;
- To avoid vortex mixing and increase the surface/volume ratio, an aspirate/dispense cycle is repeated about 15 times (Figure 39);
- Plates can be centrifuged;
- Organic layer is transferred using 8 channel-Head;
- Aspiration height is 3mm above the plasma layer surface.

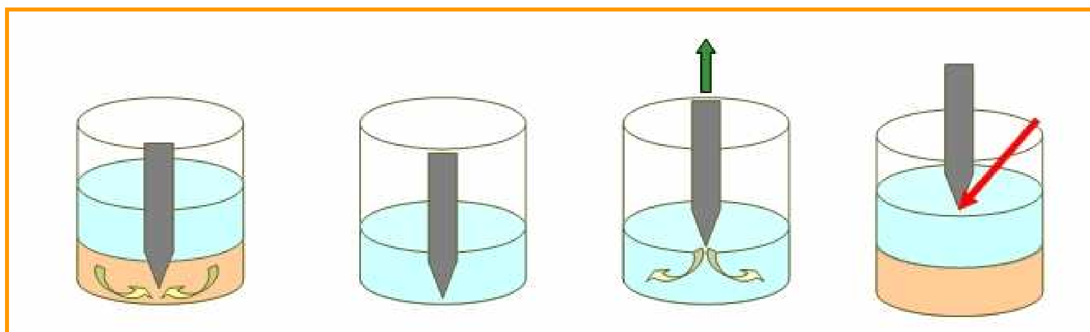

Figure 39: An aspirate/dispense cycle.

Since during the extraction about 10% less out of the total of EA was transferred the extraction recovery could be therefore corrected for the volume change.

10.2.3. Results

A fully automated high throughput Liquid Liquid Extraction has been developed for preparation of biological samples for method validation using a 96-well plate and an 8-channel robotic liquid handling workstation.

The results obtained during the validation of the assay fulfilled all requirements and recommendations regarding linearity, accuracy and precision generally accepted for bioanalytical studies.

The major time saving of using automation is at the sample aliquoting and extraction step. All the samples are vortex at the same time, manually instead each sample is singularly vortexed. To prevent the tip clogging a sample centrifugation step is needed for the automated sample aliquoting. The time saved of manual and automated process is described in Table 22.

Table 22: manual and automated time comparison

	Manual	Automated
Tube labeing	**20 min**	None
Centrifugation	None	**20 min**
Sample aliquoting	**75 min**	30 min
Extraction	**100 min**	**20 min**
Evaporation	15 min	15 min
Reconstitution and transfer	**30 min**	**10 min**
Total time	4 h	1 h 35 min
Analyst time	**3 h 45 min**	**50 min**

The time with bold line indicates the presence of analyst is required.

10.2.3.1. Linearity

The linearity of the method for Cladribine and 2-chloroadenine will be assessed by analyzing calibration samples at various concentrations between the lower (LLOQ) and upper limit of quantification (ULOQ). One set of calibration standards at 9 different non-

zero concentration levels including the LLOQ and ULOQ will be analysed in one validation batch.

The calibration curve for linearity assessment was established using $1/x^2$ weighted linear regression.

Acceptance criteria:

For calibration standards, the deviation of the back-calculated values from the nominal concentration must be within ±15% for all concentrations higher than the LLOQ and within ±20% at the LLOQ to be included in the regression line.

75 % of the calibration standards will have to fulfill the above-mentioned acceptance criteria. At least 6 calibration levels have to remain in the calibration line.

The correlation coefficient (r) for the calibration line must be ≥ 0.990.

Table *23*: Linearity urine, Linearity plasma dog

Cladribine in urine

	Slope	Mean	%Diff Slope (*) ≤ 25.0
Run 01	0.0563		8.157
Run 03	0.0584	**0.0613**	4.731
Run 04	0.0691		12.724

	Run 01	**Std 1**	**Std 2**	**Std 3**	**Std 4**	**Std 5**	**Std 6**	**Std 7**	**Std 8**	**Std 9**
		0.25	**0.50**	**1.00**	**2.50**	**5.00**	**10.00**	**25.00**	**50.00**	**100.00**
Back calculated		0.26	0.45	1.01	2.52	4.96	10.080	26.06	49.79	99.34
Acc%		104.00	90.00	101.00	100.80	99.20	100.80	104.24	99.58	99.34

	Run 03	**Std 1**	**Std 2**	**Std 3**	**Std 4**	**Std 5**	**Std 6**	**Std 7**	**Std 8**	**Std 9**
		0.25	**0.50**	**1.00**	**2.50**	**5.00**	**10.00**	**25.00**	**50.00**	**100.00**
Back calculated		0.24	0.52	1.05	2.40	5.29	9.98	23.88	48.76	99.43
Acc%		96.00	104.00	105.00	96.00	105.80	99.80	95.52	97.52	99.43

	Run 04	**Std 1**	**Std 2**	**Std 3**	**Std 4**	**Std 5**	**Std 6**	**Std 7**	**Std 8**	**Std 9**
		0.25	**0.50**	**1.00**	**2.50**	**5.00**	**10.00**	**25.00**	**50.00**	**100.00**
Back calculated		0.25	0.52	0.99	2.49	5.01	10.17	24.53	50.33	99.00
Acc%		100.00	104.00	99.00	99.60	100.20	101.70	98.12	100.66	99.00

2-chloroadenine in urine

	Slope	Mean	%Diff Slope (*) ≤ 25.0
Run 01	0.0408		1.449
Run 03	0.0439	**0.0414**	6.039
Run 04	0.0395		4.589

	Run 01	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
		0.25	**0.5**	**1**	**2.5**	**5**	**10**	**25**	**50**	**100**
Back calculated		0.25	0.5	0.98	2.27	5.12	10.3	26.65	50.69	97.06
Acc%		100.00	100.00	98.00	90.80	102.40	103.00	106.60	101.38	97.06

	Run 03	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
		0.25	**0.5**	**1**	**2.5**	**5**	**10**	**25**	**50**	**100**
Back calculated		0.27	0.44	0.97	2.42	5.15	10.42	25.37	50.75	101.33
Acc%		108.00	88.00	97.00	96.80	103.00	104.20	101.48	101.50	101.33

	Run 04	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
		0.25	**0.5**	**1**	**2.5**	**5**	**10**	**25**	**50**	**100**
Back calculated		0.28	0.43	0.86	2.43	5.14	10.42	25.17	53.86	104.91
Acc%		112.00	86.00	86.00	97.20	102.80	104.20	100.68	107.72	104.91

Cladribine in plasma dog

	Slope	Mean	%Diff Slope (*) ≤ 25.0
Run 07	0.0681		0.344
Run 08	0.0679	0.06787	0.049
Run 09	0.0676		0.393

	Run 07	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
		0.25	**0.5**	**1**	**2.5**	**5**	**10**	**25**	**50**	**100**
Back calculated		0.25	0.49	1.21	2.57	5.14	9.63	24.35	50.48	101.38
Acc%		100.00	98.00	N.R.	102.80	102.80	96.30	97.40	100.96	101.38

N.R. = the std 3 not met the acceptance criteria

	Run 08	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
		0.25	**0.5**	**1**	**2.5**	**5**	**10**	**25**	**50**	**100**
Back calculated		0.25	0.38	1.13	2.27	4.45	10.45	24.02	47.81	113.06
Acc%		100.00	N.R.	113.00	90.80	89.00	104.50	96.08	95.62	113.06

N.R. = the std 2 not met the acceptance criteria

	Run 09	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
		0.25	**0.5**	**1**	**2.5**	**5**	**10**	**25**	**50**	**100**
Back calculated		0.25	0.48	1.13	2.38	4.97	9.6	24.86	43.49	114.42
Acc%		100.00	96.00	113.00	95.20	99.40	96.00	99.44	86.98	114.42

2-chloroadenine in plasma dog

	Slope	Mean	%Diff Slope (*) ≤ 25.0
Run 07	0.124		4.202
Run 08	0.118	0.119	0.840
Run 09	0.115		3.361

	Run 07	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
		0.25	0.5	1	2.5	5	10	25	50	100
Back calculated		0.24	0.54	1.04	2.55	5.06	9.80	25.22	49.06	92.82
Acc%		96.00	108.00	104.00	102.00	101.20	98.00	100.88	98.12	92.82

	Run 08	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
		0.25	0.5	1	2.5	5	10	25	50	100
Back calculated		0.25	0.36	1.02	2.35	5.12	10.28	24.26	49.45	102.81
Acc%		100.00	N.R.	102.00	94.00	102.40	102.80	97.04	98.90	102.81

N.R. = the std 2 not met the acceptance criteria

	Run 09	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
		0.25	0.5	1	2.5	5	10	25	50	100
Back calculated		0.26	0.45	1.05	2.39	4.98	10.01	25.84	46.89	107.84
Acc%		104.00	90.00	105.00	95.60	99.60	100.10	103.36	93.78	107.84

10.2.3.2. Accuracy and Precision

Intrabatch accuracy and precision of Cladribine and 2-chloroadenine in dog urine and plasma samples higher than LLOQ level will be determined by measuring 3 sets of quality control samples at three concentration levels (low, medium and high) representing the entire range of calibration curve, per validation run (intrabatch accuracy and precision).

The concentration of low QC is 0.75ng/ml, near the lower limit of quantitation but no more than 3 times the LLOQ concentration; the medium QC concentration is 3ng/ml about in the middle of the calibration range it has to be near the geometric mean of the low and high QC concentrations; the high QC is 80ng/ml and is the upper end of the calibration curve, within the upper quartile of the calibration range.

The analyses will be performed in 3 validation runs (on 3 different days) in order to assess the overall accuracy and precision.

Any additional validation batches should contain at least duplicate QC samples (depending on batch size) at each of four concentrations higher than LLOQ. Intrabatch statistics will be calculated for these QC samples and they will be included in the overall statistics.

From the results (see Table 24) is possible to notice that the mean intra-batch as well as the overall accuracy is within 85-115% at QC levels higher than LLOQ.

The intra-batch and overall precision, expressed as CV%, do not exceed 15% at QC levels higher than LLOQ, as suggests from the guidance results validation lines.

For additional validation batches containing duplicate or triplicate QC samples, there are the following acceptance criteria to apply: at least 2/3 of the quality control samples must be within 85-115% of the nominal concentrations including at least 50% of the analyzed samples at each concentration level.

Table 24: Intra run Accuracy and Precision

Cladribine in urine

Run 01	**True Conc. ng/ml**	**Estimated Conc. ng/ml**	**Bias%**	**Mean Bias% each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low	0.62	-17.3		82.7			
	0.75	0.70	-6.7	**14.2**	93.3	**7.6**		
		0.61	-18.7		81.3			
	SS-Medium	2.88	-4.0		96.0			
	3.00	2.72	-9.3	**7.3**	90.7	**3.1**	**10.5**	**5.5**
		2.74	-8.7		91.3			
	SS-High	74.55	-6.8		93.2			
	80.00	70.22	-12.2	**9.9**	87.8	**3.1**		
		71.36	-10.8		89.2			
Run 03	**True Conc. ng/ml**	**Estimated Conc. ng/ml**	**Bias%**	**Mean Bias% each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low	0.72	-4.0		96.0			
	0.75	0.74	-1.3	**4.0**	98.7	**2.8**		
		0.70	-6.7		93.3			
	SS-Medium	2.87	-4.3		95.7			
	3.00	2.88	-4.0	**3.7**	96.0	**0.9**	**4.3**	**1.9**
		2.92	-2.7		97.3			
	SS-High	76.96	-3.8		96.2			
	80.00	74.25	-7.2	**5.4**	92.8			
		75.91	-5.1		94.9	**1.8**		
Run 04	**True Conc. ng/ml**	**Estimated Conc. ng/ml**	**Bias%**	**Mean Bias% each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low	0.80	6.7		106.7			
	0.75	0.75	0.0	**0.5**	100.0	**6.0**		
		0.71	-5.3		94.7			
	SS-Medium	2.93	-2.3		97.7			
	3.00	2.90	-3.3	**5.0**	96.7	**4.0**	**4.2**	**5.4**
		2.72	-9.3		90.7			
	SS-High	72.25	-9.7		90.3			
	80.00	75.01	-6.2	**8.0**	93.8	**1.9**		
		73.58	-8.0		92.0			

Mean ACC% 85.0%-115.0%	SD ACC%	Mean ACC% each day 85.0%-115.0%	SD ACC% each day
85.80	**6.56**		
92.67	**2.90**	**89.51**	**4.89**
90.07	**2.80**		
Mean ACC% 85.0%-115.0%	**SD ACC%**	**Mean ACC% each day 85.0%-115.0%**	**SD ACC% each day**
96.00	**2.70**		
96.33	**0.85**	**95.65**	**1.83**
94.63	**1.72**		
Mean ACC% 85.0%-115.0%	**SD ACC%**	**Mean ACC% each day 85.0%-115.0%**	**SD ACC% each day**
100.5	**6.01**		
95.0	**3.79**	**95.8**	**5.21**
92.0	**1.75**		

Cladribine in plasma

Run 07	True Conc. ng/ml	Estimated Conc. ng/ml	Bias%	Mean Bias% each level ≤20.0	ACC%	CV% ACC% ≤15.0%	Mean Bias% each day ≤15.0	CV% ACC% each day ≤15.0
	SS-Low	0.82	9.3		109.3			
	0.75	0.84	12.0	**10.2**	112.0	**1.4**		
		0.82	9.3		109.3			
	SS-Medium	2.72	-9.3		90.7			
	3.00	2.75	-8.3	**6.4**	91.7	**4.4**	**2.4**	**7.5**
		2.95	-1.7		98.3			
	SS-High	82.04	2.6		102.6			
	80.00	84.54	5.7	**3.4**	105.7	**2.0**		
		81.40	1.8		101.8			
Run 08	**True Conc. ng/ml**	**Estimated Conc. ng/ml**	**Bias%**	**Mean Bias% each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low	0.70	-6.7		93.3			
	0.75	0.83	10.7	**2.7**	110.7	**12.2**		
		0.66	-12.0		88.0			
	SS-Medium	3.01	0.3		100.3			
	3.00	3.08	2.7	**0.4**	102.7	**2.2**	**1.4**	**7.3**
		2.95	-1.7		98.3			
	SS-High	86.16	7.7		107.7			
	80.00	87.14	8.9	**6.3**	108.9	**3.3**		
		81.94	2.4		102.4			
Run 09	**True Conc. ng/ml**	**Estimated Conc. ng/ml**	**Bias%**	**Mean Bias% each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low	0.80	6.7		106.7			
	0.75	0.78	4.0	**4.5**	104.0	**2.0**		
		0.77	2.7		102.7			
	SS-Medium	3.04	1.3		101.3			
	3.00	2.93	-2.3	**0.1**	97.7	**1.9**	**3.7**	**3.6**
		3.02	0.7		100.7			
	SS-High	88.22	10.3		110.3			
	80.00	84.44	5.6	**6.9**	105.6	**2.8**		
		83.78	4.7		104.7			

Mean ACC% 85.0%-115.0%	SD ACC%	Mean ACC% each day 85.0%-115.0%	SD ACC% each day
110.2	1.56		
93.6	4.13	102.4	7.64
103.4	2.06		
Mean ACC% 85.0%-115.0%	**SD ACC%**	**Mean ACC% each day 85.0%-115.0%**	**SD ACC% each day**
97.3	11.88		
100.4	2.20	101.3	7.43
106.3	3.46		
Mean ACC% 85.0%-115.0%	**SD ACC%**	**Mean ACC% each day 85.0%-115.0%**	**SD ACC% each day**
104.5	2.04		
99.9	1.93	103.8	3.69
106.9	3.01		

2 ChloroAdenine in urine

Run 01	True Conc. ng/mL	Estimated Conc. ng/mL	Bias%	Mean Bias% (*) each level ≤20.0	ACC%	CV% ACC% ≤15.0%	Mean Bias% (*) each day ≤15.0	CV% ACC% each day ≤15.0
	SS-Low 0.75	0.91	21.33		121.33			
		0.77	2.67	14.22	102.67	8.83		
		0.89	18.67		118.67			
	SS-Medium 3.00	3.53	17.67		117.67			
		3.04	1.33	8.56	101.33	7.67	8.22	7.90
		3.20	6.67		106.67			
	SS-High 80.00	83.75	4.69		104.69			
		79.78	-0.27	1.87	99.73	2.50		
		80.95	1.19		101.19			
Run 03	**True Conc. ng/mL**	**Estimated Conc. ng/mL**	**Bias%**	**Mean Bias% (*) each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% (*) each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low 0.75	0.70	-6.67		93.33			
		0.78	4.00	0.00	104.00	5.81		
		0.77	2.67		102.67			
	SS-Medium 3.00	3.05	1.67		101.67			
		2.89	-3.67	0.22	96.33	3.00	0.65	3.52
		3.04	1.33		101.33			
	SS-High 80.00	78.69	-1.64		98.36			
		77.09	-3.64	1.72	96.36	1.91		
		80.09	0.11		100.11			
Run 04	**True Conc. ng/mL**	**Estimated Conc. ng/mL**	**Bias%**	**Mean Bias% (*) each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% (*) each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low 0.75	0.78	4.00		104.00			
		0.79	5.33	4.00	105.33	1.28		
		0.77	2.67		102.67			
	SS-Medium 3.00	3.01	0.33		100.33			
		3.21	7.00	3.22	107.00	3.31	4.20	2.02
		3.07	2.33		102.33			
	SS-High 80.00	83.84	4.80		104.80			
		84.95	6.19	5.38	106.19	0.68		
		84.11	5.14		105.14			

Mean ACC% 85.0%-115.0%	SD ACC%	Mean ACC% each day 85.0%-115.0%	SD ACC% each day
114.22	10.09		
108.56	8.33	108.22	8.55
101.87	2.55		
Mean ACC% 85.0%-115.0%	**SD ACC%**	**Mean ACC% each day 85.0%-115.0%**	**SD ACC% each day**
100.00	5.81		
99.78	2.99	99.35	3.50
98.28	1.88		
Mean ACC% 85.0%-115.0%	**SD ACC%**	**Mean ACC% each day 85.0%-115.0%**	**SD ACC% each day**
104.00	1.33		
103.22	3.42	104.20	2.10
105.38	0.72		

2-chloroadenine in plasma dog

Run 07	True Conc. ng/ml	Estimated Conc. ng/ml	Bias%	Mean Bias% each level ≤20.0	ACC%	CV% ACC% ≤15.0%	Mean Bias% each day ≤15.0	CV% ACC% each day ≤15.0
	SS-Low	0.72	-4.0		96.0			
	0.75	0.80	6.7	**0.0**	106.7	**5.8**		
		0.73	-2.7		97.3			
	SS-Medium	2.74	-8.7		91.3			
	3.00	2.98	-0.7	**4.7**	99.3	**4.2**	**2.4**	**4.2**
		2.86	-4.7		95.3			
	SS-High	78.00	-2.5		97.5			
	80.00	77.57	-3.0	**2.6**	97.0	**0.3**		
		78.05	-2.4		97.6			
Run 08	**True Conc. ng/ml**	**Estimated Conc. ng/ml**	**Bias%**	**Mean Bias% each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low	0.71	-5.3		94.7			
	0.75	0.67	-10.7	**7.1**	89.3	**3.4**		
		0.71	-5.3		94.7			
	SS-Medium	2.92	-2.7		97.3			
	3.00	3.05	1.7	**0.1**	101.7	**2.3**	**2.0**	**4.6**
		3.02	0.7		100.7			
	SS-High	81.16	1.5		101.5			
	80.00	82.60	3.2		103.3	**2.2**		
		79.13	-1.1	**1.2**	98.9			
Run 09	**True Conc. ng/ml**	**Estimated Conc. ng/ml**	**Bias%**	**Mean Bias% each level ≤20.0**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0**	**CV% ACC% each day ≤15.0**
	SS-Low	0.81	8.0		108.0			
	0.75	0.76	1.3	**7.1**	101.3	**5.1**		
		0.84	12.0		112.0			
	SS-Medium	2.94	-2.0		98.0			
	3.00	3.36	12.0	**5.3**	112.0	**6.7**	**4.2**	**5.2**
		3.18	6.0		106.0			
	SS-High	79.86	-0.2		99.8			
	80.00	80.95	1.2	**0.3**	101.2	**0.8**		
		79.96	-0.1		100.0			

Mean ACC% 85.0%-115.0%	SD ACC%	Mean ACC% each day 85.0%-115.0%	SD ACC% each day
100.0	5.84		
95.3	4.00	97.6	4.09
97.4	0.32		

Mean ACC% 85.0%-115.0%	SD ACC%	Mean ACC% each day 85.0%-115.0%	SD ACC% each day
92.9	3.12		
99.9	2.31	98.0	4.47
101.2	2.21		

Mean ACC% 85.0%-115.0%	SD ACC%	Mean ACC% each day 85.0%-115.0%	SD ACC% each day
107.1	5.41		
105.3	7.02	104.2	5.39
100.3	0.76		

For both intra- and inter- run precision and accuracy, all QC samples, including those failed with no assignable cause, should be used for calculation, those that failed for an assignable cause (eg. Rejected chromatography or sample extraction problem) should be excluded from the calculation.

Table 25: Inter run Accuracy and Precision

Matrix urine

Overall Mean Bias% (*) ≤ 10.0	Overall CV% ACC% ≤15.0	Overall Mean ACC%	SD ACC%
6.4	5.4	93.7	5.07

Overall Mean Bias% (*) ≤ 10.0	Overall CV% ACC% ≤15.0	Overall Mean ACC%	SD ACC%
4.35	6.18	103.92	6.42

Matrix plasma dog

Overall Mean Bias% (*) ≤ 10.0	Overall CV% ACC% ≤15.0	Overall Mean ACC%	SD ACC%
4.54	6.18	102.49	6.33

Overall Mean Bias% (*) ≤ 10.0	Overall CV% ACC% ≤15.0	Overall Mean ACC%	SD ACC%
3.17	5.46	99.93	5.46

10.2.3.3. Sensitivity (LLOQ)

The target of the lower limit of quantification will be 0.1ng/ml for Cladribine and 2-chloroadenine. Five samples spiked at this concentration were analyzed per batch together with the accuracy and precision levels.

The analyte response has to be at least 5 times the response observed in blank samples.

The mean intra-batch as well as the overall accuracy is within 80-120% at the LLOQ QC level.

The intra-batch and overall precision, expressed as CV%, do not exceed 20% at the LLOQ QC level.

Table 26

Run 04	True Conc. ng/ml	Peak LLOQ Area	Peak Matrix Area	% Area Ratio ≤20.0%	Estimated Conc (ng/ml)	Bias%	Mean Bias% (*) ≤20.0%	CV% ACC% ≤15.0%	ACC%	Mean ACC% 80.0%-120.0%	SD ACC%
	0,25	1128,0	0,0	0,0	0,24	-4,0			96,0		
		1130,9	0,0	0,0	0,24	-4,0			96,0		
		1086,4	0,0	0,0	0,22	-12,0	**6,0**	**8,4**	88,0	**94,0**	**7,90**
		1023,2	0,0	0,0	0,22	-12,0			88,0		
		1041,0	0,0	0,0	0,22	-12,0			88,0		
		1264,9	0,0	0,0	0,27	8,0			108,0		

N.R. Clean-up error

10.2.3.4. Selectivity

The selectivity of the method towards endogenous compounds was established by analyzing blank plasma or urine samples. Selectivity of the method towards possible impurities or degradation products of the IS and of the analyte was so established by analyzing blank samples spiked with the molecules of interest, the good results obtained are shown in Table 27

Table 27: Test selectivity

Run 04	Replicate	Peak Matrix Area	Peak LLOQ Area	Average peak areas LLOQ samples	SELECTIVITY% ≤20.0%
	1	0,0	1128,0		0,0
	2	0,0	1130,9		0,0
	3	0,0	1086,4	1112,41	0,0
	4	0,0	1023,2		0,0
	5	0,0	1041,0		0,0
	6	0,0	1264,9		0,0

10.2.3.5. Carryover

Carryover is calculated considering the area of the chromatographic peaks at AN, MET and IS retention time of the DBK sample placed after the ULOQ.

The area of the chromatographic peaks at the AN and MET retention time in the sample DBK should be less than or equal to 50% of the area of the chromatographic peaks at AN and MET retention time of the LLOQ.

The area of the chromatographic peak at the IS retention time in the sample DBK should be less than or equal to 20% of the area of the chromatographic peaks at IS retention time of the BK sample.

If in a run the concentration of an unknown sample exceeds the ULOQ, the influence on the carryover of such a concentration will be evaluated.

Peak areas of peaks eluted at the retention time of Cladribine and 2-chloroadenine following an ULOQ sample of Cladribine and 2-chloroadenine, resonse. will be expressed as percentage of the response at the LLOQ (obtained during linearity assessment).

In Table 28 and Table 29 are reported carryover results. The response for the analyte in the solvent samples following the ULOQ sample is less than 50% of the mean response at the LLOQ. Also the parameter for IS carryover are respected.

If carryover is observed exceeding the afore-mentioned levels, one or more blank samples have to be placed directly following a high concentration sample.

Table 28: Carryover analyte

Run 04	Average Area AN in LLOQ	DBK after ULOQ	Criteria: ≤50.0%
	1112,4	0,0	**0,0**

Table 29: Carryover IS

Run 04	Average Area IS in Calibration Curve	DBK after ULOQ	Criteria: ≤20.0%		
	53779,5	3382,1	**6,3**		52622,3
					52318,8
					53770,7
					53155,9
					53967,6
					54943,5
					56020,1
					51858,8
					55358,2
				Mean	**53779,5**

10.2.3.6. Stability

Long term stability

After the validation steps I determined the long-term stability of the analytes in the matrix. At the beginning of the validation a sufficient number of QC samples at the required long-term storage temperature were stored.

I analyzed 3 aliquots at low and high concentrations with fresh standard curves and I compared these against intended (nominal) concentrations. I will show in the tables the results of the 3, 6, 9 months long-term stability.

Table 30

Cladribine in urine				2-chloroadenine in urine			
Run 06	**True Conc. (ng/ml)**	**Estimated Conc. t = 0 h (ng/ml)**	**Mean**	**Run 06**	**True Conc. (ng/ml)**	**Estimated Conc. t = 0 h (ng/ml)**	**Mean**
	SS-Low	0.69			**SS-Low**	0.70	
	0.75	0.76	**0.74**		**0.75**	0.72	**0.73**
		0.76				0.78	
	SS-High	84.16			**SS-High**	78.27	
	80.00	82.20	**83.22**		**80.00**	77.35	**77.52**
		83.29				76.95	
Run 06	**Estimated Conc. 3 months -20℃ (ng/ml)**	**Mean**	**STAB % ≥ 85.0 - ≤ 115.0**	**Run 06**	**Estimated Conc. 3 months -20 ℃ (ng/ml)**	**Mean**	***STAB % ≥ 85.0 - ≤ 115.0***
	0.85				0.68		
	0.77	**0.79**	**106.76**		0.69	0.71	**97.26**
	0.75				0.77		
	84.83				79.34		
	82.72	**84.05**	**101.00**		78.43	79.69	**102.80**
	84.59				81.29		

Cladribine in plasma

Run 11	True Conc. (ng/ml)	Estimated Conc. t = 0 h (ng/ml)	Mean
	SS-Low	0.84	
	0.75	0.78	**0.77**
		0.70	
	SS-High	75.77	
	80.00	76.13	**75.39**
		74.28	
Run 11	**Estimated Conc. 6 months -20 °C (ng/ml)**	Mean	**STAB % ≥ 85.0 - ≤ 115.0**
	0.71		
	0.74	**0.76**	**98.70**
	0.82		
	76.63		
	88.78	**82.60**	**109.56**
	82.40		
Run 12	**True Conc. (ng/ml)**	**Estimated Conc. t = 0 h (ng/ml)**	**Mean**
	SS-Low	0.72	
	0.75	0.63	**0.67**
		0.67	
	SS-High	72.20	
	80.00	71.99	**71.85**
		71.35	
Run 12	**Estimated Conc. 9 months -20 °C (ng/ml)**	**Mean**	**STAB % ≥ 85.0 - ≤ 115.0**
	0.67		
	0.69	**0.68**	**101.49**
	0.68		
	72.84		
	71.09	**71.43**	**99.42**
	70.35		

2-chloroadenine in plasma

Run 11	True Conc. (ng/mL)	Estimated Conc. t = 0 h (ng/mL)	Mean
	SS-Low	0.80	
	0.75	0.89	**0.84**
		0.84	
	SS-High	87.91	
	80.00	86.58	**88.31**
		90.45	
Run 11	**Estimated Conc. 6 months -20 °C (ng/mL)**	**Mean**	**STAB % ≥ 85.0 - ≤ 115.0**
	0.82		
	0.69	**0.75**	**89.29**
	0.74		
	74.01		
	74.74	**75.58**	**85.58**
	77.99		
Run 12	**True Conc. (ng/ml)**	**Estimated Conc. t = 0 h (ng/ml)**	**Mean**
	SS-Low	0.84	
	0.75	0.84	**0.82**
		0.79	
	SS-High	83.33	
	80.00	84.71	**83.28**
		81.81	
Run 12	**Estimated Conc. 9 months -20 °C (ng/ml)**	**Mean**	**STAB % ≥ 85.0 - ≤ 115.0**
	0.79		
	0.76	**0.79**	**96.34**
	0.83		
	82.06		
	84.29	**83.58**	**100.36**
	84.40		

10.2.4. Conclusions

The results obtained during the validation of the assay fulfilled all requirements and recommendations regarding linearity, accuracy, and precision generally accepted for bioanalytical studies.

The fully automated LLE methodology avoids several disjointed steps involved in manual or semiautomated LLE method, leading to significantly reduced sample preparation time (Figure 40), increased sample throughput, and clean sample extracts for improved detection.

Moreover the use of Ultra Performance Liquid Chromatography (UPLC), a chromatographic system designed in a special way to withstand high system back-pressures, gives new possibilities in liquid chromatography, especially concerning decrease of time and solvent consumption.

UPLC advantages are clearly observed. The separation mechanisms is still the same, chromatographic principles are maintained while speed, sensitivity and resolution is improved. This all supports easier method transfer from HPLC to UPLC and its

revalidation. The main advantage was particularly a significant reduction of analysis time, which meant also reduction in solvent consumption.

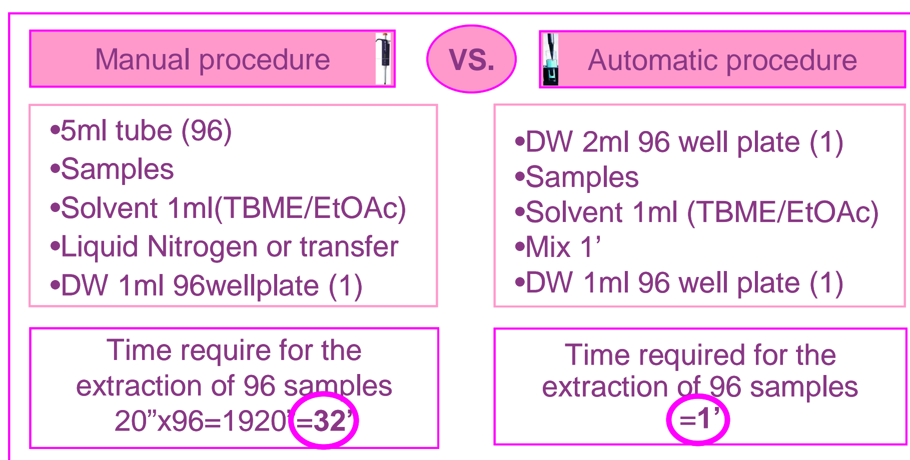

Figure 40: time saved in vortexing 96 samples using robotic workstation

The automated method produced considerable improvements over the manual method with respect to the recovery and repeatability values obtained with the different clean up techniques. Subsequent testing of the method with real samples demonstrated its applicability.

General strategies for automating liquid-liquid extractions have been achieved using the 96-well format. The resulting assays drastically reduce sample preparation time (at least 3-fold faster when compared to manual extraction), decrease material and labor costs and increase throughput in clinical studies.

- Faster - At least 3 - fold faster when compared to manual extraction
- Analyst time used more effectively
- Increased throughput of clinical samples
- Traditionally liquid-liquid extractions associated with being cumbersome, automation reduces many repetitive activities
- Decreased material and labor costs

11. Solid Phase Extraction (SPE)

11.1. Introduction

Solid Phase Extraction (SPE) is versatile and selective methods of sample preparation in which analytes are bound onto a solid support, interferences are washed off and analytes are selectively eluted for further workup and analysis. The many different choices for sorbent chemistry that make SPE a very powerful method for sample preparation also create many different ways in which sorbents can be used[51].

11.2. Fundamental Principles

SPE is a specific type of sample preparation in which an analyte, contained in a liquid phase, comes in contact with a solid phase (solid particles usually in column or disk, in this case in the wells of a 96 well plate) and is selectively absorbed in into the surface of that solid phase. All other materials not absorbed by chemical attraction or affinity remain in the liquid phase and go to waste.

Generally a wash solution is then passed through the sorbent bed to remove any adsorbed contaminants from the sample matrix, yet retain the analyte of interest on the solid phase. Finally, an eluting solvent (usually an organic solvent such as methanol or acetonitrile that may be modified with acid or base) is added to the sorbent bed. This solvent disrupts the attraction between analyte and solid phase causing desorption, or elution, from the sorbent. Liquid processing through the sorbent bed can be accomplished via vacuum or positive displacement[52].

In Figure 41are represented the basic steps for solid-phase extraction:

- (a) conditioning the sorbent bed;

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51 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. (2003), cap 11, page 361.

52 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. (2003), cap 2, pages 49, 50.

- (b) loading analytes;
- (c) washing away interferences;
- (d) selective elution for further workup and analysis.

The SPE product format shown is for a disk in a cartridge or column although the procedure is generally similar regardless of format[53].

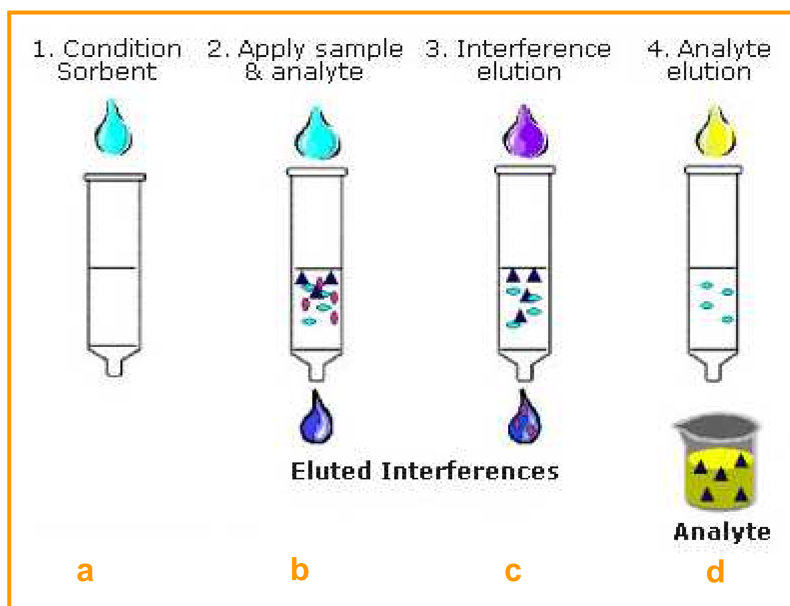

Figure 41: the basic steps for solid-phase extraction.

High-throughput solid phase extraction utilizes 96 well microplate formats.

Although the samples can be processes manually liquid handling workstation is preferred; the use of the automatic workstation results in very high throughput. Many sorbent chemistries and formats are available in the plates to meet most needs[54].

11.3. Advantages

This technique has numerous advantages:

53 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. (2003), cap 11, page 362.

54 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. (2003), cap 2, pages 49, 50.

- It provides very selective extracts reducing the potential for ionization suppression from matrix materials.
- A wide variety of sample matrices can be accepted.
- It permits high recoveries with good reproducibilities.
- Analytes can be concentrated.
- High recoveries with good reproducibilities.
- Low solvent volumes.
- No emulsion formation as with LLE technique.

The technology has been improved in recent years with the introduction of more selective but also more generic solid sorbent chemistries, improved disk based SPE devices, smaller bed mass sorbent loading, improved plate formats for using smaller volumes, and faster and more efficient automation hardware[55].

11.4. Disadvantages

Many of the disadvantages for SPE are directed toward the perceived difficulty to master its usage. The great selectivity and the many choices for manipulating pH and solvent conditions make it difficult to grasp the chemistry of the technique. An important argument is related to slow or difficult flow of sample through the particle bed; precipitated fibrins, thrombins or other particles in the biological are often at fault. In order to avoid this potential problem, samples are usually centrifuged or filtered prior to extraction and this step is especially important with the use of automated method. Sometimes a larger particle size packing (e.g., 100µm instead of 40µm) is used to improve flow characteristics.

Also the development process of the method takes too much time.

The cost of performing a solid phase extraction procedure can be greater than that of other techniques such as PPT or LLE. However any estimate of cost should not include

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55 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. (2003), cap 11, page 364.

only the consumable items needed to perform the extraction but also must factor in the time saving that the approach offers[56].

11.5. Sorbent Chemistries and Attraction Mechanisms

11.5.1. Nature of the Sorbent Particle

Two major types of sorbent particles are used for SPE: silica and polymer.

A wide range of surface areas and pore diameters can be prepared surface area is a function of the average pore diameter: the smaller the pore diameter, the higher the surface area.

Particle size used affects flow characteristics and performance. In the case of biological matrices that are viscous a larger particle size is required to provide better flow characteristics.

11.5.2. Attraction Mechanisms

Four primary modes of analyte attraction to solid sorbent particle are:

- Reversed phase (also called non polar).
- Ion exchange.
- Mix mode (also called mixed phase).
- Normal phase (also called polar).

11.5.3. Packed Particles Beads

The traditional format for SPE has been single disposable columns, also called cartridges, filled with solid sorbent particles (from 25 to 500mg) held between two polyethylene frits.

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56 D.A. Wells, High Throughput Bioanalytical Sample Preparation, Methods and Automation Strategies, Elsevier. (2003), cap 11, page 365.

The pharmaceutical industry responded to a challenge of higher throughput sample preparation by utilizing solid-phase extraction in a 96-well format. This microplate format presents many efficiencies of operation such as ease of labeling, sealing and manipulation. I decided to combine this format with multiple tip Hamilton Workstation in order to dramatically faster pipetting throughput via parallel processing of samples.

11.5.4. High-Throughput and new Applications

This application will describe an automated solid phase extraction method coupled with HPLC analysis to determine a peptides mixture in order to achieve results more accurately with more speed and less efforts.

Automation is an effective tool in achieving all three of these goals.

When the sample preparation step became rate limiting automation could be seen as the more efficient and faster way to work with greater number of samples cleaned up in a 96-well format by the use of a Robotic Workstation and the ability of the operator.

The evaporation and reconstitution steps not only take time and effort, but can also lead to loss of valuable sample. Therefore, the ability to elute in very small volumes of solvent is desirable to minimize the amount of time required.

Currently, there are two technologies Disc and. μElution.

In the μElution design, a much smaller sorbent mass (e.g., 2 mg) is packed into an internally tapered well with an aspect ratio of 1.15. The μElution technology functions more like a chromatography column and enhances the capture of target analytes. It also helps to prevent breakthrough during the load and wash steps. The hold-up volume for the μElution technology was determined to be as little as 15μL.

A small hold-up volume is important for achieving a low elution volume. Larger hold-up volumes result in larger elution volumes.

11.6. Experimental

11.6.1. Objective

The aim of this project was the automation of the sample preparation in the study for the evaluation of the stability assessment of a mixture of four natural peptides in human blood (derived from Myelin Basic Protein) using the UPLC-MS/MS technique.

The study was conducted according to MSR-QMS.

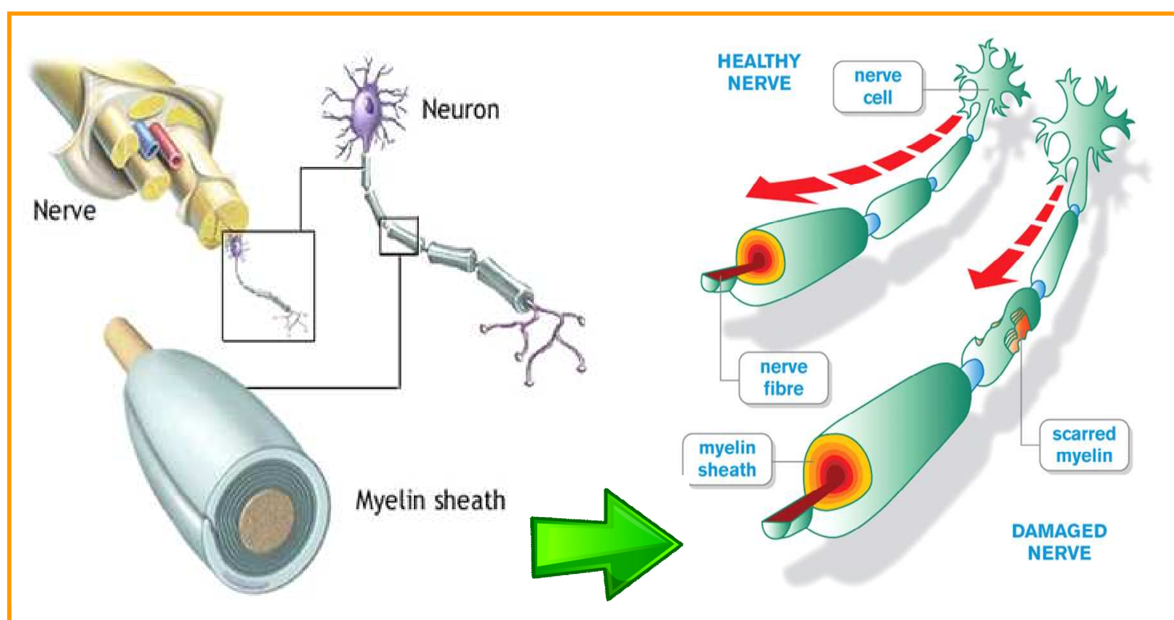

Figure 42: In Multiple Sclerosis the myelin sheath which is a single cell whose membrane wraps around the axon, is destroyed with inflammatory and scarring.

This mixture is designed to induce immunological tolerance of the body's T-cells to key auto-antigens involved in the pathogenesis of Multiple Sclerosis (Figure 42).

Oligodendrocytes form a protective sheath, known as myelin that insulates the fibrous cables, or axons, radiating from nerve cells.

In multiple sclerosis, the immune system's T cells and B cells attack oligodendrocytes, ultimately damaging the myelin sheath to the point that the electrical signals transmitted by the axons beneath it are disrupted.

11.6.2. Instrumentation

11.6.2.1. Reagents and chemicals

Analyte is a mixture of four linear peptides: Myelin Basic

The peptides were provided by Bachem AG – Bubendorf, Switzerland. Human blood was collected from healthy volunteers (AVIS Torino and Ivrea, Italy). Acetonitrile and Methanol were HPLC-grade and purchased from Merck kGaA (Darmstadt, Germany). Protease inhibitor cocktail tablets, Complete Mini (Roche Diagnostics, Mannheim, Germany) without ethylenediaminetetraacetic acid (EDTA), were used to prevent peptide degradation in biological matrixes. Formic acid (FA) and Phosphoric Acid were obtained from Sigma-Aldrich (St.Louis, MO, USA). Ultrapure water was produced by a Milli-Q Plus, Millipore system (Billerica, MA, USA).

11.6.2.2. Equipment

MICROLAB STARlet Liquid Handling Workstation Hamilton Robotics.

Oasis HLB: Hydrophilic-Lipophilic Balance Sorbent reversed-phase sorbent for all compounds (e.g. parent drug and its polar metabolites). Water-wettable sorbent, no impact of sorbent drying.

The SPE Strata-X (Reversed Phase polymeric sorbent) µElution 96-well plate were purchased from Phenomenex (Torrance, California, USA).

The UPLC/MS system used for the assay comprised an Acquity UPLC system (Waters Corporation; Milford, MA, USA) coupled with a triple quadrupole mass spectrometer equipped with a turbo ion spray source (API 4000; Applied Biosystems, Toronto, Canada) and with an Acquity BEH C18 column *(2.1x100 mm, dp=1.7 mm;* Waters*)*.

Data were processed using Analyst software (version 1.4.2) from Applied Biosystems.

11.6.3. MS/MS detection

Peptides were infused into the triple quadrupole mass spectrometer equipped with an ESI source operated in the positive ion mode for identification. Mass spectra were obtained infusing the mixture of peptides at a concentration of 10µg/ml in water/ACN (50:50, v/v) with 0.1% FA using a syringe pump operated at a flow rate of 5µl/min and

setting the UPLC flow rate at 200µl/min. For each peptide, the most abundant ion produced in positive mode was $[M+3H]^{3+}$.

11.6.4. Automated SPE

The most logical method was created on Vector. It allows changes and modifications during the set up of the method in order to evaluate and establish the best conditions.

It is possible the complete control of rates for aspiration and dispensing of the solutions/samples onto the SPE plates including the time associated with the air push and the pressure equilibrium.

The method set-up was thoroughly investigated in order to obtain the best SPE conditions. During this phase, different SPE plates and different solvent mixtures were tested to obtain the best results in term of recovery, robustness and reproducibility of the extraction method.

On the whole, analysis sampling and sample preparation are the most important steps of analytical procedures. Due to its flexibility, SPE is a multivariate process whose optimization should be approached from a multivariate perspective. For the optimization of this SPE procedure we investigated the selection of the most appropriate sorbent, the design of the SPE bed, the determination of the volume of the sample to load and the determination of the nature and volumes of solvents first used to wash the column and then to elute the analyte. Microelution SPE was used in order to avoid solvent and sample spending.

Strata™-X plates have been engineered to allow higher loading volumes than Oasis® HLB plates, which helps to avoid limits on loading volumes that can lead to problems with lower limit of quantification (LLOQ) and lower limit of detection (LLOD) of low concentration analytes.

The choice of sorbent is the key point in SPE because it can control parameters such as selectivity, affinity and capacity.

This choice depends strongly on the analytes of interest and the interactions of the chosen sorbent through the functional groups of the analytes.

In designing the automation strategy for Solid Phase Extraction there are many parameters that the operator has to consider.

In first has to be found the most convenient SPE condition, than it is important to planning of the most appropriate deck layout which has to be different from the other before described because of the needs of this technique.

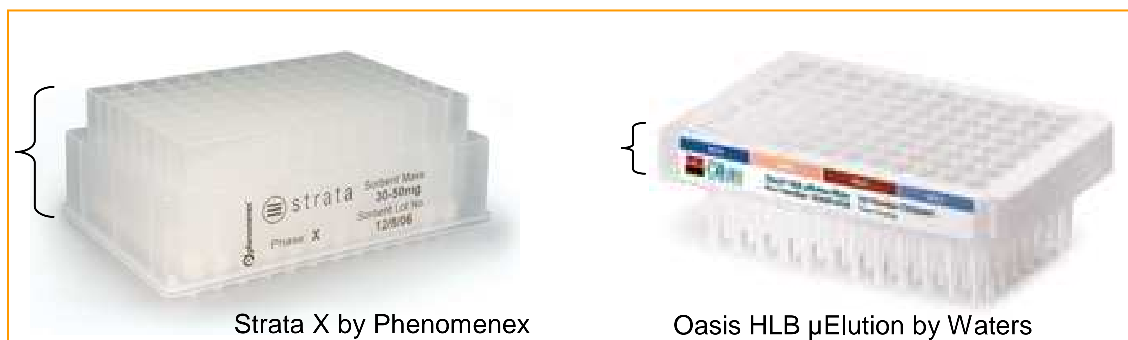

Figure 43: Different types of 96-SPE-well plates tested.

With the Oasis HLB plate, are possible robust SPE methods without having to endure irreproducible results and low recoveries caused by undesirable silanol activity, sorbent drying, pH limitations, and breakthrough of polar compounds/metabolites. The technology incorporate the first hydrophilic-lipophilic-balanced water-wettable copolymer optimal for any sample cleanup and are unique in their purity, reproducibility, stability, and retention characteristics.

At this point the microplate can be placed onto the workstation deck for all subsequent pipetting tasks.

Once the sample plate has been prepared and place onto the deck the first pipetting action by the instrument is to aspirate the solvents for conditioning the plate. These solvents are sequentially aspirated from a reagent reservoir and dispensed into all the wells of the plate. If needed a vacuum manifold step is performed between.

It is important to evaluate the correct measure of the vacuum to apply. It has to be not too strong but not too low.

Than a volume of sample matrix is next aspirated from the sample plate, a vacuum manifold step follows.

A clean collection plate is placed inside the manifold.

In first was used an Oasis HLB μElution plate, 1/pk [186001828BA]. Plates and theie characteristics are shown in Figure 43, Figure 44 and .Figure 45.

This plate can be correctly placed in a good position for dispensation and vacuum without moving any plate.

In fact this plate has a low high and the axes of the workstation were inside the settled parameters.

But during the clean up was noticed that there were some percolation problems.

For this reason we decided to use Strata X plate but the deck layout positions had to be changed for the plate dimension. It was used a different carrier to avoid the collision between the tips and the plate.

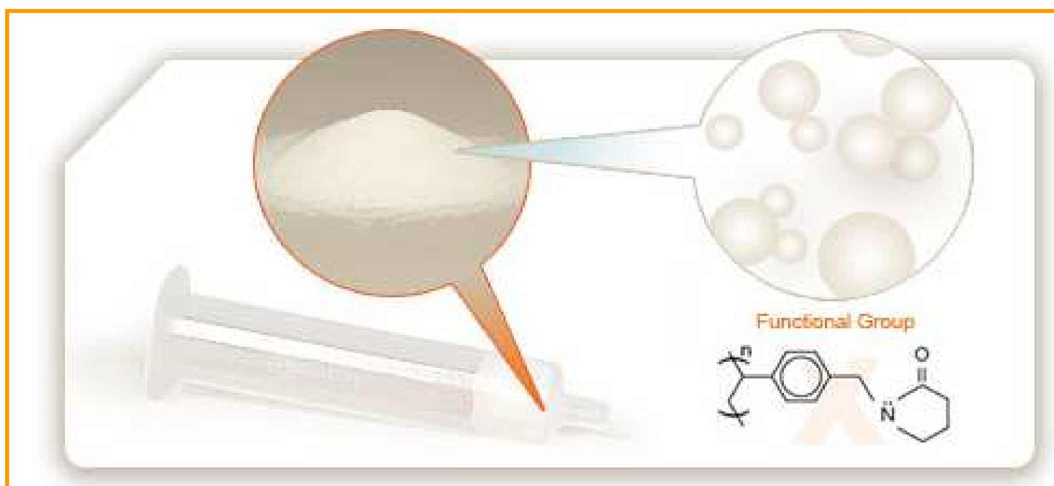

Figure 44

Strata X: Material Characteristics

- Particle Size (µm) 33
- Pore Size (Å) 85
- Surface Area (m2/g) 800
- pH Stability 1-14

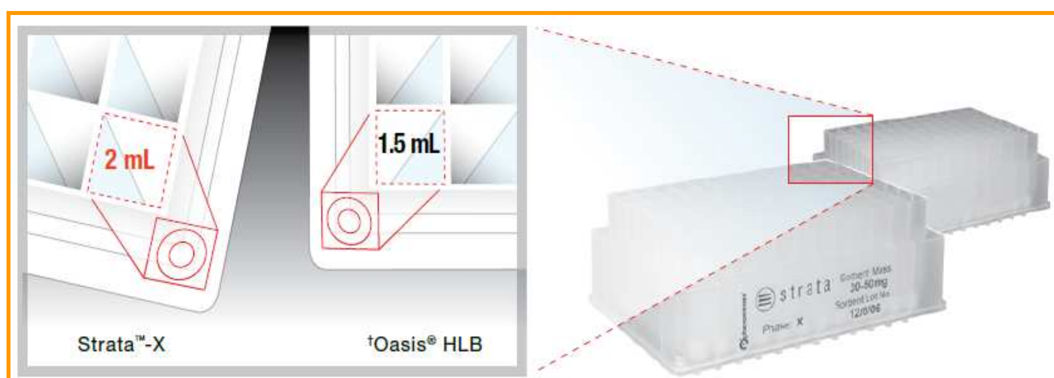

Figure 45

The final SPE conditions were the following:

- sample pre-treatment: 100µl of sample + 400µl of 0.5% Phosphoric Acid solution,
- conditioning with 200µl of methanol;
- equilibrating with 200µl of water;
- pre-treated sample loading;
- washing with 200µl of 10% methanol solution, drying for one minute and eluting with 100µl methanol/acetonitrile (1:1, v/v).

11.6.5. Peptide Stability Investigation

The samples used to investigate peptide stability in human blood were prepared by adding the peptides working solution to blood in order to achieve final concentrations of 50.0 and 100.0ng/ml. The spiked blood samples were stored at 37°C (in order to simulate the human physiological conditions) and at different time points (5-15-30-60-120 min) samples were centrifuged at 2500xg for 10min at 4°C. Subsequently samples were extracted via SPE procedure and analyzed by LC-MS/MS technique.

11.6.6. Results

Results obtained using the better SPE conditions for all peptides: recovery and choice of the better clean-up are described below in Table 31 and in Figure 46.

In table 8 is shown the extraction efficiency, a ratio of the detector response from the extracted sample to the detector response from an unextracted containing the same amount of analyte. This represents 100% recovery during extraction. In this case we have extraction efficiency over 90% for three of the compounds; only for the compound C it is below 90%. However extraction efficiency need not always to be very high but it should be consistent, precise and reproducible.

Table 31

Compounds	Average SPE Recovery	Inter-day% RSD
Compound A	92.20%	0.01
Compound B	98.90%	1.45
Compound C	84.50%	1.45
Compound D	91.60%	2.71

The clean up step has some relevance in determining the repeatability in routine analyses, where a quite large number of different samples are run within the same session. This point could be even more critical for those multi-residue approaches, where the elution of several compounds of interest could last several minutes, thus enhancing the risk of progressive dirtiness of the interfaces.

In an SPE procedure, choosing right washing solvent composition is a difficult task. A good washing solvent should be able to eliminate the matrix interferences as much as possible without eluting the analytes. In this part of the work, different washing solvent compositions are studied as shown in Figure 46.

As a preliminary study to choose solvent, different concentrations of methanol as washing solvent are studied with Oasis HLB plates.

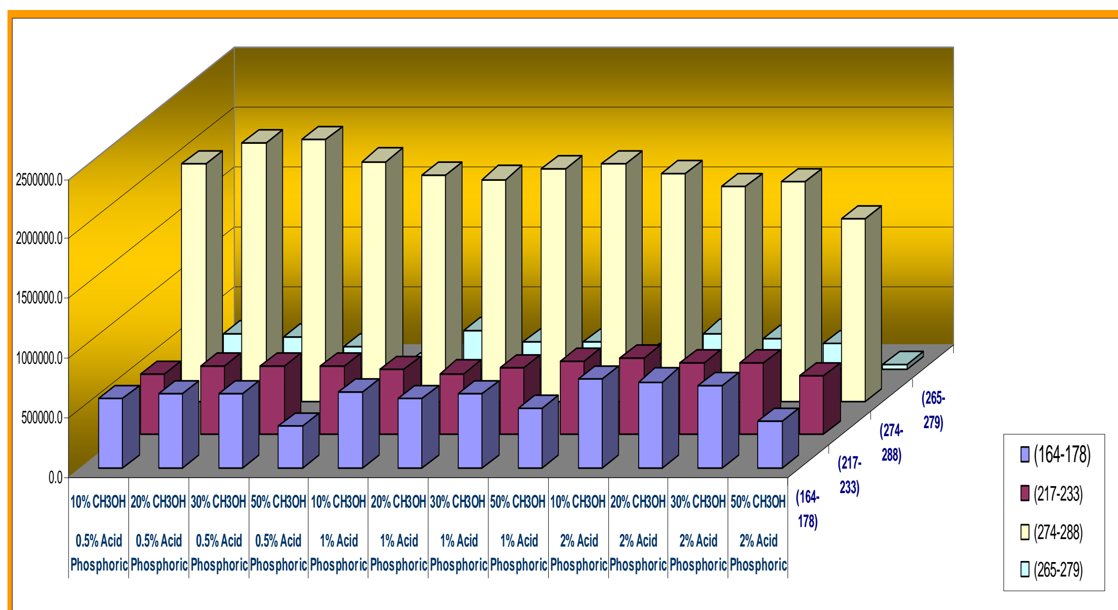

Figure 46: Choice of the better clean-up.

11.6.7. Linearity, Sensitivity and Recovery: comparison between Manual and Automated procedure

The linearity of results for both automated and manual methods demonstrates that drug samples in plasma can be reliably prepared by the Hamilton Robotic Workstation.

The high degree of linearity indicates that the purified samples generated following this automated procedure allow accurate LC-MS/MS analysis across a wide concentration range. The comparison is shown in. Figure 47 and Figure 48.

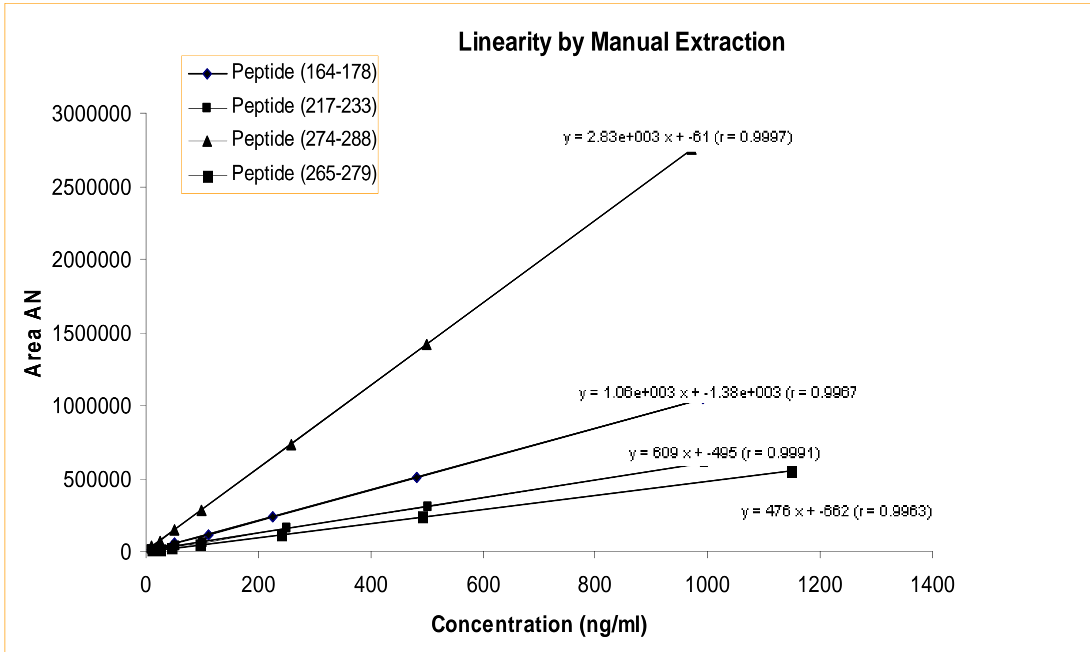

Figure 47

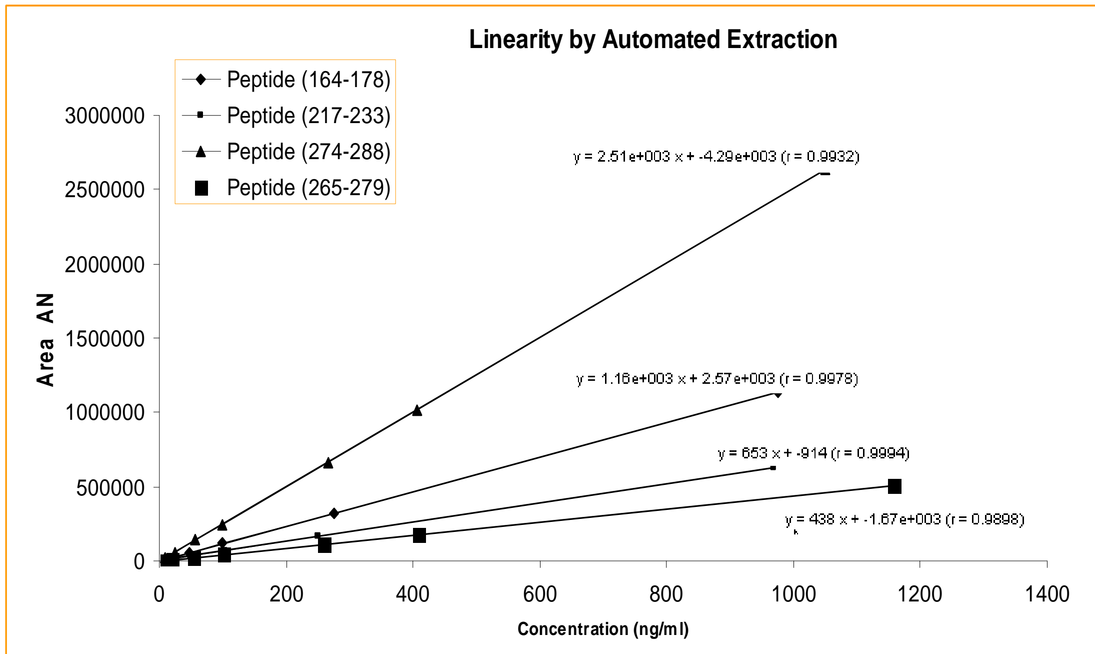

Figure 48

Comparable chromatographic performance to the manual were achieved using the robotic workstation.

A quick automated SPE sample preparation has been developed. This method compared to the manual procedure brings the same good results but also eliminates the need for time consuming sample preparation procedures.

Chromatograms are shown in Figure 49 and. Figure 50.

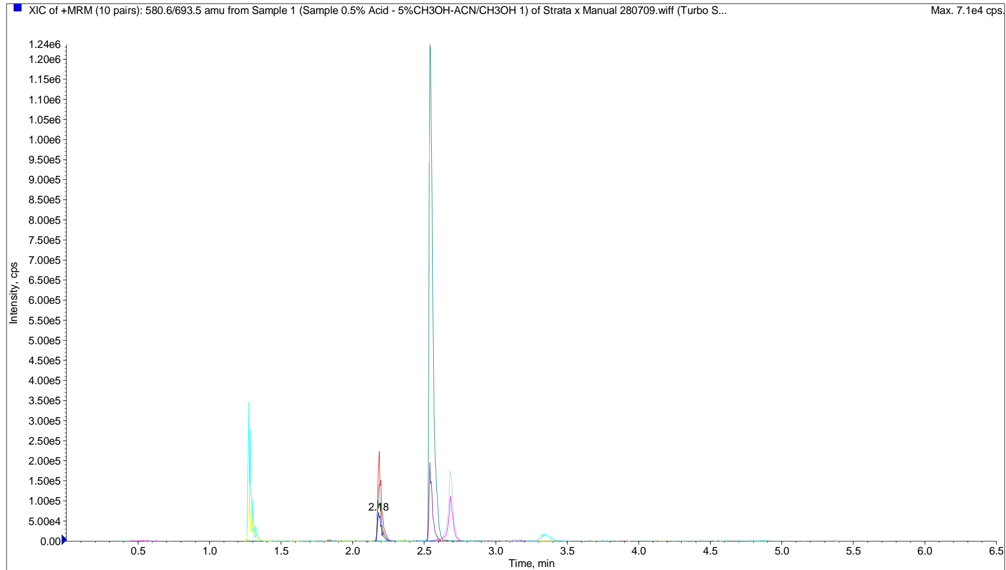

Figure 49: Chromatograms of the four peptides by manual extraction.

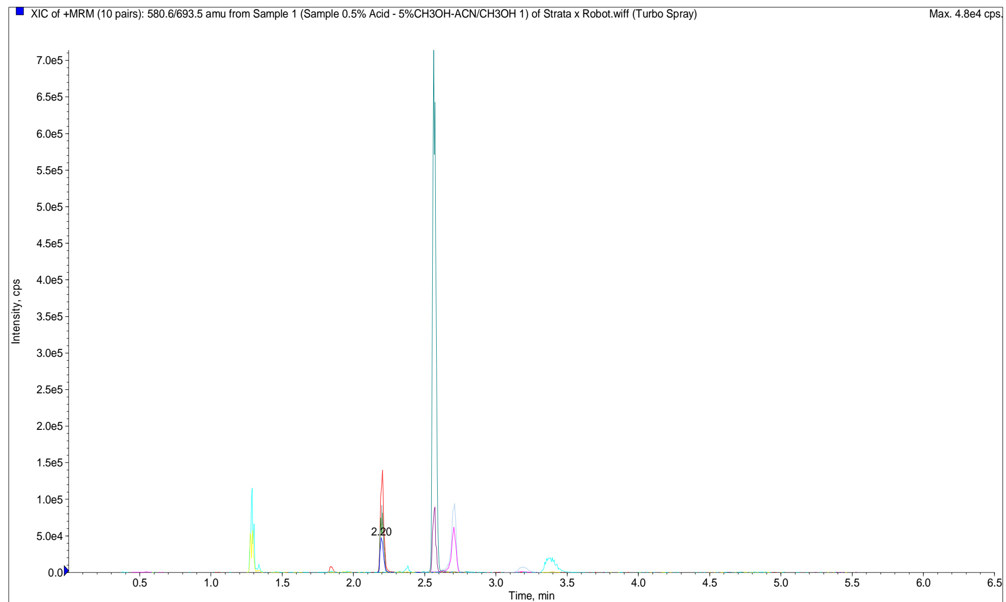

Figure 50: Chromatograms of the four peptides by automated extraction.

Figure 51 shows the recoveries obtained in the manual and automated extraction procedures.

The recoveries are equivalent for one of the peptides of the mixture, and better for two of them. However recoveries are quite always greater or near 90% in the automated way.

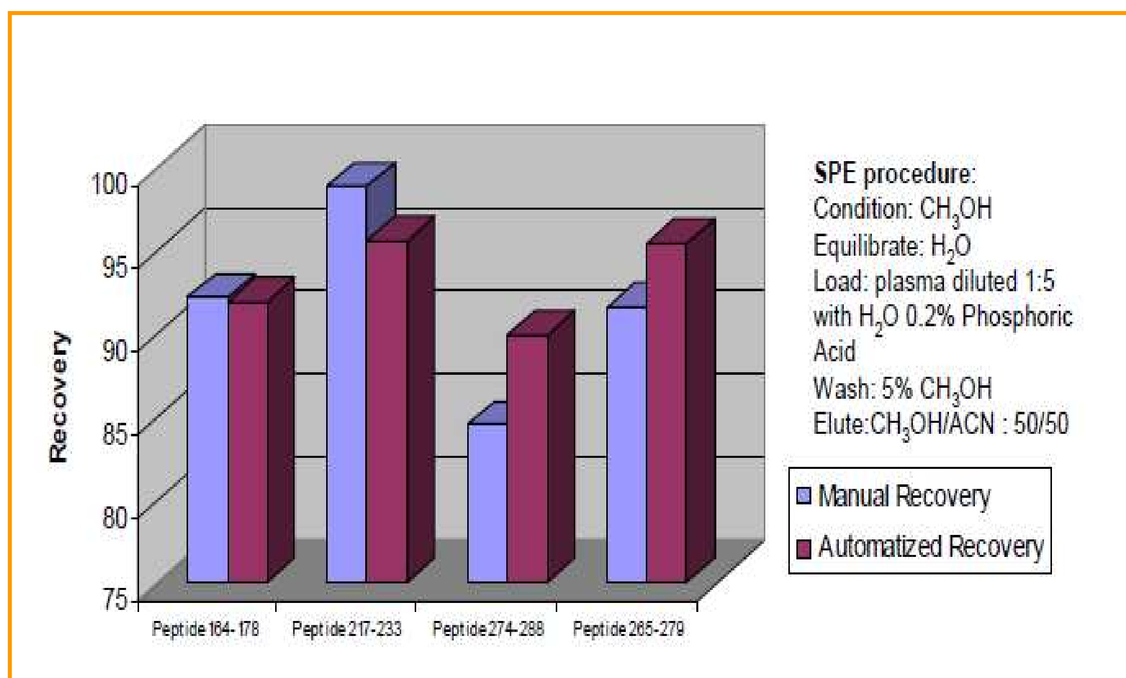

Figure 51: Recovery comparison between Manual and Automated Extraction.

11.6.8. Conclusions

Although the results obtained during stability assessment showed a rapid enzymatic degradation of the four peptides in human blood the use of liquid handling to automate the extraction was successful.

The recoveries obtained in the manual and automated extraction procedures and chromatograms are comparable.

It shows that all liquid handling steps can be performed by the workstation proving to be quick and labor saving so the future SPE-LC-MS-MS procedure could eliminates the human factors of specimen handling, extraction, and derivatization, thereby reducing labor costs and rework resulting from human error or technique issues.

In conclusion, the automated extraction of peptides from blood samples has been shown to be feasible using the Hamilton robotic workstation.

Compared with manual extraction methods reported previously the method has a shorter processing time while maintaining equivalent linearity, sensibility and recovery.

“UPLC-MS/MS strategy to quantify a mixture of four shirt myelin basic peptides in human plasma samples”

Poster Authors:

Rita Mastroianni[2], Paolo Frascarolo[2], Maria Chiara Zorzoli[3], Luca Barbero[3], Simona D’Urso[2], Luigi Colombo[1]

1 Global Bioanalytics, 2 NCEs Laboratory, 3 Automation Laboratory.

12. Dried Blood Spots (DBS)

Plasma has been the mainstay matrix for measurement of systemic concentration of compounds used in the assessment and evaluation of pharmacokinetics (PK) and pharmacodynamics (PD), both efficacy and safety, in drug discovery and development. An alternative, dried blood spots (DBS), has recently gained increasing popularity, with some distinct practical advantages but also technical issues, although the method was employed in pediatrics as early as 1963[57].

12.1. Introduction

DBS sampling is an innovative technique that reduces sample volume requirements and offers significant ethical and economical benefits, Figure 52.

Figure 52: Dried Blood Spot cards

57 Use of Dried Blood Spots in Drug Development: Pharmacokinetic Considerations Malcolm Rowland and Gary T. Emmons, 25 March 2010. The AAPS Journal.

12.2. History

The concept that capillary blood, obtained from pricking the heel or finger and blotted onto filter paper, could be used to screen for metabolic diseases in large populations of neonates was introduced in Scotland by Robert Guthrie in 1963. Neonatal screening for phenylketonuria became nationwide in 1969-70. Since then, Guthrie card samples have been collected routinely from infants in over 20 countries to screen for phenylketonuria and more recently for congenital hypothyroidism, sickle cell disorders and HIV infection. The limitations of sensitivity and specificity when screening such small volumes of blood restricted the use of dried blood spots for many years. However, recent advances such as the production of monoclonal antibodies, expression of synthetic proteins, and the introduction of the polymerase chain reaction have overcome many of these problems[58].

DBS is an FDA listed class II medical device.

A medical device is a product which is used for medical purposes in patients, in diagnosis, therapy or surgery. If applied to the body, the effect of the medical device is primarily physical, in contrast to pharmaceutical drugs, which exert a biochemical effect. Specific regional definitions of medical device vary slightly as detailed below. The medical devices are included in the category: Medical technology.

Medical devices include a wide range of products varying in complexity and application. Examples include tongue depressors, medical thermometers, blood sugar meters, total artificial hearts, fibrin scaffolds, stents and X-ray machines.

12.3. Bioanalytics

12.3.1. Preclinical Study

Although very few applications of DBS in preclinical study have been published on toxicokinetic or pharmacokinetic assessment of drug or drug candidates in small animals, the potential benefits of using this technique is too pronounced to be ignored by

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58 Parker SP, Cubitt WD (September 1999). "The use of the dried blood spot sample in epidemiological studies". J. Clin. Pathol. 52 (9): 633–9.

pharmaceutical industry. The small blood volume (<100µl for multiple spots) used for DBS sampling makes its use possible for serial bleeding from small animals[59].

12.3.1.1. Animal use

DBS technology brings opportunities for animal use reduction and refinement.

In 1986 the European Council of Ministers adopted Directive 86/609/EEC on 'the protection of animals used for experimental and other scientific purposes'. The Directive sought to improve the controls on the use of laboratory animals, set minimum standards for housing and for the training of those handling animals and supervising the experiments.

The Directive also aimed to reduce the numbers of animals used for experiments by requiring that an animal experiment should not be performed when an alternative method exists, and by encouraging the development and validation of alternative methods to replace animal methods.

This legislation is largely a framework, and laws governing animal experiments in the UK, for example, are very much stricter.

A few years ago, it became clear that officials within the European Commission wished to revise the Directive to promote improvements in the welfare of laboratory animals and to further encourage the development of alternative methods. Since 1986 important progress has been made in science and new techniques have become available, such as use of transgenic animals, xenotransplantation, and cloning. According to the Commission, these require specific attention, which the old Directive does not provide.

The process of revising the Directive has been going on for several years, and draft legislation was only available to the European Parliament and the public at the end of 2008. The Parliamentary process can take at least two years, and implementation in the Member States a further two, so national legislation to implement the Directive may not be in place for some time.

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59 Barfi eld et al., 2008; Beaudette and Bateman, 2004.

12.3.2. Clinical Study and Therapeutic Drug Monitoring

DBS-LC-MS/MS has been increasingly employed in clinical study and therapeutic drug monitoring for the analysis of a wide spectrum of drug molecule.

Because DBS samples could be collected by patients themselves or their guardians with minimum training, this opens up the possibilities of collecting clinical pharmacokinetic samples not only from various in-patients, but also from out-patients, especially those from remote areas.

In all cases where serum or plasma, instead of DBS, has been used as the matrix for quantitative analysis of drugs during discovery and development, it would be necessary to determine the relationship between the analyte concentration in the two matrices (plasma/serum vs. DBS). Hematocrit is an important parameter that needs to be considered.

Current analytical methods require relatively large volume of plasma. Typical blood sample volume is 250µl for rat and 200µl for mouse.

Mouse sampling frequently has to be terminal. Profiles frequently are limited by allowable blood sample volume and large number of mice and juvenile rats are added to studies to generate TK data.

Blood is centrifuged to produce plasma and practically it is very hard to centrifuge small volumes of blood to yield plasma.

12.3.3. Fundamental Principles

There are different types of cards that can be used in DBS technology.

S&S 903, Whatman manufactured from 100% pure cotton linters with no wet-strength additives added.

FTA and FTA Elute cards, and recently FTA DMPK-A®, FTA DMPK-B®, FTA DMPK-C® also manufactured by Whatman (now part of GE Healthcare). These are designed for nucleic acid analysis. According to the manufacturer, some of these cards are chemically treated with proprietary reagents that upon contact, lyse cells, denature proteins and prevent the growth of bacteria and other microorganisms.

All the cards have been employed in combination with LC-MS/MS for the quantitative analysis of small drug molecules and their metabolites in preclinical and clinical studies.

Card choice is dictated by a combination of handling and performance criteria. Handling requirements may be influenced by operational or safety factors, while performance depends on many factors such as the analyte chemical structure, extraction solvent, and analysis workflow, which are usually determined empirically. To identify appropriate cards, normally the analyte recovery from all types of cards is investigated using, in part, the "Instruction for Use" protocol provided with the cards.

FTA DMPK-A

- Blood spots dry within 2 h;
- Blood spot area is ~20% smaller than DMPK-B or DMPK-C cards;
- Protein denaturing activity will inactivate endogenous enzymes;
- Cell lysis releases endogenous cellular materials onto card;
- Stabilization of DNA allows resampling of blood spot for pharmacogenomics;
- Impregnated chemicals may interfere with mass spectrometry detection e.g. ion suppression.

FTA DMPK-B

- Blood spots dry within 2 h;
- Protein denaturing activity will inactivate endogenous enzymes;
- Cell lyses releases endogenous cellular materials onto card;
- Stabilization of DNA allows resampling of blood spot for pharmacogenomics;
- Impregnated chemicals may interfere with mass spectrometry detection e.g. ion suppression.

FTA DMPK-C

- Blood spots dry within 2 h;
- No impregnated chemicals to interfere with analysis;
- Proteins are not denatured so cards may be better suited for protein based biomolecules.

12.3.4. Blood Collection

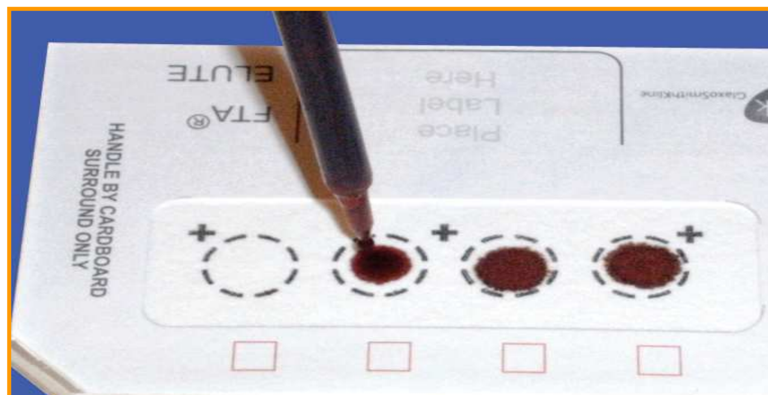

Figure 53

In preclinical studies, small animals (e.g. mouse, rat, etc.) can be tail bled. After the pricks, the blood drop is directly applied onto the sampling paper/card within a pre-marked circle, ideally one drop per spot. Touching the circle area should be avoided, especially before the blood is applied and dries completely. The predefined circle must be homogenously and symmetrically filled and both sides of the card/paper must show the same red color. Samples indicating contamination or hemolysis or with insufficient volume collected are deemed not suitable

The blood sample can also be applied with a calibrated pipette (Figure 53) onto the sampling paper/card, thus avoiding potential variability in blood samples due to hematocrit effect, blood volume influence, possible uneven distribution of blood on the card/paper and other sampling errors. The pipette tip should be a few millimeters above the card/paper. As the blood drop touches the card/paper, it should be swiftly expelled[60].

12.3.4.1. Examples of Invalid Spots and Invalid Collections of DBS

Examples of invalid spots and invalid collections on card are shown.

60 Dried Blood Spot sampling in combination with LC-MS/MS. Biomed. Chromatogr.2010; 24, 50.

In Figure 54 the specimen is invalid because the specimen was not dry before mailing. DBS must dry a minimum of 4 hours (possibly overnight in area with high humidity) before packaging and shipping.

Figure 54: Spot with insufficient quantity of blood.

In Figure 55 is possible to see that the sample is not completely dried before the placement in the storage bag.

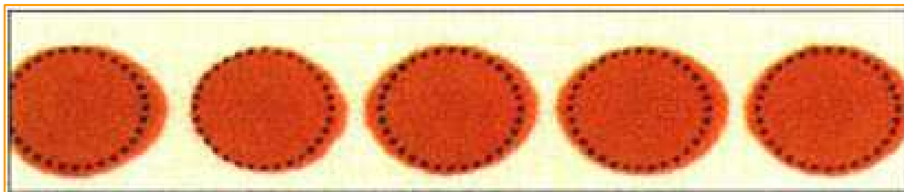

Figure 55: Sample not allowed to air dry before placing in storage bags.

In Figure 56 the specimen is invalid because the specimen appears clotted or layered. The volume of specimen will not be uniform between spots resulting in errors during the testing process.

This may have been caused by:

- touching the same circle on the filter paper to blood drop several times;
- filling circle on both sides of filter paper.

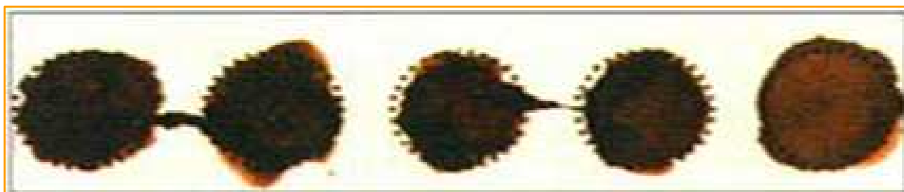

Figure 56: Sample where blood was clotted and did not soak into paper.

In Figure 57 and Figure 58the specimen is invalid because the specimen exhibits emolization and serum rings i.e. serum becomes separate from cells.

This may have been caused by the following:

- Not allowing alcohol to dry at puncture site before making skin puncture.
- Allowing filter paper to come in contact with alcohol, hand motion, etc.
- Squeezing area surrounding puncture site excessively.
- Drying specimen improperly.
- Applying blood to filter paper with a capillary tube.

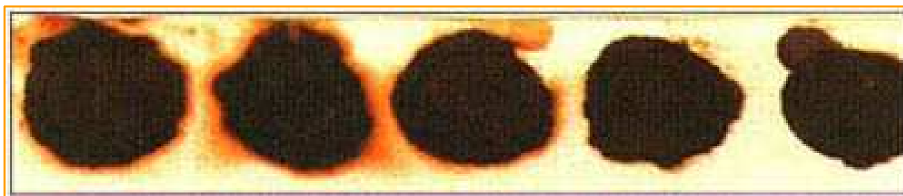

Figure 57: DBS with contamination or hemolyzed blood.

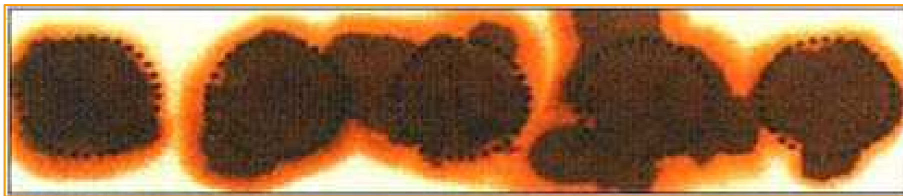

Figure 58: DBS where serum separated from cells.

12.3.5. Drying, Storage and Transportation

It is very important to dry blood spots completely before storage or transportation. In general, a minimum of 2–3 h drying in an open space at room temperature (15–22°C) is recommended. However, the drying time depends on the type of paper/card and the blood volume applied.

The samples should not be heated, stacked or allowed to touch other surfaces, and should be kept away from direct sunlight or daylight if needed.

Thus, after drying, the DBS samples should be protected against humidity and moisture by covering them with a paper overlay and packing them in low gas-permeable zip-closure bags with desiccant packages. Humidity indicator cards should be included in the storage package. DBS samples protected in this manner may be stored at room temperature for many weeks, months or years, depending on the analyte stability. However, samples that contain unstable compounds should be stored at a lower temperature in order to enhance stability. DBS samples that have been packed as described above can be transported through the mail in a high-quality bond envelope[61].

12.3.6. Punching out prior analysis

After drying at room temperature a disc is punched out from the dried blood spot using a specific manual puncher as shown in Figure 59. It is also possible to automate this operation with the use of BSD 600/1000 robotic puncher.

Figure 59

12.3.7. Off -line extraction

For quantitative analysis, one or more DBS disks are punched from DBS card/paper into assay tubes or the appropriate wells of 96-well microtiter plate for extraction. The

61 Dried Blood Spot sampling in combination with LC-MS/MS. Biomed. Chromatogr.2010; 24, 51.

extraction procedure is usually carried out by adding a certain amount of extraction solvent (methanol, acetonitrile or a mixture of water/organic) containing the internal standard(s). The extraction solvent must be strong enough to interrupt the binding of analyte to protein in the matrix or the paper material. The analyte of interest is then extracted with gentle shaking or vortexing. Sonication may be necessary for better extraction efficiency[62]. After centrifugation, the resulting extracts are transferred manually or by an automated liquid handler to new tubes or microtiter plates. The extracts can be directly injected onto the LC-MS/MS system for analysis, or dried for reconstitution using an MS-friendly solvent prior to analysis.

12.3.8. Stability

The DBS technique provides another dimension for the storage of biological samples, even in the absence of refrigeration in some cases. Several applications have demonstrated the advantages of DBS over other means in preserving some unstable analytes from degradation or delaying the degradation process[63]. Room temperature is the intended storage condition for most DBS samples. However, a lower temperature may be necessary for longer stability coverage for some analytes.

12.3.8.1. Approaches for enhanced stability

DBS is not a miraculous process for all unstable compounds. Necessary, approaches should be undertaken to extent the stability coverage of any unstable compound. Three main approaches are suggested:

- Temperature adjustment: lowering the temperature is very common for stabilizing small molecules in DBS. The degradation reaction (reduction, oxidation or hydrolysis) is expected to be 10 times slower when the temperature is decreased from 22 to 0°C.

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62 Damen et al., 2009; Wilhelm et al., 2009; van der Heijden et al., 2009

63 Alfazil and Anderson, 2008; Garcia Boy et al., 2008

- Maintaining dryness: the water present in DBS samples plays a critical role in enzymatic and chemical hydrolysis. Minimizing water content by drying the DBS card completely, changing desiccant bag or applying vacuum to DBS sample containers during storage would be a good combination.

However this effect is apparently compound dependent.

12.3.9. Advantages

Blood is directly spotted onto collection cards it is air dried and stored with desiccated at room temperature.

The process is simplified and the possibility to obtain better quality is higher.

It is possible to avoid:

- Centrifugation
- Sub-aliquoting
- Freezing
- Defrosting

Samples volume passed from 300µl to 10-20µl, in consequence:

- Reduction of the number of animals used.
- Reduced costs
- Shipping
- Storage

12.3.10. Disadvantages

The use of DBS bears some apparent disadvantages. Because the collected blood volume is very small, assay sensitivity is always a challenge for the determination of some analyte at low circulating level.

Unlike samples from aqueous matrix such as whole blood, plasma or serum, for which homogeneity could be easily ensured during collection and prior to analysis, the quality of DBS samples is often a concern for absolute quantification due to possible DBS

card/paper quality issue, possible no-compliance in sample collection, drying, storage and transportation.

Manual punching is very time consuming and is definitely not suited for the current high throughput environment. A punch device could be a source of assay error and/or contamination.

With a good quality control of blood sampling (procedure standardization and adherence to the established procedure), implementation of automation for DBS sample punching and extraction, improved chemistry of pretreated card/paper for enhance stability of unstable compounds, and an higher analysis throughput via DBS-BSD-Hamilton Robotic Workstation-UPLC-MS/MS is expected to play an increasingly important role in the quantitative analysis of drugs and metabolites in blood[64].

BSD (Figure 60) is a fully automated walk away punch instrument designed specifically for high-throughput. It automatically punches without the need for the operator to insert the framed sample card. The instrument has been designed to accommodate internationally accepted and standardized framed cards.

Figure 60: BSD throughput punch instrument

64 Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small moleculesWenkui Li and Francis L. S. Tse

12.3.10.1. Benefits in High-Throughput of automated DBS sampling

- Very small sample volume per subject required (only 10-20 µl). This makes it far easier and faster to extract samples, and also allows multiple samples to be extracted from the same animal.
- DBS is suitable for both preclinical (animal) samples and clinical (human) samples.
- The minimal sample volume required makes it possible to do serial bleeds from one lab animal for a more accurate pharmacokinetic profile.
- No centrifugation or other preparatory handling steps are needed as with traditional plasma samples.
- Faster throughput at both the sample collection point and at the Bioanalytical lab.
- Cards are stored and handled at room temperature in ambient atmospheric conditions. No refrigeration needed.
- DBS cards containing samples ship using standard carriers. No expensive refrigerated shipment by courier or other special handling required.
- Enthalpy can process DBS samples rapidly and with great precision using robotic systems specially developed for the purpose. Enthalpy has automated the process of generating DBS QC samples for calibration, which makes sample throughput faster while maintaining required levels of accuracy.
- Analytical results obtained from DBS samples can be more meaningful than those from conventional liquid plasma samples because DBS uses whole blood.

12.3.11. Experimental

A new small chemical entity (NCE) was used to evaluate the possible application of the technique.

An NCE is a molecule developed by the innovator company in the early drug discovery stage, which after undergoing clinical trials could translate into a drug that could be a cure for some disease.

It was evaluated the possibility for DBS technique to bring advantages in Merck Serono PK processes.

It was evaluated:

- the sample processing procedure in order to find the most suitable type card, the appropriate solvent and mixing extraction time.
- the accuracy and precision, using QC samples at three different concentrations, the matrix effect and the recovery.
- the reduction of the punched area diameter maintaining the same MS peak area ratio with the purpose to enable the automation of the extraction process.
- finally it was investigated if the molecule of interest is equally distributed onto the blood spot.

12.3.12. Materials

- Analyte with IS available and analytical validated method;
- Small organic molecule for cancer disease and Internal Standard;
- FTA® DMPK-A Cards, Lot No. FE6847009 (1);
- FTA® DMPK-B Cards, Lot No. FE6847069 (2);
- FTA® DMPK-C Cards, Lot No FE6847509 (3);
- Extraction solvents. CH_3OH - CH_3OH/CH_3CN - $CH_3OH/ACN/H_2O$ - CH_3CN;
- Harris UNI-CORE 6.0-3.0mm W/Mat (5), Harris cutting Mat (8), 903® Dry Rak (9), Plastic Storage Bags;
- Eppendorf tubes (4), 96 Deep Well Plate 1ml (6);
- Microtiter Stirrer model 715+, cod 29970005, Tecnovetro (7);
- Termomixer comfort, Eppendorf 1.5ml (10).

All the instrumentation is shown in Figure 61.

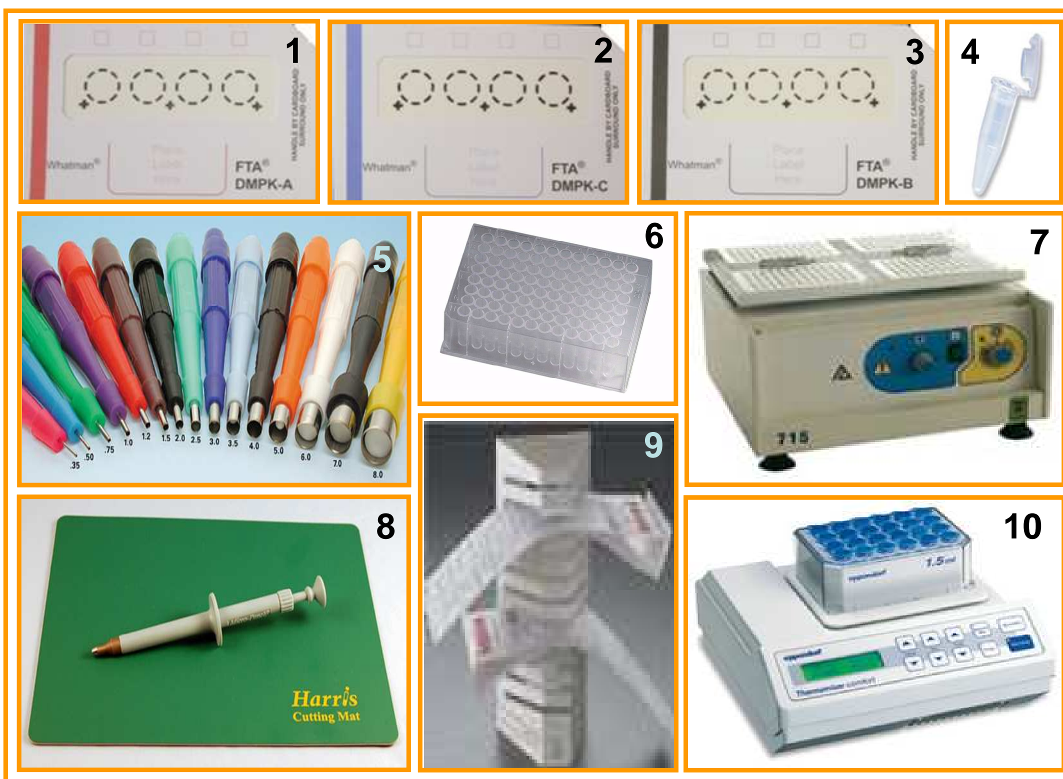


Figure 61

12.3.13. Sample Preparation and Automation

We developed a DBS extraction method on the base of a LC-MS/MS method developed for the determination the molecule as free base in rat, dog, rabbit and monkey plasma using a deuterated internal standard, in the concentration range of 1.0ng/ml to 500.0ng/ml further extended to 250,000.0ng/ml.

In origin plasma samples were extracted using protein precipitation with precipitation reagent in Sirocco filter plates.

12.3.14. Instrumentation, Chromatographic conditions and Mass Spectrometry conditions

MICROLAB STARlet Liquid Handling Workstation Hamilton Robotics.

The chromatography has been achieved using an Agilent 1100 Series pump equipped with a Waters Sunfire column, 50 x 2.1 mm, 5.0µm.

An Applied Biosystems API4000 triple quadrupole mass spectrometer equipped with turbo ion spray source (TIS) operating in positive ion mode has been used.

HPLC/MS system: Autosampler: CTC HTS PAL, HPLC pump: Agilent HP 1100 binary pump Guard column: Frit 2.1 mm for narrow bore column Column Waters Sunfire, *5 μ*m 50 x 2.1 mm Switching valve: Valco 2 position.

Mass spectrometer. SCIEX API 4000 triple quadrupole equipped with a turbo ion spray source (TIS).

Data system: Analyst version. 1.4.2.

12.4. Results

12.4.1. Sample preparation

We decided to prepare spiked samples with the molecule of interest, and then we directly applied the sample onto the sampling card with a calibrated Gilson 20µl pipette.

We spotted 15µl of sample in the pre-marked circle of the spot.

The pipette tip should have been a few millimeters above the card, as the blood drop touches the card it should have been swiftly expelled.

Touching the circle area is avoided especially before the application of the blood and his completely drying.

12.4.2. Choice of the optimal extraction conditions

The extraction method is the solution containing the IS to be added on the punched spot.

For quantitative analysis the DBS were punched from the cards using the Harris cutting Mat into Eppendorf tubes or wells of 96-well microtiter plates for extraction. The extraction procedure was carried out by adding a certain amount of different extraction solvents containing IS.

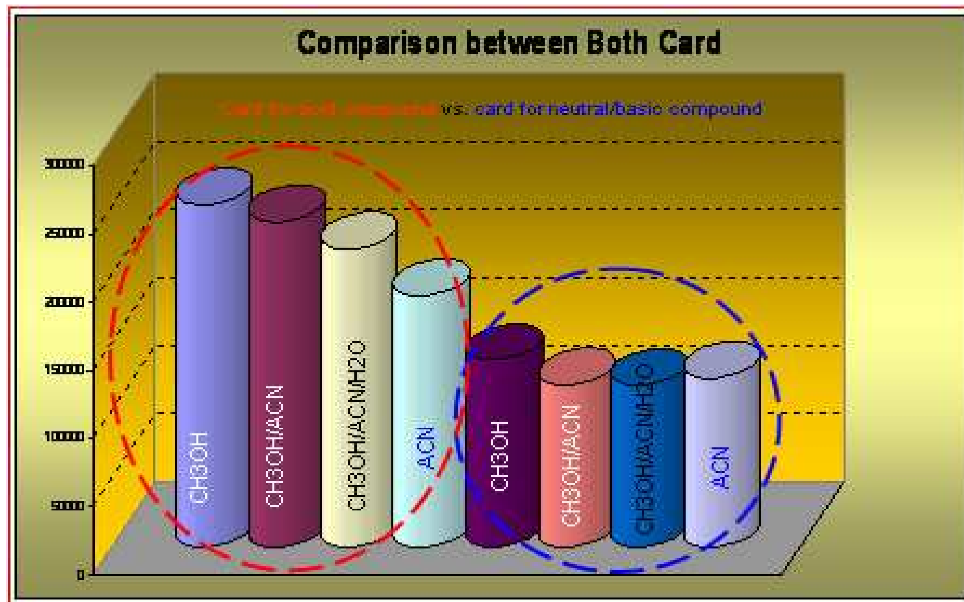
The extraction solvent must be strong enough to interrupt the binding of the analyte to protein in the matrix or paper material.

The different solvents we tested on FTA-DMPK-A and FTA-DMPK-B cards are:

- CH_3OH;
- ACN/CH_3OH 50/50;

- ACN/CH_3OH/H_2O 1/1/1;
- ACN (after extraction an equivalent volume of H_2O was added);
- ACN/H_2O 60/40;
- ACN/H_2O 70/30.

The analyte was extracted with gentle shaking. Sonication may be necessary for better extraction.



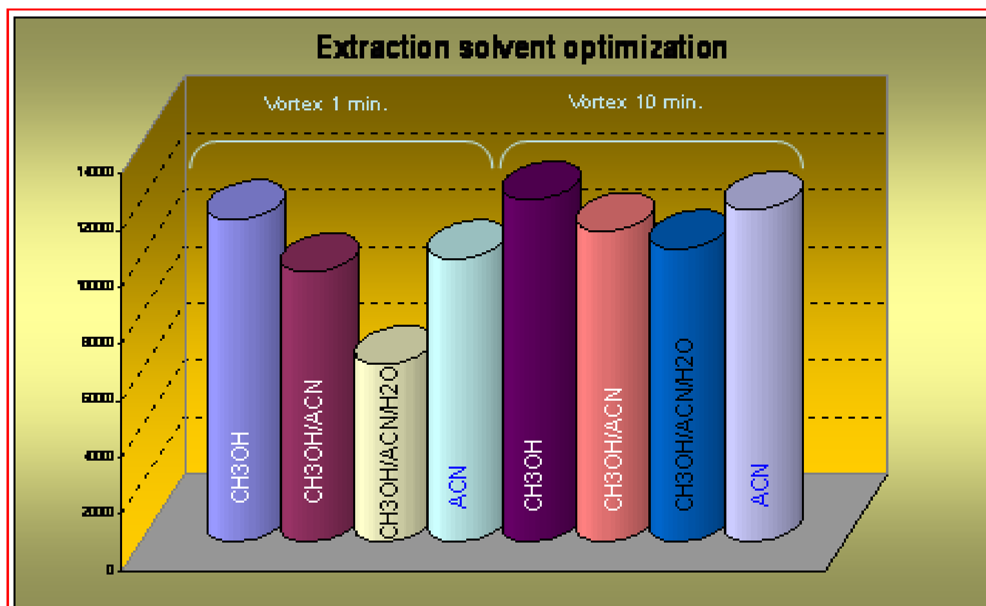


Figure 62

As we can see in Figure 62 the best extraction solvent is CH_3OH and the most appropriate card is FTA-DMPK-A. Vortexing for 10 minutes improves the extraction, but is not a determinant parameter.

12.4.3. Linearity

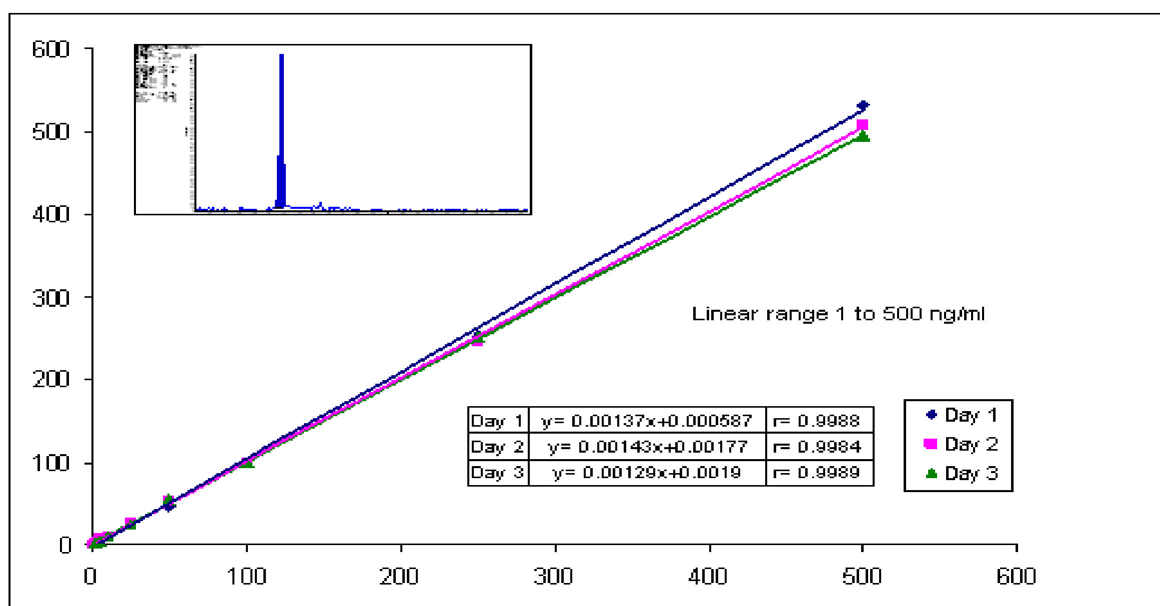


Figure 63

Calibration plots of the analyte peak area ratio to that of the internal standard versus the nominal concentration in blood were constructed and the results are shown in Figure 63 and in Table 32.

Linearity was tested by preparing and analyzing three calibration curves in the range of 1.0 to 500ng/ml and the result was that linear responses were observed for DBS over the range. This is represented by the following linear regression equation for each day: y = 0.0137x + 0.000587, r = 0.9988; y = 0.0143x + 0.000177, r = 0.9984; y = 0.0129x + 0.0019, r = 0.9989 where y represents the peak area ratio of the molecule to that of IS and x represents the concentration of the molecule in ng/ml.

Table 32

Run 01	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
	1.0	**2.5**	**5.0**	**10.0**	**25.0**	**50.0**	**100.0**	**250.0**	**500.0**
	1.0	2.6	5.4	9.2	24.7	54.2	94.7	239.0	499.0
	100.0	104.0	108.0	92.0	98.8	108.4	94.7	95.6	100.0

Run 02	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
	1.0	**2.5**	**5.0**	**10.0**	**25.0**	**50.0**	**100.0**	**250.0**	**500.0**
	1.0	2.6	5.3	8.8	25.2	53.2	98.0	242.2	504.1
	100.0	104.0	106.0	88.0	100.8	106.4	98.0	96.9	100.8

Run 03	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8	Std 9
	1.0	**2.5**	**5.0**	**10.0**	**25.0**	**50.0**	**100.0**	**250.0**	**500.0**
	1.0	2.8	4.9	9.8	23.7	53.5	98.4	246.5	489.6
	100.0	112.0	98.0	98.0	94.8	107.0	98.4	98.6	97.9

12.4.4. Accuracy and Precision QC samples stored at RT for 1 month (Stability)

Table 33

True Conc. ng/ml	Estimated Conc. ng/ml	Bias%	Mean Bias% each level ≤20.0%	ACC%	CV% ACC% ≤15.0%	Mean Bias% each day ≤15.0%	CV% ACC% each day ≤15.0%
SS-Low	3.3	10.0		110.0			
3.0	2.8	-6.7	**7.8**	93.3	**12.5**		
	3.6	20.0		120.0			
SS-Medium	40.4	1.0		101.0			
40.0	40.7	1.8	**0.5**	101.8	**1.5**	**1.6**	**8.5**
	39.5	-1.3		98.8			
SS-High	373.8	-6.6		93.5			
400	384.6	-3.8	**3.6**	96.2	**3.2**		
	398.4	-0.4		99.6			
True Conc. ng/ml	**Estimated Conc. ng/ml**	**Bias%**	**Mean Bias% each level ≤20.0%**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0%**	**CV% ACC% each day ≤15.0%**
SS-Low	3.1	3.3		103.3			
3.0	3.4	13.3	**2.2**	113.3	**11.4**		
	2.7	-10		30.0			
SS-Medium	40.5	1.3		101.3			
40.0	40.3	0.7	**1.5**	100.8	**0.9**	**0.1**	**6.6**
	41.0	2.5		102.5			
SS-High	375.3	-6.2		93.8			
400.0	384.6	-3.8	**3.5**	96.2	**3.0**		
	398.4	-0.4		99.6			
True Conc. ng/ml	**Estimated Conc. ng/ml**	**Bias%**	**Mean Bias% each level ≤20.0%**	**ACC%**	**CV% ACC% ≤15.0%**	**Mean Bias% each day ≤15.0%**	**CV% ACC% each day ≤15.0%**
SS-Low	3.5	16.7		116.7			
3.0	3.3	10.0	**5.6**	110.0	**13.2**		
	2.7	-10.0		90.0			
SS-Medium	42.9	7.3		107.3			
40.0	39.5	-1.3	**2.3**	98.8	**4.3**	**4.2**	**7.4**
	40.4	1.0		101.0			
SS-High	431	7.8		107.8			
400.0	426.7	6.7	**4.8**	106.7	**4.1**		
	399.7	-0.1		99.9			

The intra- and inter-day accuracy and precision of the method of DBS method were evaluated using QC samples at three different concentrations analyzed against calibration curve, which was constructed using calibration standards prepared from a separated stock solution.

The accuracy, the percent deviation from theoretical concentration, was generally within 10% for all QC samples.

DBS technique provides with another dimension for storage of biological samples, even in absence of refrigeration. This application has demonstrated the advantages of DBS in preserving analytes from degradation or delaying the degradation process.

In this case samples have been stored at room temperature for 1 month. However, a lower temperature may be necessary for longer stability coverage for some analytes.

12.4.5. Punch position and size comparison

Although good accuracy and precision date were obtained from the DBS assay, important parameters that may potentially affect the assay performance, such as punch position, punch size, different extraction volume etc, were investigated.

Spots of 10 and 50µl of LLOQ from two different donors were added to the center on the printed circle on FTA Elute cards and allowed to diffuse. These samples were allowed to dry for at least 3h at room temperature prior to analysis. Before analysis the DBS samples were sealed in a plastic bag with desiccant at room temperature.

In this test was evaluated if a 10µl of blood sample was sufficient for making an area of 3mm diameter.

Moreover 50µl of sample blood volume was chosen to allow more than one punch for a 3mm disk to be taken from the same blood spot; an example of the procedure is shown in Figure 64.

The influence of the following parameters on the assay performance was then evaluated:

- Different punch position;
- Different punch size (for automation);
- Different volume of extraction solvents;
- LLOQ Comparison between: Different cards, Blood samples from different donors, Different punch size.

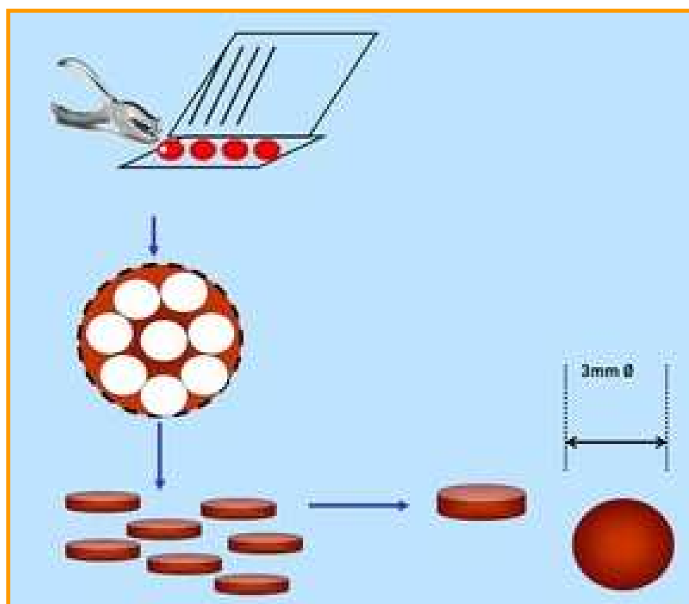


Figure 64

In Table 34 are described the results of sampling location. Usually the 3mm or 6mm sample disks are punched out from the center area of the blood spots.

But we wanted to test if the sample taken from the center area were different from those collected away from the center area.

A drop of 50µl at LLOQ concentration was spotted on the card.

We punched out five 3mm sample disks. One was punched out from the center area and four from the peripheral area.

The one taken from the center area as three taken from the peripheral area were extracted with 100µl CH_3OH plus IS.

It was found that there is no significant difference between the center spots and peripheral spots for the tested molecule: all Areas ratio are comparable.

Than the fifth 3mm disk was extracted with 50µl of CH_3OH plus IS. This volume was tested in order to evaluate the possibility of an automated extraction. The results confirmed this possibility.

Table 34

FTA® DMPK-A punch diameter 6mm, extraction solvent 200µl		
*Remaining peripheral area after the first punched step		
Parameter	Results in Area Ratio (AU)	Recovery (LLOQ Area Ratio %)
Mean LLOQ Donaor A n=2	0.004	100
Mean LLOQ Donaor B n=2	0.004	100
Mean LLOQ Donaor A n=2*	0	0
Mean LLOQ Donaor B n=2*	0	0
FTA® DMPK-C punch diameter 6mm, extraction solvent 200µl		
*Remaining peripheral area after the first punched step		
Parameter	Results in Area Ratio (AU)	Recovery (LLOQ Area Ratio %)
Mean LLOQ Donaor A n=2	0.004	100
Mean LLOQ Donaor B n=2	0.0036	90
Mean LLOQ Donaor A n=2*	0.001	25
Mean LLOQ Donaor B n=2*	0.001	28
Direct comparison results of LLOQ samples punched from the center area and non center area of the spot		
Puncher 3mm, extraction solvent 100µl		
Area Ratio	Donor A FTA® DMPK-A	Donor B FTA® DMPK-A
Center	0.003	0.002
No center n=3	0.002	0.0023
Punch diameter 3mm, extraction solvent 50µl in order to obtain automatic extraction in 96 DW plates		
Area Ratio	Donor A FTA® DMPK-A	Donor B FTA® DMPK-A
No center	0.004	0.004

12.5. Conclusions

A simple and rapid assay has been developed using whole blood dried onto filter paper cards. The collection of samples is easier, and sample preparation prior to analysis is less time-consuming and requires fewer resources, as it involves the simple extraction of compound from a disc punched from a dried blood spot into LC mobile phase solvent.

The method is both precise and accurate, selective and linear over the investigated concentration range with acceptable inter and intra-assay variability.

We selected the correct card type for our molecule, the best extraction solvent and the appropriate mixing time. We did not detect the influence of hematocrit (patient A and B) composition. The blood spots proved to be stable during a period of 1 month at ambient temperature. The intra- and inter-run accuracy and precision are within the method acceptance criteria. It has been found that sampling location and punching position on the spot do not significantly affect the precision and accuracy of the assay. We have been able to use 3mm punched spots with 50µl of extraction solvent in order to enable the automation of the extraction process in future.

In conclusion we developed a method suitable for analyzing our molecule and DBS technique can be considered as alternative technique for PK/TK Regulated Bioanalytical studies giving results comparable to the standard methods, but with significant savings in time and resources.

These findings were presented in poster sessions at the following meetings:

"EBF Congress: Connecting Strategies on Dried Blood Spots", June 17-18 2010. Sheraton Brussels Hotel, Brussels, Belgium.

"R&D Science Day", Thursday, September 30th 2010 NCD Day. Merck Serono Headquarters. 9, Chemin des Mines CH-1202 Geneva.

"6th MS-Pharmaday", Milan, October 6-8 2010, Università degli Studi di Milano.

Poster Title:

"Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of new chemical entities"

Poster Authors:

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13. LIMS

13.1. Definition

A Laboratory Information Management System (LIMS) is a software system used in laboratories for the management of samples, laboratory users, instruments, standards and other laboratory functions such as invoicing, plate management, and workflow automation.

Laboratory Information Management Systems, specifically designed for this field, are highly beneficial in speeding up all involved steps, from protocol design to the production of the final pharmacokinetic report.

LIMS are database systems, designed to combine study and sample information with acquired data from laboratory instruments in order to reduce administration and speed the production of the final report".

Most commercial LIMS have been developed for quality control and clinical chemistry applications. Because of their sample oriented design, these systems do not meet in a straight forward way requirements important for protocol driven and highly organized kinetic studies. In contrast, kinetic LIMS are study oriented, in the sense that interest is focused on the study in total and not just on a single sample. These systems 'fully understand' the design of the study and, therefore, are able to take over various study activities from the user, like managing involved samples, coordinating the sample analyses, reducing acquired raw data to concentration-time profiles, reporting final data and exporting profiles to pharmacokinetic calculation modules. Once entered or acquired, information about the study, samples and analytes is available during all subsequent study phases, thus speeding up all involved steps, from protocol design to production of the final kinetic report.

13.1.1. Data model

All study and sample information is stored in the LIMS database according to a data model specifically designed for kinetic studies and reflecting all the complex relationships between study parameters and samples observed in practice. The simplified data model

contains all relevant study elements as entities, namely involved treatments, species, subjects, matrices, study days and sampling times, as well as the resulting study samples. The arrows between the entities describe the relationships between the different study elements. Matrices and species are selected from pre-defined dictionaries.

13.1.2. Functionality and Traceability

- When the sample arrived.
- Who received it, who accepted it and under what kind of contract conditions.
- Where the sample was conserved initially.
- How the sample was distributed.
- Where the sample was conserved until the moment of the analysis.
- If someone split the sample or sampling procedure.
- What analytic procedure was followed (with detail, for example, of the conditions of use of equipment).
- Quality parameters of the analytic method used.
- Technicians that carried out the analyses (training and qualifications).
- What reagents were used (with detail, for example, of reception date and of opening date).
- What standards / strains were used (with the supplier's certificate or all the relative information in its preparation was).
- What equipment was used (maintenance, calibration and verification).
- Who and how the specifications of the equipments, reagents, cultivation means and materials used were checked to their reception.
- What mechanisms for the evaluation of the quality of the results were used.
- What were the environmental conditions.
- Who validated the results.

13.1.3. Initialization

During the initialization phase, a new kinetic study is defined, which means, that information concerning the study design, involved samples (including calibration standards and QC samples), and analytes to be measured are entered into the LIMS. First,

all involved study elements like treatments, subjects, collected matrices, nominal sampling times, etc. are entered and linked according to the study plan. Using this information, the system is able to calculate the total number of samples to be collected and to describe each sample by treatment, dose, study day, species, subject, matrix and sampling time. A flexible procedure then allows the automatic generation of sample identification codes for all kinds of coding conventions, which are in use in the different preclinical and clinical centers.

After initialization all the samples expected are described in the LIMS by a combination of study parameters. Each combination is unique and is identified internally by a sample number. Generation of sample codes is initiated by providing the system with the desired syntax.

The study undergoes a number of consecutive phases during LIMS treatment. During initialization, the study is completely open for any modification or extension. After approval, the study is ready for sample log-in and data acquisition and the study protocol is available as a printout. Completed studies are “frozen” to prevent any further handling on reported and already interpreted bioanalytical data and than archived for long-term data storage. In case of limited storage capacity, archived studies are removed from the on-line database leaving only some key data in the LIMS to allow management of archived studies.

13.1.4. Sample management

Study samples undergo a certain life cycle during LIMS treatment which can easily be monitored by providing each sample with a status. The status changes each time a certain study activity has been completed for a sample. Versatile management functions update the user at any time about the current sample situation, with regard to sample definition, receipt, availability, analyses, storage and disposal.

During initialization the samples remain in the status ‘expected’ and various outputs, like sample lists, sample labels, etc. can be generated, thereby using information readily available from the study initialization. Labels contain a detailed description of the sample to be collected together with the sample identification code. Modem systems even provide bar-codes on the labels for quicker and more reliable sample identification. After receipt, the samples are logged into the LIMS, either manually or by means of a bar-code reader.

Even the manual procedure allows a quick sample log-in due to a simple three-step procedure: retrieving codes for all delivered samples from the database, changing the status from 'expected' to 'received' and again storing the updated status for each sample in the database. Log-in of samples which are 'missing' for various reasons is performed accordingly. During log-in, all relevant information about sample source, collection, storage and condition is registered and stored in the LIMS database as part of the sample description. Additionally, all effective time values documented during sample collection, as well as subject initials, urine volumes, effective dosing times, etc. will be entered into the LIMS during this phase. Input of sample information can be very time-consuming, in particular for large studies with manual data entry. For this reason, some companies store all patient related information in a special database which is connected to the kinetic LIMS for rapid data transfer. During sample analysis, the samples are in 'progress'. When sample analysis has been 'completed', the samples are 'archived' for a certain time and, because of the limited lifetime, finally "disposed" of.

For management of calibration standards and QC samples a less sophisticated LIMS functionality is required. Usually, only details about preparation, biological matrix, storage, analyte lots, etc. are stored in the database to provide sample reports with appropriate header information.

13.1.5. Worksheets, data capture

Electronic generation of worksheets represents one of the central LIMS activities. Sample codes are transferred into an empty worksheet, supplemented with vial numbers, dilution factors, etc. and then downloaded via local network into the analytical instrument. During sample analysis, the worksheet file is filled with acquired data and finally uploaded to the LIMS, which closes the worksheet circle. Since no manual data transfer is involved during worksheet generation and data acquisition, considerable time is saved daily during sample analysis, reducing significantly the overall time required for a kinetic study.

During sample analysis, acquired data are transferred to the worksheet file and linked to the corresponding sample codes. Usually, raw data such as chromatograms, spectra, etc. remain in the analytical instrument and are maintained and archived there, while processed data such as retention times, peak heights, areas, optical densities are imported by the LIMS for further data reduction.

13.1.6. Pharmacokinetic evaluation

Approved concentration-time profiles are the input data for all pharmacokinetic evaluations. Graphical representations visualize the input data and help to understand derived kinetic interpretations. Pharmacokinetic parameters are released in the form of kinetic summary tables.

Usually, kinetic evaluation starts when sample analysis is finished and all profiles are complete. However, in special circumstance, it might be helpful to obtain preliminary kinetic information during an ongoing sample analysis. In this case, the LIMS should release warnings that the profile is incomplete and that kinetic calculations have to be repeated after the sample analysis has been completed.

The user should have access to the usual model-dependent and model-independent calculation routines. However, special packages, such as population kinetics, are rarely integrated into pharmacokinetic LIMS. For this reason, the LIMS should obtain an interface for exporting concentration-time profiles to external pharmacokinetic programs. Since the input routines of such commercial packages are not standardized, the LIMS must be able to export the concentration-time profiles in different output formats as Excel table.

13.1.7. Good Automated Laboratory Practices

All computerized systems which are involved in pivotal studies are subject to the principles of Good Automated Laboratory Practice (GALP).

Treatment of non-GLP studies, now called QMS, follows the common scientific standard: a study can be directly used after initialization, data correction is possible without special restriction, corrective actions do not need to be documented and final data do not have to meet specific acceptance criteria.

For GLP studies, additional effort is needed to restrict access to the study, to handle data according to agreed procedures, to maintain a constant quality of final concentrations and to assure that the history of study; sample and data treatment will be transparent during study audits to drug regulatory authorities.

To meet these goals, access to the study should be carefully controlled, in such a way that only personnel responsible for the study, together with their deputies and seniors, are authorized to enter, edit or delete study elements, samples and data.

In conclusion the use of a LIMS Software, in my particular case the use of Watson LIMS by Thermo, is very useful in fact it allows better planning work, it facilitates the registration of primary data and its traceability in general; it is also able to save it.

13.1.8. Creation of an Excel File containing macro

In order to optimize and enhance an important existing manual application, a particular Excel File containing macros was created.

This File (Figure 65) consists of 13 sheets.

It can be used for a maximum of 1000 samples

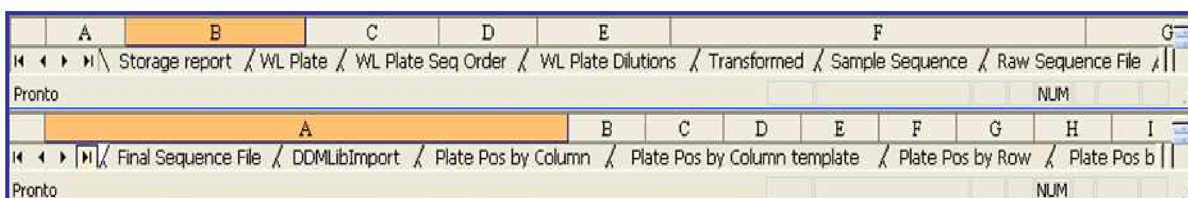

Figure 65

It allows the creation of analytical sequences with the vial position number automatically assigned without manual intervention. It interfaces directly the LIMS with Analyst without the necessity of any further operation.

In the previous situation, the process required the operator manually reformatted plates to produce the correct analytical sequence.

This file provides a flexible way to import a variable number of samples into Analyst. It illustrates an innovative application and adaptation of existing concepts in response to a specific need.

It is also used to interface the dilution factors selected into the LIMS with the Hamilton Vector Software.

Although the file was developed for a particular application, we believe it can be easily applied to other circumstances.

13.1.8.1. General Instruction

- Creation of different folders (Figure 66).

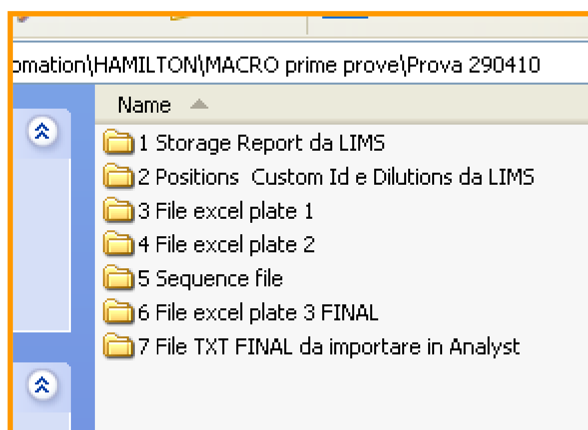

Figure 66

- Into LIMS "*Study Storage Report Selection*" select container and box of interest.
- Copy as an Excel file the "*Storage Report*" in the first sheet of the Excel file.
- The same operations have to be done also for "*Samples Position*" and "*Samples Custom ID*".
- After the compilation of the first four sheets on the fifth called "*Transformed*" the macro is activate as shown in Figure 67, Figure 68 and Figure 69.

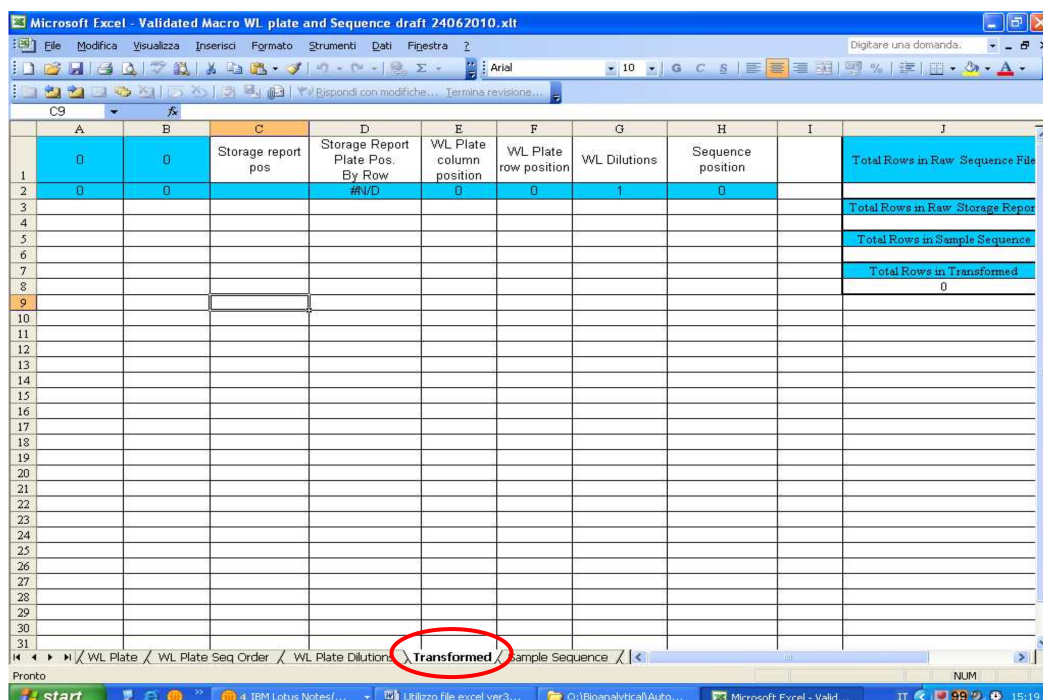

Figure 67

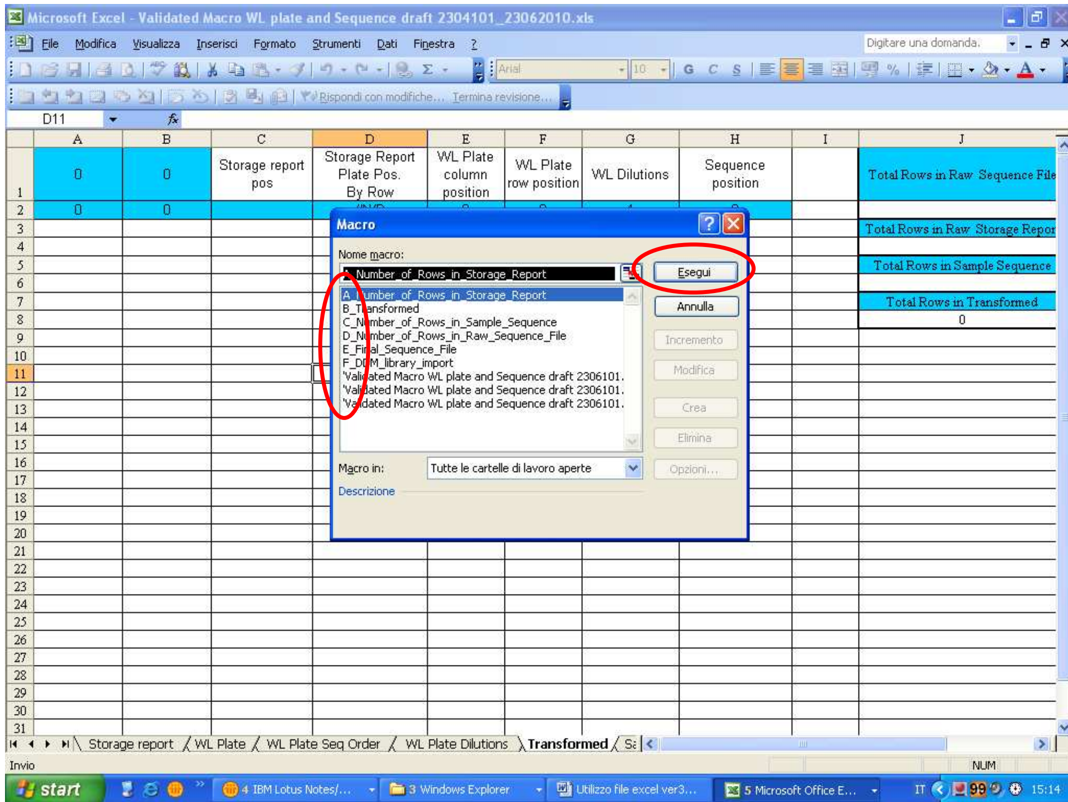

Figure 68

Microsoft Excel - Validated Macro WL plate and Sequence draft 230410A.xls

	A	B	C	D	E	F	G	H	I	J
1	Watson Id	Custom Id	Storage report pos	Storage Report Plate Pos. By Row	WL Plate column position	WL Plate row position	WL Dilutions	Sequence position		Total Rows in Raw Sequence File
2	31070000137	31070000137	A4	4	6	2	1	32		
3	31070000138	31070000138	B4	16	5	4	1	55		Total Rows in Raw Storage Repor
4	31070000139	31070000139	C4	28	4	5	1	66		73
5	31070000140	31070000140	D4	40	9	5	1	71		Total Rows in Sample Sequence
6	31070000141	31070000141	A12	12	11	2	1	37		
7	31070000142	31070000142	B12	24	12	2	1	38		
8	31070000143	31070000143	C12	36	2	3	1	40		
9	31070000144	31070000144	D12	48	4	4	1	54		
10	31070000145	31070000145	E5	53	7	2	1	33		
11	31070000146	31070000146	F5	65	6	4	1	56		
12	31070000147	31070000147	G5	77	5	5	1	67		
13	31070000148	31070000148	H5	89	10	5	1	72		
14	31070000153	31070000153	E7	55	8	2	1	34		
15	31070000154	31070000154	F7	67	7	4	1	57		
16	31070000155	31070000155	G7	79	6	5	1	68		
17	31070000156	31070000156	H7	91	11	5	1	73		
18	31070000161	31070000161	A9	9	9	2	1	35		
19	31070000162	31070000162	B9	21	8	4	1	58		
20	31070000163	31070000163	C9	33	7	5	1	69		
21	31070000164	31070000164	D9	45	12	5	1	74		
22	31070000169	31070000169	E10	58	10	2	1	36		
23	31070000170	31070000170	F10	70	9	4	1	59		
24	31070000171	31070000171	G10	82	8	5	1	70		
25	31070000172	31070000172	H10	94	1	6	1	75		
26	31070000497	31070000497	E4	52	6	1	1	20		
27	31070000498	31070000498	F4	64	12	1	1	26		
28	31070000499	31070000499	G4	76	3	3	1	41		
29	31070000500	31070000500	H4	88	8	6	1	82		
30	31070000501	31070000501	E12	60	1	3	1	39		
31	31070000502	31070000502	F12	72	2	4	1	52		

WL Plate / WL Plate Seq Order / WL Plate Dilutions / Transformed / Sample Sequence

Figure 69

By the creation and the export from LIMS of a "*Work list/ Sequence File*" pasted in the sheet "*Raw Sequence File*", the residual macro steps are activate. The final sheet is obtained and it will be imported into Analyst software. The analysis can start without the manual or automated effective reorganization of the plate. This operation allows the operator to save time.

14. Conclusions

In this PhD project in Pharmaceutical Analysis the manual sample preparation processes for LC/MS analysis have been automated using highthroughput techniques, and the developed process has been enclosed in the frame of a pharmaceutical bioanalytical process managed by a LIMS system in Merck Serono S.A.

Through use the Hamilton MicroLab Star liquid handling workstation, the most important sample preparation techniques were successfully automated and implemented for several biological matrix samples and analytes. This in turn has greatly reduced the intensive manual labor as well as the possibilities of systematic error associated with the manual volumetric transfers. Significant advantages in terms of efficiency and throughput have now been achieved by this new automated process while maintaining the integrity of the precision, accuracy and quality of the produced data.

- Over 12 studies and 5000 samples have been prepared using **Protein Precipitation Technique** in the Automation Laboratory since implementation in the first six months. The original goal to automate the sample workload using this approach was met.
- A fully automated high throughput **Liquid Liquid Extraction** method validation has been developed. The results obtained during the validation of the assay fulfilled all requirements and recommendations regarding linearity, accuracy and precision generally accepted for bioanalytical studies.
- **Solid Phase Extraction** procedure can be performed by the workstation proving to be quick and labor saving so in the future it could eliminate the human factors of specimen handling, extraction, and derivatization, thereby reducing labor costs and rework resulting from human error or technique issues. The automated extraction of peptides from blood samples has been shown to be feasible using the Hamilton robotic workstation. Compared with manual extraction methods reported previously the method has a shorter processing time while maintaining equivalent linearity, sensibility and recovery.

- It has been demonstrated also that **Dried Blood Spot** technique is useful to eliminate critic processing aspects and could become of key importance in providing breakthrough in the time required to develop a drug by increasing the number/time of analyzed samples; avoiding blood centrifugation to produce plasma; simplifying collecting, shipping & storing blood samples; reducing the number of animals. The preliminary tests demonstrated the feasibility and applicability of this technique in Regulated Bioanalytics. It was found that with this innovative technique all the steps before the analysis were simplified and the capability to obtain better result quality was higher.

Comparative evaluations between manual and automated sample preparation techniques for new chemical entities in different matrixes have been done. The results in terms of accuracy and precision are at a very high level.

In addition, several advantages over conventional manual methods were confirmed, such as reducing "working hour" for analysts, eliminating "working-hour" restriction, and decreasing drastically lag time for each prepared sample toward HPLC analysis.

Automation Laboratory can work as a bridge between the sample management system, New Chemical Entities Laboratory and the information management system (LIMS), and it will reduce drastically budgets and time frame for development of pharmaceutical product.

We have developed a fully automated laboratory robotic system for sample preparation capable of conducting most of routine experiments. Main objectives for developing this novel robotic system are to streamline the analytical tasks by automating sample preparations and analytical procedures, and to ensure the safety of analysts by assigning risk-involving procedures, such as handling of highly dangerous substances.

Final conclusions:

- Automated PPT has been utilized for many studies in Merck Serono S. A.;
- LLE for biological sample extraction has been validated for Clarity study;

- SPE, and DBS for biological sample extraction can be automated;
- Automation **saves time** and **increases the quality of data**, extraction steps are more reproducible/reliable if computer controlled;
- It is important to automate without loosing control of the process and keeping the possibility of method changes if necessary;
- Risk of cross contamination due to human mistakes is avoided;
- HTBSP can be used in **GLP** and **GCLP** environments.

Author in GxPDocs of the following documents:

- **WI**: “Centrifuga Allegra 6 R Beckman Coulter, 5810 R Eppendorf, GR. 4.22 Jouan”.
- **WI**: “File Excel: Gestione dei campioni tra i sistemi Watson LIMS, Vector (Hamilton) e Analyst”.
- **SOP**: “Sistema robotico Integrato Hamilton Microlab STARlet”.