# Small Molecule Bioanalysis by LC-MS and LC-MS/MS

Purospher® STAR RP-18 endcapped and SeQuant® ZIC®-HILIC HPLC columns for analysis of endogenous and exogenous compounds in biological samples

# Content

# Application Index

| Molecule Name | Column Used | Page |
|---|---|---|
| MMA in Plasma/Serum | SeQuant® ZIC®-HILIC (3 µm, 100Å) PEEK 100 × 2.1 mm | 11–16 |
| Catecholamines in Urine/plasma/serum | SeQuant® ZIC®-cHILIC (3 µm, 100Å) PEEK 100 × 2.1 mm | 17–26 |
| Ethyl glucuronide and Ethyl sulfate in Urine | SeQuant® ZIC®-HILIC (3 µm, 100Å) PEEK 150 × 2.1 mm<br>SeQuant® ZIC®-HILIC (guard fitting) PEEK 14 × 1.0 mm | 27–29 |
| Cortisol in saliva and urine | Purospher® STAR RP-18 endcapped (2µm) 100 x 2.1 mm | 30–32 |
| Vitamin D2 and D3 in plasma/serum | Purospher® STAR RP-18 endcapped (2 µm) 100 x 2.1 mm | 33–36 |
| Metformin in plasma | SeQuant® ZIC®-HILIC (5µm, 200Å) PEEK 100 × 2.1 mm | 38–40 |
| Tramadol in urine | Purospher® STAR RP-18 endcapped (2 µm) 50 x 2.1 mm | 41–43 |
| Buprenorphine in urine | Purospher® STAR RP-18 endcapped (2 µm) 50 x 2.1 mm | 44–46 |
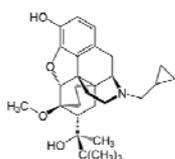


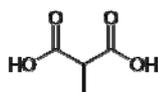Buprenorphine
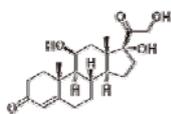
Cortisol
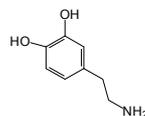
Dopamine
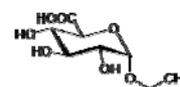
Ethyl glucuronide (EtG)
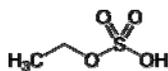
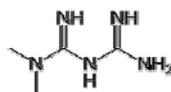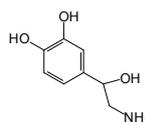Ethylsulfate (ES)
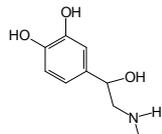
Epinephrine
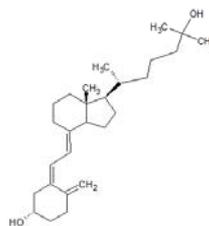
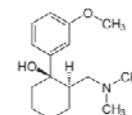25-OH-Vitamin D3
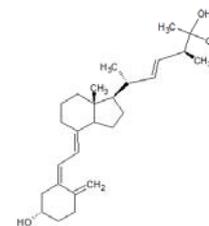
25-OH-Vitamin D2

Methylmalonic Acid (MMA)

Metformin

Norepinephrine

Tramadol

# Introduction

Many parties (companies, institutes and hospitals) require methods for accurate quantification of drugs and endogenous substances in biological samples to provide qualitative and quantitative measures of the active drug and/or its metabolite(s) for the purpose of pharmacokinetics, toxicokinetics, bioequivalence and exposure–response (pharmacokinetics/pharmacodynamic studies). In addition, bioanalysis also applies to drugs used for illicit purposes, forensic investigations, anti-doping testing in sports, and environmental concerns.
Bioanalysis is thus a discipline within analytical chemistry aiming at quantitative measurement of drugs and their metabolites, biological and biotics (macromolecules, proteins, DNA, large molecule drugs, metabolites) in biological samples. The term bioanalysis traditionally refer to the measurement of small molecules in biological fluids but over the past two decades this discipline has expanded substantially because of the increased interest in biopharmaceuticals (e.g. proteins and peptides).

## Bioanalytical Organisations
There are several national and international bioanalytical organizations active in pharmaceuticals sciences in general and/or bioanalytical chemistry in particular, for example:

AAPS – American Association of Pharmaceutical Scientists
BSAT/APA – Applied Pharmaceutical Analysis
CVG – Canadian LC-MS Group
EIP – European Immunogenicity Platform
EUFEPS – European Federation for Pharmaceutical Scientists
FABIAN (Dutch Bioanalytical Society)
GBC – Global Bioanalysis Consortium
JBF – Japan Bioanalysis Forum

On the following pages we have included a number of applications with relevance to clinical bioanalysis, focusing on a number of molecules either being endogenous (naturally occurring) or molecules being administered to humans (exogenous). As you will experience there is both examples for hydrophobic molecules (candidates for reversed phase mode) and those being more polar and hydrophilic, thus candidates for HILIC. This compilation presents analytical solutions with complete experimental details for your needs in small molecule bioanalysis.
EMD Millipore offers virtually everything but the instrument to successfully implement the highlighted methods in your laboratory.

# Choosing the Best Analytical Technique

Choosing the best analytical technique, with following method development/modification, is not difficult when a literature reference can be found to same or similar needs. Methods are published in pharmacopeia, in application databases and as scientific studies. This can provide good guidance for the work, but what happens when no relevant information exist?

Method development means to define needs, set goals, and make experimental plans, then to carry out the practical work and finally validate and put the new method into routine work.
For these reasons, method development should be started at the desk, and not in the laboratory.
A number of questions should be addressed and answered.

- Is the primary goal quantitative or qualitative analysis?
- If quantitative analysis is requested what levels of accuracy and precision are required?
- Are standards available?
- Do we need to perform detection of one or many analytes?
- Is it necessary to resolve all sample components?
- How many different sample matrices is the method designed for?
- How many samples will be analysed at one time?

These initial questions will define the method goal, and to find out requirements of the new method. Do you really need high resolution (in separation and detection), short analysis time, maximum sensitivity, long column lifetime, a column with wide pH stability or will the method be used at neutral pH and under non-aggressive conditions. True optimization of a method is a balance between selectivity, speed and efficiency, in order to produce resolution that fits the purpose of the application. Ideally, the development should result in a robust method that gives the laboratory a low overall price-per-injection and ultimately a cost-efficient assay.

Common mistakes in method development are; inadequate formulation of method goals, insufficient knowledge of chemistry, use of the first reversed phase HPLC column available, trial and error with different columns and mobile phases, and use of wrong instrument set-up. These mistakes often results in laborious, time consuming projects that lead to methods which fail to meet the needs of the laboratory. After defining the goal of the method development, specific information of the sample and the analytes should be sought. Listed are some of the most common parameters; nature of the sample (urine/plasma/serum/whole blood etc), number of compounds/analytes present, chemical structure (functionality), analyte molecular weight, pKa values , Log P and/or Log D values (hydrophilicity/hydrophobicity), expected concentration ranges, sample matrix and sample solubility. Think about the sample as being the central part during all steps. When selecting the most suitable mode of separation, it is dependent on sample solubility and how the analytes of interest differ from other compounds or matrix in sample, where biological samples are per definition mainly aqueous.
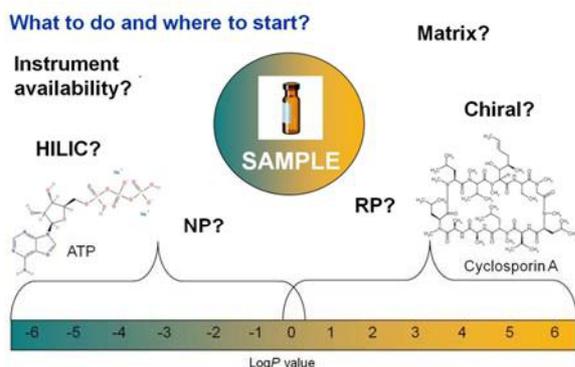
Figure1. Sample in the centre when defining the method goal.

## Selection of initial conditions – mode of separation, column and mobile phase

In reversed phase (RP) mode the mobile phase is polar and the stationary phase is less polar. The major distinction between analytes is their hydrophobicity where samples should be soluble in water or a polar organic solvent. In hydrophilic interaction liquid chromatography (HILIC) mode, the mobile phases are the same as for reversed phase, but with the opposite elution strength. The major distinction between analytes is their hydrophilicity and the sample should be soluble in a polar organic solvent or organic solvent - water mixtures. For polar and hydrophilic compounds the traditional approach utilized reversed phase ion-pairing and were used for analytes being ionic or potentially ionic. In this situation the mobile phase contains a buffer, an ion-pair reagent and a polar organic solvent. Today, reversed phase ion-pairing methods can easily be replaced with HILIC with the benefit of having more robust and sensitive methods without the need of using ion pairing reagent. This is helpful in LC-MS analysis where use of ion-pair reagents is not wanted.

## Stationary Phase Selection

The initial column selection is very important and it is not advisable to use the first reversed phase HPLC column available. Reversed phase liquid chromatography is indeed a workhorse and a particulate RP-18 column is often the first choice, but many methods are developed not utilizing best or most appropriate selectivity. If the sample is mainly of hydrophobic character, having positive log P values and mainly having hydrophobic functional groups, a reversed phase column is the right choice. Select a C18 or C8 bonded phase for good retention and resolution. Methods intended for bioanalysis (analysis of dirty samples) and/or where proper sample preparation is unwanted/not possible, a monolithic reversed phase column (e.g. Chromolith) could be a superior choice over a particulate column. Monolithic Chromolith® RP-18 columns have very good matrix tolerability and long column lifetime. If samples are cleaner or good sample preparation will be included in the final method, and very high peak capacity is needed, a particulate column, such as Purospher® STAR RP-18 endcapped with small particles may be more useful. Choose bonded phases based on high purity, low acidity silica for best peak shape and lowest detector noise. A HILIC column such as SeQuant® ZIC®-HILIC should be selected if the sample is consisting of polar hydrophilic molecules.

# Biological Samples (Matrix)

The liquid part of blood, devoid of cells and platelets, is termed either plasma or serum depending on how the sample has been prepared. Since some confusion seems to exist in the literature, a definition could be appropriate. Blood plasma is the liquid portion remaining after the cellular components have been removed from the blood by centrifugation. An anticoagulant must be added to prevent the blood from clotting before the separation takes place, and the kind of anticoagulant used may be important to know for the analyst. Anticoagulants usually interfere with the clotting process by binding calcium ions, and examples of anticoagulants are sodium citrate, EDTA, potassium oxalate, and heparin. If no anticoagulant is added, clotting will start within minutes and the fibrin clot formed will contain within it the cellular components of the blood. The liquid remaining when the clot is removed is blood serum, and is equivalent to blood plasma, except that it lacks the plasma components that have taken part in the clotting process, mainly the protein fibrinogen. Tissue is defined as a group or layer of similar cells united to perform specific functions. Urine is the excrementitious fluid secreted from the blood by the kidneys, passed through the ureters, stored in the bladder, and discharged through the urethra wherefore a wide variety of metabolic products in both conjugated and unconjugated form are present.

### Sample Storage

Plasma, serum and tissue samples are comparatively stable wherefore no special precautions must be taken, and samples are normally stored in plastic containers at temperatures down to –20 °C. Urine is perhaps the most complicated biological matrix available and will normally need the addition of preservatives to prevent bacterial degradation.

# Sample Preparation

Different approaches can be used for sample pre-treatment and purification. A common feature is that loss of analyte during the work-up procedure is inevitable and the actual analyte recoveries must be determined while developing a new method, or otherwise the data produced by the method will be questionable. The most common practice for determining the analyte recovery is internal standard (I.S.) addition, where the I.S. should be added to the matrix at the start of the analysis. When working with biological matrices one important criterion for the I.S. is that binding strength of the analytes and the I.S. to proteins and other components of the matrix should be similar. The I.S. should also have physiochemical properties that are comparable to the analyte and thus behave similar to the analyte during sample pre-treatment (i.e., extraction and purification), yet the properties must be sufficiently unique to allow the I.S. to be clearly discerned from the analyte in the quantitation step. When using LC-MS or LC-MS/MS, isotope dilution is the preferred route for determining analyte recoveries. Deuteriated analytes are the ideal internal standards, whose identical chemical properties make them behave exactly as the analytes, but the mass difference makes them easily identifiable in the quantitation procedure.

## Liquid-Liquid Extraction (LLE)

LLE was the predominant technique for extraction of molecules of interest from biological matrices in the early ages of small molecule bioanalysis, but its popularity has declined as other, more efficient, techniques have emerged. If LLE is contemplated as a sample pre-treatment alternative, the polarity of the analytes and their ability to bind to proteins are important aspects when selecting solvents for the extraction procedure. Thus, to quantitatively extract the analyte(s), the chosen solvent should not only dissolve the compounds of interest completely, but also be capable of breaking associations to proteins. These requirements are seldom satisfactorily met in practice and low extraction efficiencies are therefore often seen. Another obstacle is that LLE is an inherently non-selective procedure whereby lipids are likely to be co-extracted and may well cause interferences in the ensuing operations. Co-extracted lipids can be removed to some extent, but when LLE is used, the user should expect a significant presence of lipids, even in a purified sample. The limited compatibility of solvents with plastic vessels is another problem, which makes glass one of the few extraction reservoir materials possible. But since certain groups of molecules tend to bind to glass surfaces it is recommended that all glassware should be silanized prior use or to work with plastic material. Another problem often associated with LLE is the necessity of using large amounts of toxic and/or flammable solvents, leading to complications with handling and waste disposal. Despite all drawbacks LLE will also in future be chosen for sample purification.

## Protein Precipitation/Protein Crash

Addition of solvent, acids, bases, salts or mixtures thereof to a biological sample will stimulate precipitation of proteins. It is an efficient approach in cleaning up samples and commonly referred to as protein crash. An optimized protocol can remove up to 95% of all proteins in a sample. It is a "quick-and-dirty" approach wherefore other matrix components, lipids and minerals, will remain and could be potential causes of column problems (reducing column life-time, selectivity shifts), ion-suppression/ion-enhancement and thereby lead to inconsistencies or inaccuracies in the detection and quantitation of analytes of interest. When using protein precipitation the underlying mechanism is to alter the solvation potential of the solvent by lowering the solubility of the solute by addition of another reagent. The solubility of proteins depends on the distribution of hydrophilic and hydrophobic amino acid residues on the protein's surface. Proteins that have high hydrophobic amino acid content on the surface have low solubility in an aqueous solvent. Charged and polar surface residues interact with ionic groups in the solvent and increase the solubility of a protein. Addition of miscible solvents such as methanol to a solution may cause proteins in the solution to precipitate. Miscible organic solvents decrease the dielectric constant of water, which in effect allows two proteins to come close together. In this compilation we have included a method, **Determination of Methylmalonic acid (MMA) in plasma** to exemplify how protein precipitation can be optimized and how efficient it can be applied in your laboratory.

## Solid Phase Extraction (SPE)

Over the last three decades, the use of SPE has gained in popularity and is today considered as one of the more common techniques of choice for sample work-up. SPE offers several benefits over to LLE, such as improved recoveries, less solvent consumption, smaller sample volumes, and increased sample throughput. SPE is relatively easy to use and the technique can be incorporated in dedicated fully automated sample preparation systems of various formats. Another important advantage with SPE is the wide variety of sorbents commercially available with a variety of functionalities that allow users to choose from different modes of interaction when performing sample purification. The development of new sorbents of various formats (i.e., discs, syringes, membranes, etc.) is still of interest as the number of analyses demanding highly specific and efficient sample work-up increases. For more information about EMD Millipore SPE products offering, please visit http://www.emdmillipore.com/chromatography.

When implementing SPE into an analytical method some simple guidelines should be considered:

**Sample characteristics**     Analyte pKa, molecular weight, polarity, matrix, interferents, etc.

**Sorbent selection**     Reversed phase, normal phase, ion exchange, adsorption, etc.

**Solvent selection**     Prepare elution profiles for different solvents.

Method development can thereafter be carried out according to these four steps:

- Conditioning of the solid phase bed.
- Application of the sample.
- Sample pretreatment (multiple steps) to remove loosely bound matrix constituents.
- Elution of the analyte, preferentially with a minimal amount of matrix compounds co-eluting.

When a suitable procedure has been established for the compounds of interest, the breakthrough volume of the sorbent should be examined. Knowing the maximum extractable sample amount, both volumetrically and gravimetrically, linearity, repeatability, reproducibility, and recoveries may be determined in order to validate the work-up procedure.

# Endogenous Compounds

Substances being classified as endogenous are originating from an organism, tissue, or cell. Many organisms, in addition, produce small molecule secondary metabolites, biologically active compounds, that can be used as research tools (markers) and/or for drug discovery (lead molecules). A biological marker is used as an indicator of some biological state or condition, for instance B12 deficiency (see methylmalonic acid application). Biomarkers are often measured and evaluated to examine normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. In the following section, you will find a number of LC-MS and LC-MS/MS methods for the analysis of endogenous compounds.
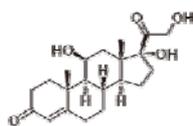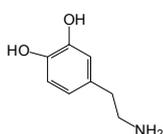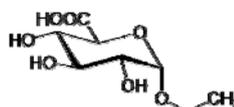
Cortisol          Dopamine          Ethyl glucuronide (EtG)          Epinephrine

25-Hydroxyvitamin D3    25-Hydroxyvitamin D2    Methylmalonic Acid (MMA)    Norepinephrine

# Methylmalonic Acid (MMA)
## – B12 deficiency biomarker

Methylmalonic acid (MMA) in serum, plasma and urine is used to monitor cobalamin (vitamin B12) deficiency and methylmalonic acidemia. Measurements of metabolites, i.e. methylmalonic acid and homocysteine have proven to be more sensitive in the diagnosis of vitamin B12 deficiency than measurement of serum B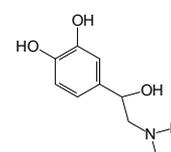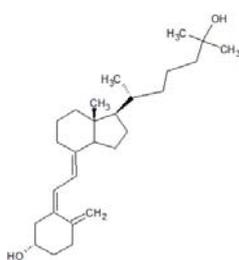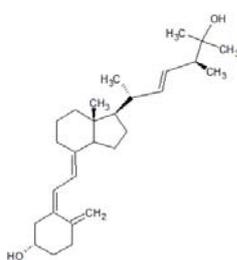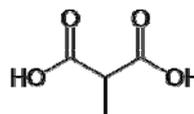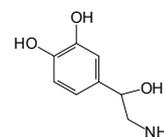12 levels alone. Different methods for MMA in serum, plasma, urine and cerebrospinal fluid have been developed including GC-MS, LC-MS-MS, HPLC, and capillary electrophoresis (CE). The main problems to overcome in method development are related to the low physiological concentrations (100-500 nM) of MMA in human serum, and the fact that MMA is a hydrophilic non-volatile compound. Retention and separation of MMA on reversed phase liquid chromatographic columns is difficult as MMA shows poor retention and the structural isomer succinic acid (SA) may interfere (ion suppression) since serum concentrations of SA are usually considerably higher than MMA. Many labs use methods that require extraction and derivatization steps to yield MMA-derivatives that are compatible with GC-MS-techniques or reversed phase mode based liquid LC-MS/MS methods where derivatives of MMA and SA may be differentiated due to different fragmentation pattern. As a consequence the costs per MMA-test are usually considerably higher than standard immunological assays for B12, and this is possibly the main reason why serum B12 assays still are used in clinical routine.

We have developed a method that combine hydrophilic interaction liquid chromatography (HILIC) with single stage negative ESI-MS. that allows analysis of up to 130 serum samples per 24 hours.* The new method take advantage of the bonded zwitterionic stationary phase ZIC®-HILIC that is an ideal tool for separation of polar compounds, such as MMA in serum/plasma. In this compilation we illustrate how to optimize protein crash for sample preparation of serum samples using experimental design and multivariate data analysis. We also show that this method successfully can be transferred from a LC-MS platform to a LC-MS/MS platform. Both methods have been fully validated, and are currently in use for clinical routine analysis of serum/plasma MMA at several hospitals in Europe and US.
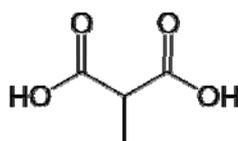


Methylmalonic Acid (MMA)

# Methylmalonic Acid (MMA) in Plasma
## SeQuant® ZIC®-HILIC

Recommended column:

SeQuant® ZIC®-HILIC (3µm, 100Å) PEEK 100 × 2.1 mm                   (1.50441.0001)

Recommended solvents and reagents

Acetonitrile:   hypergrade for LC-MS LiChrosolv®                   (1.00029)

Water:          Water for chromatography LiChrosolv®              (1.15333)
                or freshly purified water from Milli-Q® water purification system

Ammonium acetate (HPLC grade) or in-situ prepared buffer from ammonia and acetic acid

Ammonia solution 28-30% for analysis EMSURE® ACS, Reag. Ph Eur     (1.05423)
Acetic acid 96% for analysis EMSURE®                               (1.00062)
Formic acid 98-100% for analysis EMSURE® ACS, Reag. Ph Eur         (1.00264)

### Mobile phase preparation
A 100 mM ammonium acetate buffer was prepared by adjusting the pH with concentrated formic acid. The isocratic mobile phase consisted of acetonitrile and 100 mM ammonium acetate buffer adjusted to pH 4.5 with formic acid (80:20, v/v) and the column cleaning between injections were achieved by increasing the salt concentration by addition of salt from mobile phase B (100 mM ammonium acetate buffer adjusted to pH 4.5 with formic acid).

### Protein precipitation solution:
The plasma protein precipitation (PPT) solution was prepared by adding 43 µl of the 196 µmol/L D3-MMA stock solution and 250 µl concentrated acetic acid to acetonitrile to yield a total volume of 50 ml. This PPT solution thus contained 0.5 volume-% acetic acid and 0.17 µmol/L D3-MMA.

### HPLC Sample Preparation
Human EDTA or citrate plasma (200 µL) was added to 800 µL of the protein precipitation (PPT) solution in 2 ml autosampler glass vials. The vials were capped and allowed to stand on an orbital shaker for 5 minutes before centrifugation at 6200 rpm for 10 min at 15 °C and then placed in the autosampler of the LC/MS instrument.

# Methylmalonic Acid (MMA) in Plasma
## SeQuant® ZIC®-HILIC

**Optimization of sample preparation – use of protein precipitation**

The protein precipitation conditions were optimized with regard to ionization efficiency using experimental design and multivariate analysis. Acetic acid was chosen as stimulant for the protein precipitation since an acid was target molecule for the extraction. The effects of changes in the relative volume portions of plasma and PPT solution (1:4, 1:3 and 1:2) plus the concentration of acetic acid (0.3, 0.5 and 0.7 volume-%) were evaluated. Partial least squares (PLS) analysis demonstrated that highest yield could be achieved at a 3:1 ratio between PPT solution and plasma/serum sample, see figure 1. Yet, with a 4:1 ratio, it is possible to increase the column lifetime substantially and thereby attain more economical analysis. Recoveries for MMA range between 90-93% and both within-day and between-day RSD's are $\leq$ 5 %.
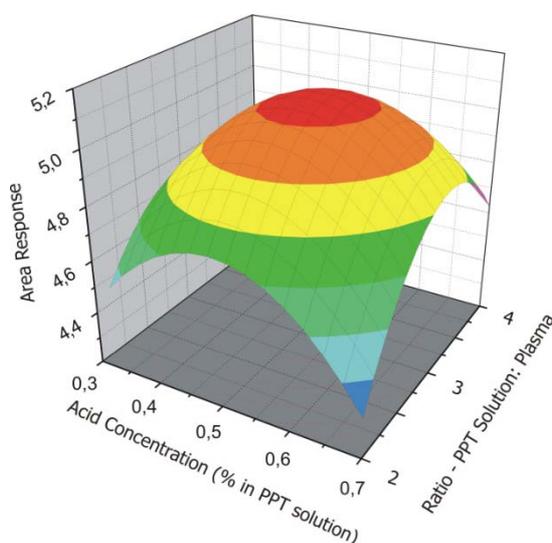


Figure 1. Response surface model, based on partial least square analysis, illustrating the extraction efficiency as a function of acid concentration in the protein precipitation (PPT) solution and the volumetric relationship between plasma and PPT solution.

# Methylmalonic Acid (MMA) in Plasma – LCMS
## SeQuant® ZIC®-HILIC

## Chromatographic Conditions

| | | |
|---|---|---|
| Column: | SeQuant® ZIC®-HILIC (3µm, 100Å) PEEK 100 × 2.1 mm | (1.50441.0001) |
| Injection: | 4 µL in mobile phase | |
| Detection: | LC-MS, negative ESI mode, SIM; (m/z 117.2 and 120.2) | |
| Flow Rate: | See table | |
| Mobile Phase: | Acetonitrile and 100 mM ammonium acetate, pH 4.5 (80:20 v/v). Total ionic strength: 20 mM | |
| Temperature: | 30 °C | |
| Sample: | Patient plasma sample treated according to sample preparation protocol. | |
| Pressure Drop: | 90 Bar (1305 psi) | |



| Time (min) | A (%) | B (%) | Flow Rate (mL/min) | Flow Direction |
|---|---|---|---|---|
| 0.00 | 80 | 20 | 0.40 | Waste |
| 1.50 | 80 | 20 | 0.40 | Ion source |
| 3.00 | 80 | 20 | 0.40 | Ion source |
| 3.01 | 55 | 45 | 0.80 | Waste |
| 5.00 | 55 | 45 | 0.80 | Waste |
| 5.01 | 80 | 20 | 0.80 | Waste |
| 9.00 | 80 | 20 | 0.80 | Waste |
| 9.01 | 80 | 20 | 0.40 | Waste |
| 10.00 | 80 | 20 | 0.40 | Waste |
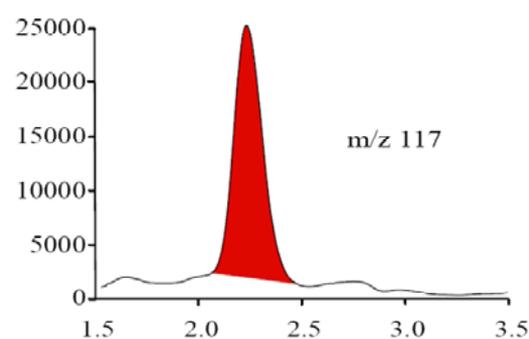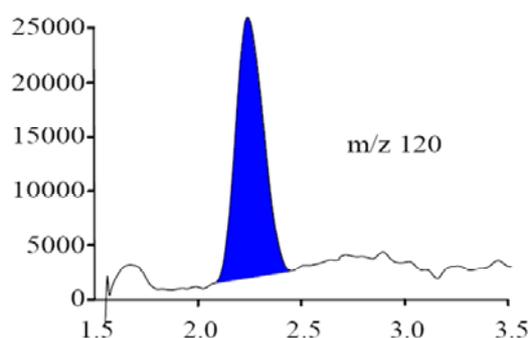
## Chromatographic Data

| No. | Compound | Retention Time (min) | m/z |
|---|---|---|---|
| 1 | Void volume | 0.5 | – |
| 2 | D3-MMA (I.S.) | 2.20 | 120.2 |
| 3 | MMA | 2.22 | 117.2 |

# Methylmalonic Acid (MMA) in Plasma – LCMS
## SeQuant® ZIC®-HILIC

$Y=0.001419X+0.2863$
$R2=0.9992$

**Excellent Linearity in Clinical Relevant Concentration Range**

$Y=0,001487 X +0,6846$
$R2=0.9982$

**Adequate Linearity in Extended Concentration Range**
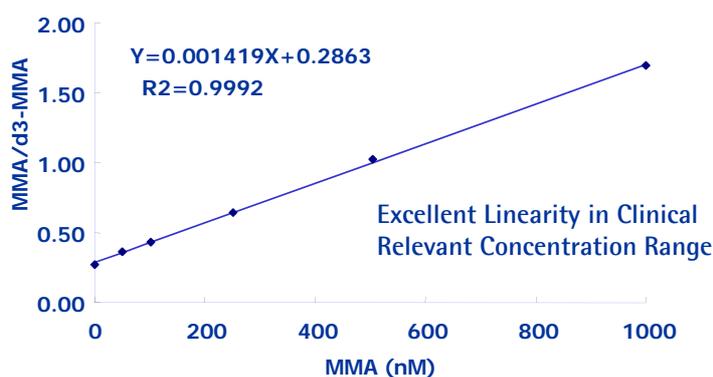
**Quantitation of MMA in plasma LC-MS (negative ESI)**

Limit of Detection = 30 nM
Limit of Quantitation = 90 nM

**Can be used for routine analysis**
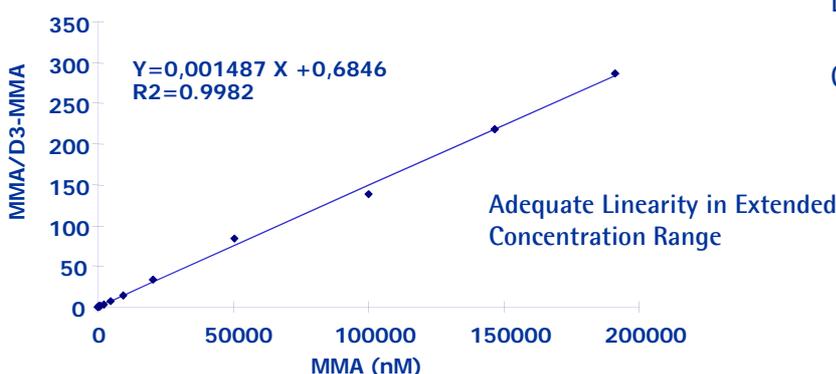
| DAY | Control Endogenous Conc (mM) | S.D | n | Control E+ 100 nM Conc (mM) | S.D | n | Control E + 500 nM Conc (mM) | S.D | n | Control E + 1000 nM Conc (mM) | S.D | n |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 169.5 | 4.7 | 18 | 256.0 | 5.1 | 18 | 629.1 | 17.6 | 18 | 1104.6 | 23.6 | 18 |
| 2 | 179.8 | 5.7 | 18 | 264.0 | 7.9 | 18 | 643.3 | 16.2 | 18 | 1126.9 | 30.5 | 18 |
| 3 | 178.7 | 4.3 | 18 | 270.3 | 8.3 | 18 | 621.8 | 10.8 | 18 | 1144.7 | 52.9 | 18 |
| 4 | 177.8 | 10.7 | 15 | 277.7 | 10.1 | 14 | 622.8 | 14.8 | 15 | 1083.2 | 22.7 | 15 |
| 5 | 169.5 | 4.7 | 18 | 256.0 | 5.1 | 18 | 629.1 | 17.6 | 18 | 1104.6 | 23.6 | 18 |
| 6 | 179.8 | 3.4 | 15 | 289.7 | 8.7 | 15 | 629.9 | 13.7 | 15 | 1100.7 | 40.1 | 15 |
| Average | 175.8 | | | 269.0 | | | 629.3 | | | 1110.8 | | |
| %RSD | 2.6 | | | 3.1 | | | 1.2 | | | 1.9 | | |

Intra-day variability estimated from patient samples, controls and standards containing 0-1.0 μmol/L, at six different days of analysis over a ten weeks period. The analytical recovery rates were between 90 and 93 %. Total number of experiments for each concentration level, n=34. More than 3000 samples, controls and standards were analysed during development and validation.

# Methylmalonic Acid (MMA) in Plasma
# Method Transfer from LC-MS to LC-MS/MS
## SeQuant® ZIC®-HILIC

### Chromatographic Conditions

| | |
|---|---|
| Column: | SeQuant® ZIC®-HILIC (3µm, 100Å) PEEK 100 × 2.1 mm            (1.50441.0001) |
| Injection: | 7 µL in mobile phase |
| Detection: | LC-MS/MS, negative ESI mode, MRM; (m/z 117.1→73.0; 117.1→55.1 and 119.9→75.9) |
| Flow Rate: | See table on page 14. |
| Mobile Phase: | Acetonitrile and 100 mM ammonium acetate, pH 4.5 (80:20 v/v). Total ionic strength: 20 mM |
| Temperature: | 40 °C |
| Sample: | Patient plasma sample treated according to sample preparation protocol. |
| Pressure Drop: | 90 Bar (1305 psi) |

**Quantitation of MMA in plasma LC-MS/MS (negative ESI)**
Limit of Detection = 5 nM
Limit of Quantitation = 15 nM
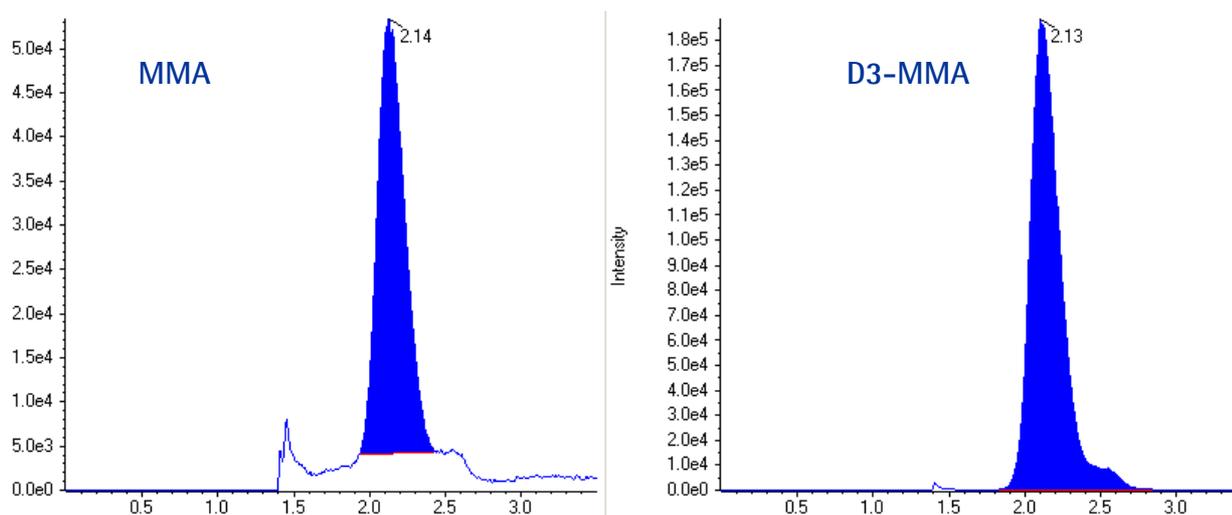


### Chromatographic Data

| No. | Compound | Retention Time (min) | m/z |
|---|---|---|---|
| 1 | Void volume | 0.5 | – |
| 2 | D3-MMA (I.S.) | 2.13 | 119.9→75.9 |
| 3 | MMA | 2.14 | 117.1→73.0 |

# Catecholamines
## – stress biomarkers

Catecholamines are basic organic compounds based on a catechol molecular backbone and a side-chain amine, and they act as neurotransmitters. Epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine are the most common endogenous catecholamines, see figure 2, all produced from phenylalanine and tyrosine and can be monitored through excreted urine. Catecholamines are water-soluble and partly bound to plasma proteins, circulating in the bloodstream; therefore ideal to separate in hydrophilic interaction liquid chromatography (HILIC) mode. pKa values, LogP, and molecular weight for each analyte can be seen in table 1. Simple sample preparation procedures using either dilution followed by filtration or protein precipitation followed by centrifugation can be applied if sample matrix is urine or plasma/serum. This application presents a stepwise method development strategy to find optimal experimental conditions for; a) UV; b) MS and c) fluorescence detection for aforementioned polar molecules useful for urine analysis. The catechol profile differ among urine and plasma/serum wherefore we also have developed a new LC-MS/MS method that allows analysis of metanephrine and normetanephrine, beside dopamine, norepinephrine and epinephrine, thus providing a solution for both saliva urine and plasma/serum analysis.
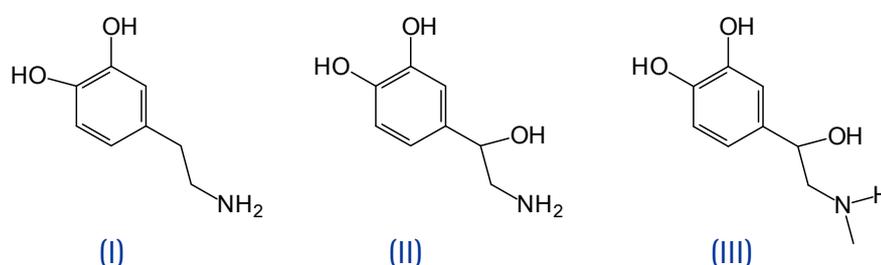


Figure 2. Chemical structure of Dopamine (I), Norepinephrine (II) and Epinephrine (III).

| Compound | pKa | LogP | Molecular weight (g/mol) | Smiles* |
|---|---|---|---|---|
| Dopamine | 8.89 | –1.0 | 153.18 | C1=CC(=C(C=C1CCN)O)O |
| Norepinephrine | 8.4 | –1.2 | 169.18 | C1=CC(=C(C=C1C(CN)O)O)O |
| Epinephrine | 8.55 | –1.4 | 183.20 | CNCC(C1=CC(=C(C=C1)O)O)O |

* The simplified molecular-input line-entry system or SMILES is a specification in form of a line notation for describing the structure of chemical molecules using short ASCII strings. SMILES strings can be imported by most molecule editors for conversion back into two-dimensional drawings or three-dimensional models of the molecules.

## Retention Prediction

Using structural information from each molecule, expressed in table 1 as their corresponding SMILES* string, retention prediction was carried out using the online SeQuant® HILIC prediction model (http://www.emdmillipore.com/chromatography). Using a ZIC®-HILIC column (100x4.6 mm, 5 µm, 200 Å) and an eluent containing 70 volume-% acetonitrile and 30 volume-% 100mM ammonium acetate buffer, pH 6.7, the model predict possible retention for dopamine and epinephrine, whereas norepinephrine would have a retention factor of 0.5. Under slightly acidic conditions (pH 5.56), but overall same conditions, the prediction model indicate less retention for norepinephrine and same possible retention for dopamine and epinephrine. An initial experiment was performed using the more beneficial neutral pH conditions but using a shorter column with only 50 mm length, see figure 3, to verify the retention prediction. Detection (UV) was set at 270 nm.



Figure 3. Separation of epinephrine and norepinephrine on a 50x4.6 mm ZIC®-HILIC column, using 70:30 (v/v) acetonitrile and ammonium acetate buffer (100 mM, pH 6.7) at a flowrate of 0.5 mL/min.

Epinephrine and norepinephrine are both well retained (retention factor around 2 for both compounds) and well resolved from each other (with a resolution factor of 2.3) under given experimental conditions. Thus it is worth pointing out that the online prediction model is only a tool to find out if a molecule has retention in HILIC mode or not. It should not be used to predict retention time. For certain molecules very accurate prediction can nonetheless be obtained. In this examples the useful information was that all molecules will have retention in HILIC mode, but this can also be seen from their LogP values.

## Organic Solvent Effect in Mobile phase Composition

In order to develop a robust and cost-effective assay for different analytical purposes, buffer salt concentration and choice of organic modifier was studied. Early findings revealed that buffer salt concentration in the mobile phase could easily be lowered without compromise on separation power or selectivity (data not shown). The choice of organic modifier was thereafter tested. Acetonitrile was replaced with methanol to find more economical conditions to lower overall analysis cost for a potential quality control method. At the same time, lower detection wavelength was also tested (210 nm instead of 270 nm) in order to get higher method sensitivity.

In figure 4, acetonitrile has been replaced with methanol (but not with adjusted volume ratio in mobile phase to compensate for a methanol's higher elution strength) and a 50% reduction of buffer salt concentration. Surprisingly, more retention is seen for both analytes, but can be explained with the lower total ionic strength in the mobile phase. More expected is the loss of resolution between epinephrine and norepinephrine; from 2.3 to 1.9 due to the effect from methanol on selectivity and separation power. UV detection at 210 nm provide higher sensitivity despite methanol has much higher cut-off than acetonitrile, and being less transparent at lower wavelengths.
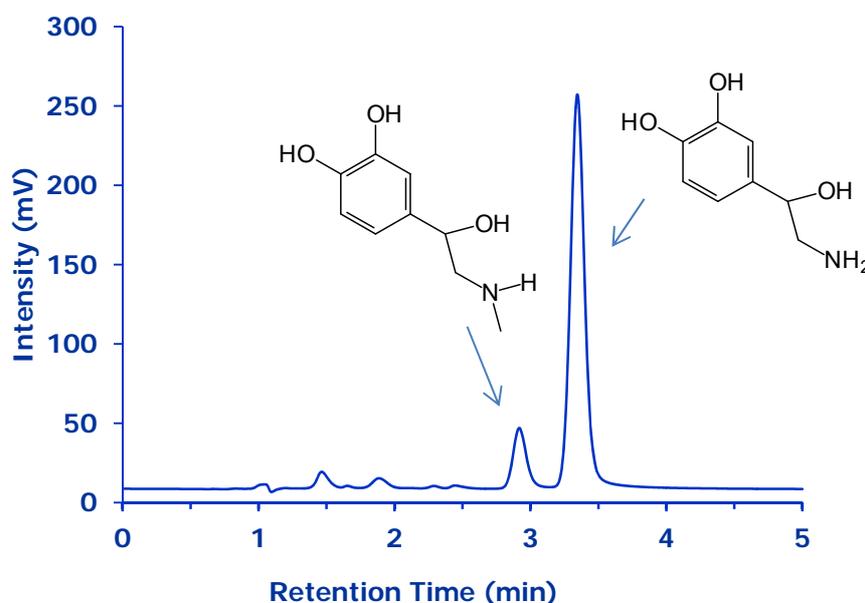


Figure 4. Separation of epinephrine and norepinephrine on a 50x4.6 mm ZIC®-HILIC column, using 70:30 (v/v) methanol and ammonium acetate buffer (50 mM, pH 6.7) at a flowrate of 0.5 mL/min.

To improve the resolution between epinephrine and norepinephrine, the mobile phase proportion of methanol was increased from 70 to 80 volume-%, and thereby also lowering overall buffer salt concentration in the mobile phase from 15 to 10 mM, see figure 5.
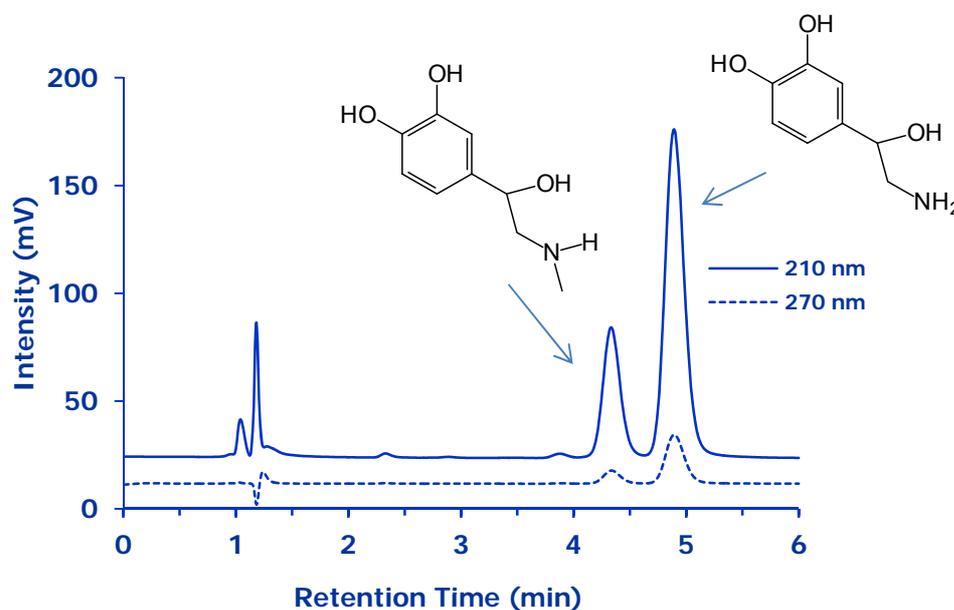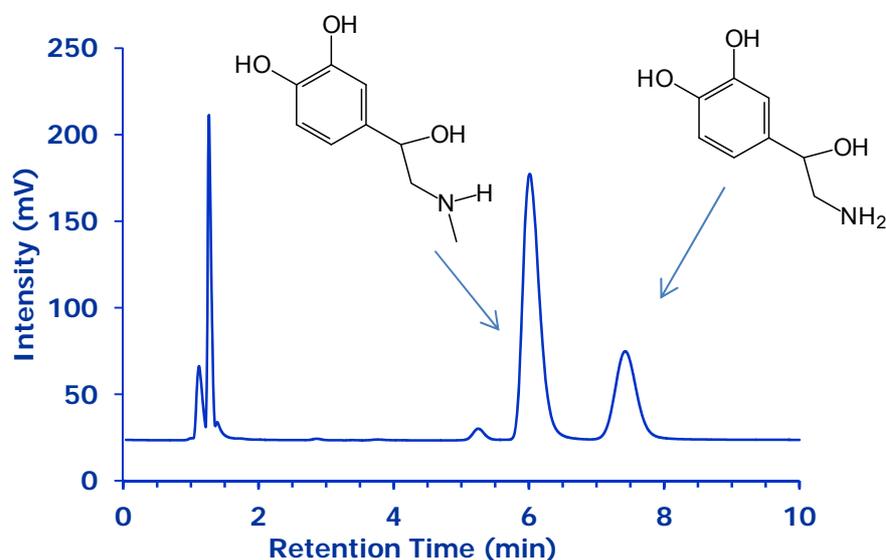
Figure 5. Separation of epinephrine and norepinephrine on a 50x4.6 mm ZIC®-HILIC column, using 80:20 (v/v) methanol and ammonium acetate buffer (50 mM, pH 6.7) at a flowrate of 0.5 mL/min.

As can bee seen in figure 5, the new experimental conditions provide almost to much retentivity to make it into an useful method, i.e. waste of chromatographic space, yet with higher resolution (2.7) between epinephrine and norepinephrine than previous chromatograms. To shorten overall run time and get maximum separation efficiency, methanol was abandoned and acetonitrile was chosen as optimal organic modifier (acetonitrile is a less viscous solvent than methanol thereby providing higher separation efficiency).

**Optimal Conditions for Analysis of Epinephrine and Norepinephrine using Standard LC–UV**
To obtain same resolution between epinephrine and norepinephrine it was found that a volume fraction of 75% acetonitrile was optimum, with constant total ionic strength in mobile phase of 10 mM buffer salt. To speed up the separation it was possible to increase the flow rate from 0.5 to 1.0 mL/min, see figure 6. These results are satisfactory, the method is robust, rapid and fit any standard HPLC system. The experimental conditions given in Figure 5, is thus recommended for a standard HPLC system with UV-detection for quantitation of relatively high concentrations of epinephrine and norepinephrine.

**Scaling of Separation – Finding Optimal Conditions for MS Detection**
To convert the application to more mass spectrometry friendly conditions, the separation in figure 5 was scaled to a 2.1 mm id column. To maximize method sensitivity and still allow a fairly large injection volume, the column length was increased from 50 to 100 mm. When scaling a separation from a 4.6 to 2.1 mm inner diameter column, the effective scaling factor is five and both flowrate and injection volume should be reduced accordingly, as can be seen in figure 7.

Figure 6. Separation of 25 ppm of each epinephrine and norepinephrine on a 50x4.6 mm ZIC®-HILIC column, using 75:25 (v/v) acetonitrile and ammonium acetate buffer (40 mM, pH 6.7; total ionic strength 10 mM) at a flowrate of 1.0 mL/min. Injection volume is 10 µL.
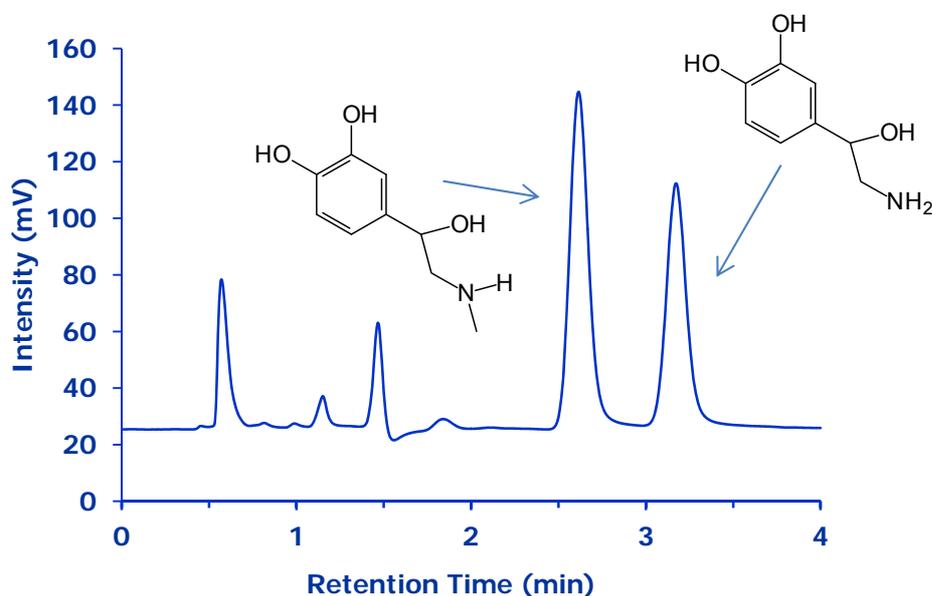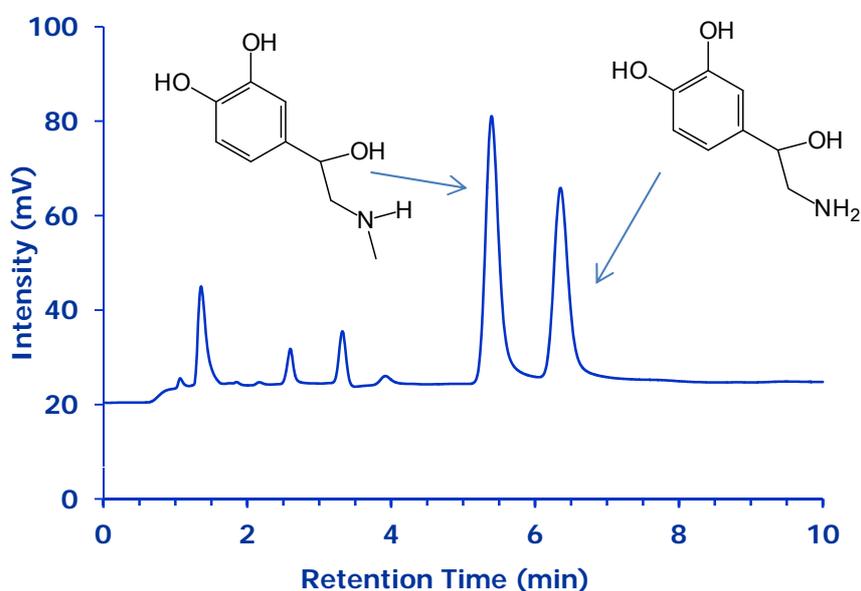


Figure 7. Separation of 25 ppm of each epinephrine and norepinephrine on a 100x2.1 mm ZIC®-HILIC column, using 75:25 (v/v) acetonitrile and ammonium acetate buffer (40 mM, pH 6.7; total ionic strength 10 mM) at a flowrate of 0.2 mL/min. Injection volume is 2 µL.

Same retention factor and same resolution is nonetheless obtained in both figure 6 and 7 (as expected for a scalable and robust stationary phase). The only differences is that the peak height is lower and the overall retention time is doubled for the narrow inner diameter column.

The experimental conditions given in figure 6 provide robust conditions for LC-MS quantitation of epinephrine and norepinephrine. However, if more resolution among the two model analytes is desired or wanted, it is possible to change the pore size of the material instead of further adjustment of mobile phase composition. All separations in figure 3-7 are generated on columns with a stationary phase having a 200 Angstrom pore size. Changing to stationary phase with a smaller pore size, the surface area will be larger, hence more retention will be attained. In figure 8, all experimental parameters are same as in figure 7, but where the stationary phase is changed to contain both a smaller particle size (from 5 to 3.5 μm) and pore size (from 200 to 100 A). This resulted in increase in both overall retention and resolution.



Figure 8. Separation of 25 ppm of each epinephrine and norepinephrine on a 100x2.1 mm ZIC®-HILIC column with 3.5 micron particle size and 100 Å pore size, using 75:25 (v/v) acetonitrile and ammonium acetate buffer (40 mM, pH 6.7; total ionic strength 10 mM) at a flowrate of 0.2 mL/min. Injection volume is 2 μL.

## Analysis of Catecholamines Using other Zwitterionic HILIC Columns

To investigate how this separation work on other HILIC columns in general and on other bonded zwitterionic HILIC stationary phases more specifically. Two columns were chosen; one 100x4.6 mm id Nucleodur HILIC column with 3 micron particles having 100 Ångstrom (Å) pore size, and one 100x4.6 mm id Shiseido PC-HILIC with 5 micron particles also having 100 Angstrom pore size. Neither of these columns were commecially available in 2.1 mm id format when this study was carried out, hence a slight discrepancy in data.
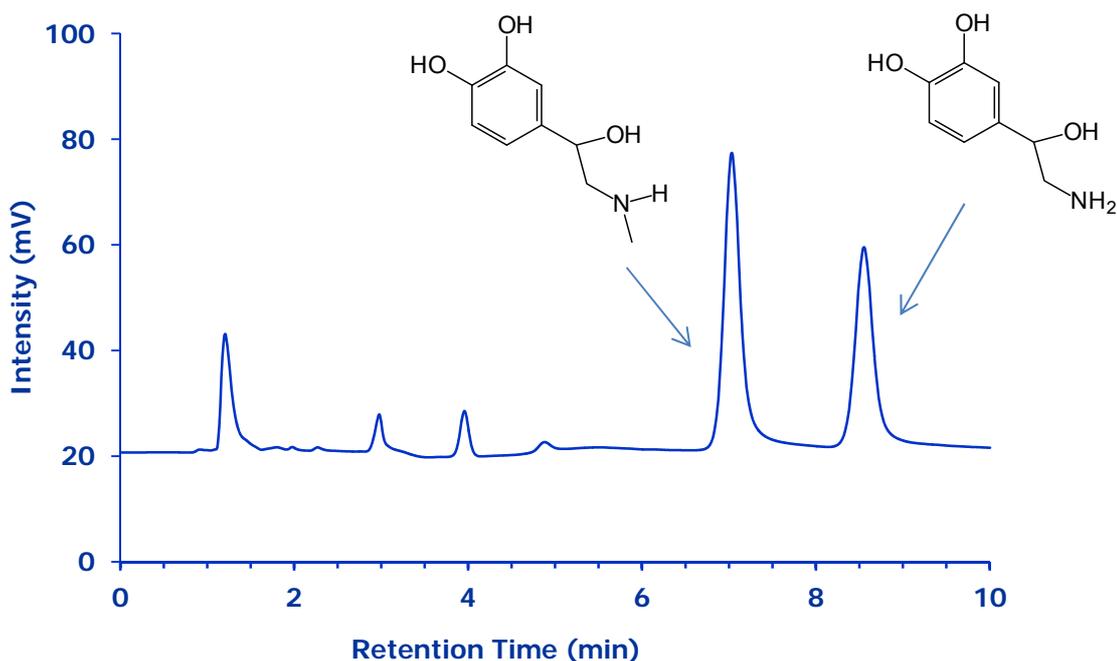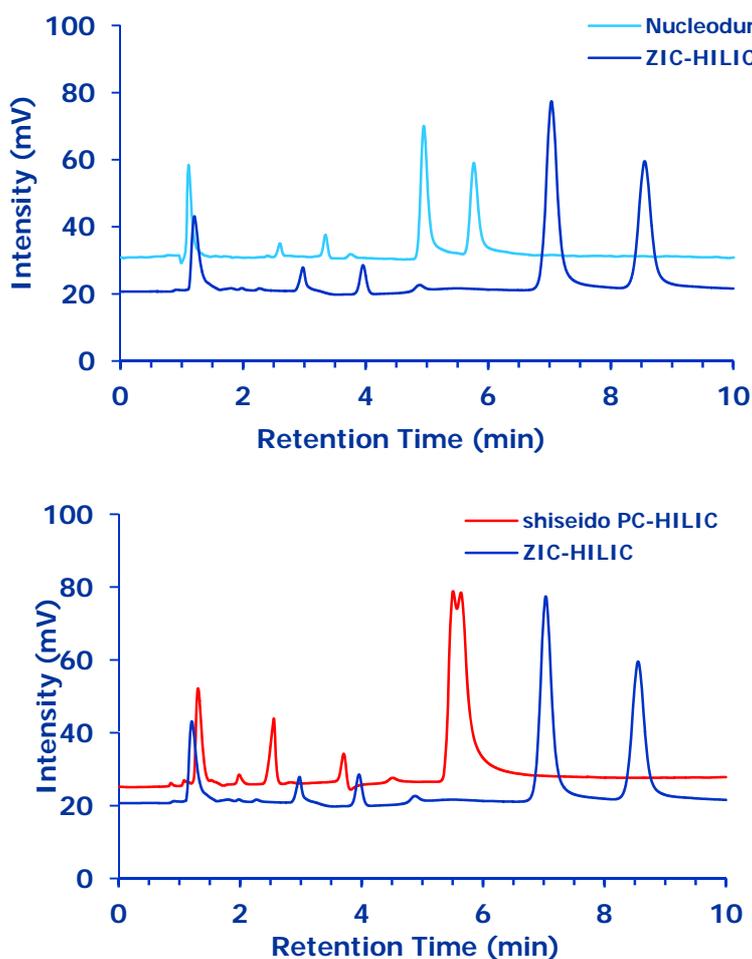
Figure 9 and 10. Separation of 25 ppm of each epinephrine and norepinephrine on a 100x2.1 mm ZIC®-HILIC column with 3.5 micron particle size and 100 Å pore size (dark blue trace), on a 100x2.1 Nucleodur HILIC column with 3.5 micron particle size and 100 A pore size (light blue trace), and on a 100x4.6 mm id Shiseido PC-HILIC with 5 micron particles also having 100 Angstrom pore size (red trace) using 75:25 (v/v) acetonitrile and ammonium acetate buffer (40 mM, pH 6.7; total ionic strength 10 mM) at a flowrate of 0.2 mL/min (ZIC-HILIC) or 1.0 mL/min (Nucleodur and Shiseido PC-HILIC) and. injection volume was 2 μL (ZIC-HILIC) or 10 μL (Nucleodur and Shiseido PC-HILIC).

## Analysis of Catecholamines in Urine with HILIC and Fluorescence Detection

Many endogenous molecules such as catecholamines have been analyzed in clinical laboratories with lengthy methods using older techniques and where the data validity could be questioned. Of this reason, there is a vast interest in developing new assays at hospitals and laboratories. In many situations, LC-MS and LC-MS/MS methods are sought for reasons of sensitivity and specificity. However, in terms of sensitivity, for some compounds fluorescence detection can be a viable alternative.

Catecholamines such as dopamine, epinephrine and norepinephrine are hydrophilic compounds, and therefore very suitable for HILIC, but they also have molecular backbones, see figure1, that would allow use of fluorescence (FL) detection. HILIC combined with FL detection can provide high sensitivity due to use of high percentage of organic solvent in mobile phase, and where the potential impurity contribution from water and reagent sources can be reduced compared with a classical reversed phase (RP) – fluorescence detection method. Obvious drawback with both RP and HILIC-FL is that positive identification as obtained with mass spectrometric detection is nonetheless not possible. For laboratories and hospitals with narrow or no budgets for expensive instruments HILIC-FL can represent a more economical approach. In figure 11, a chromatogram is shown for the separation of a standard solution containing dopamine, epinephrine and norepinephrine, and figure 12 show applicability to human urine samples diluted 100 times with mobile phase as only sample preparation method.
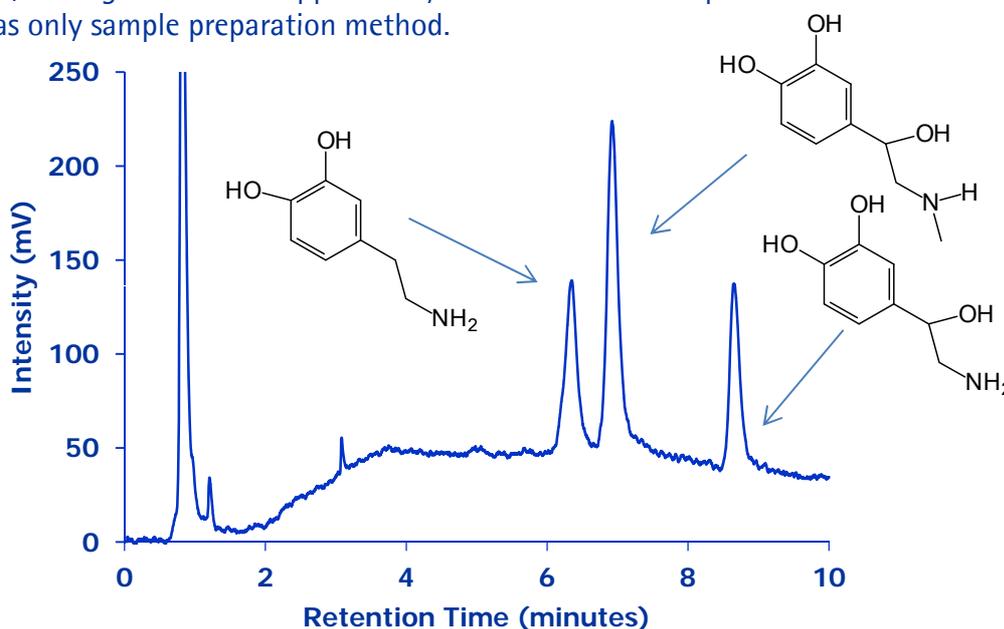


Figure 11. Separation of 50 ppb dopamine and epinephrine, and 75 ppb norepinephrine on a 150x4.6 mm ZIC®-HILIC column with 3.5 micron particle size and 100 A pore size, using gradient elution with acetonitrile and ammonium formate buffer at a flowrate of 2.0 mL/min. See experimental section for complete experimental details.
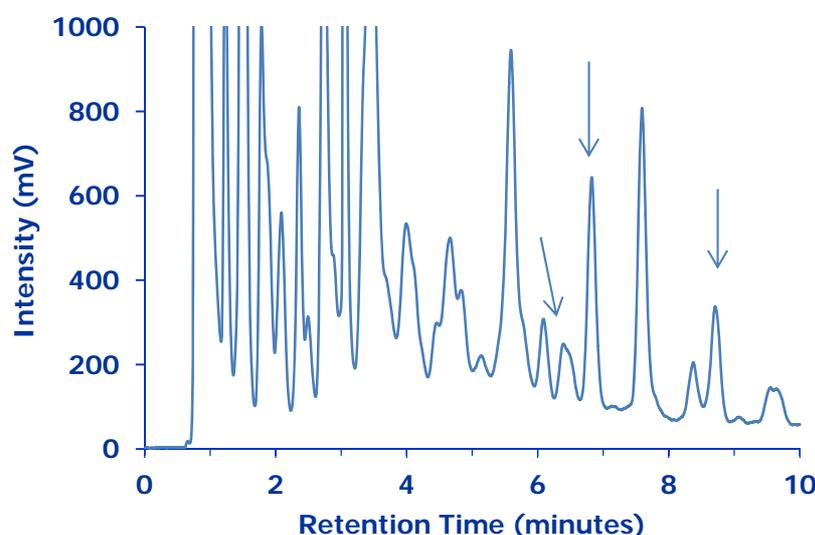
Figure 12. Analysis of a human urine sample (stored in cooler 24 hours) diluted 100X in mobile phase A on a 150x4.6 mm ZIC®-HILIC column with 3.5 micron particle size and 100 Å pore size, using gradient elution with acetonitrile and ammonium formate buffer at a flowrate of 2.0 mL/min. See experimental section for complete experimental details.

## Experimental conditions for Urine Analysis with SeQuant® ZIC®-HILIC

**Column:** SeQuant® ZIC®-HILIC (5μm, 200Å) 150 x 4.6 mm (1.51455.0001)

**Mobile phase (v/v):** A: Acetonitrile/NH4formate 25 mM, pH 6.3 (90:10 v/v)
B: Acetonitrile/NH4formate 100 mM, pH 3 (80:20 v/v)

**Gradient:**

| Time (min) | % A | % B | |
|---|---|---|---|
| 0-10 | 100→0 | 0→100 | Linear gradient |
| 10-15 | 100 | 0 | Equilibration |

**Flow rate:** 2.0 mL/min.

**Chromatographic system:** Hitachi VWR Chromaster with Fluorescence Detection (Ex=260 nm and Em=320 nm)

**Injection Volume:** 30 μL

**Temperature:** 40 °C

**Sample:** 50 ppb of Dopamine and Adrenaline, 75 ppb of Noradrenaline diluted in mobile phase A
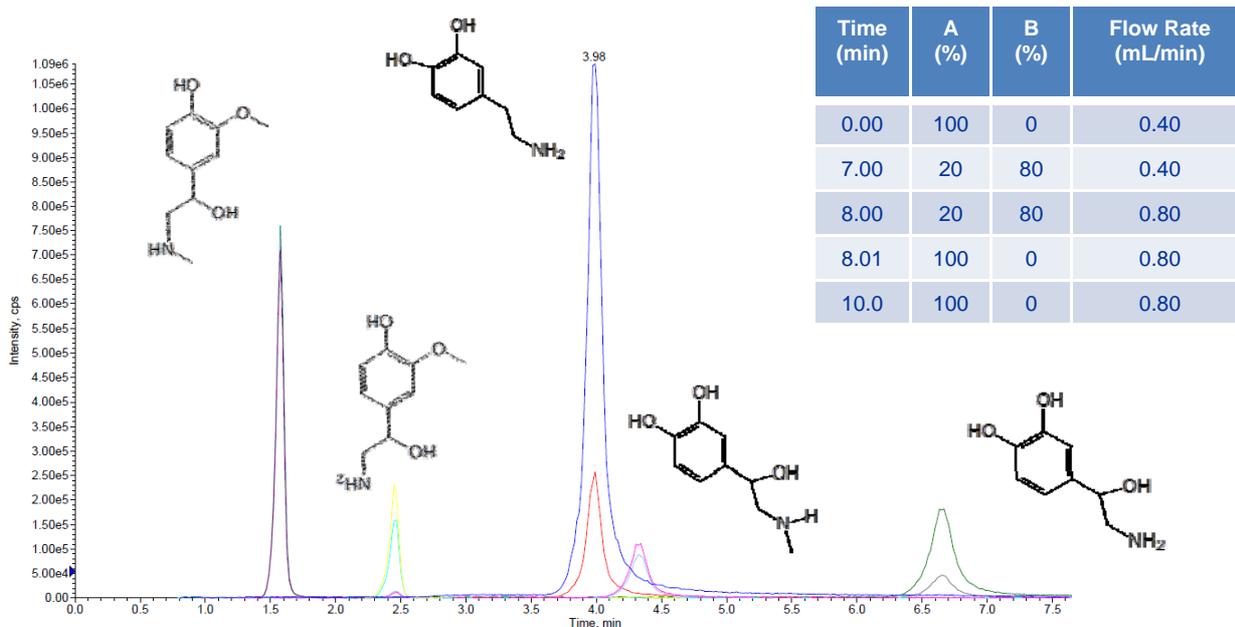
**Sample preparation:** 1. Pipette 100 μL urine and diluted to 10 mL with mobile phase A.
2. Fill autosampler vials with sample and perform direct injection.

# Catecholamines in Urine and Plasma – MS/MS
## SeQuant® ZIC®-cHILIC

### Chromatographic Conditions

| | | |
|---|---|---|
| Column: | SeQuant® ZIC®-cHILIC (3µm, 100Å) PEEK 100 × 2.1 mm | (1.50658.0001) |
| Injection: | 30 µL in mobile phase | |
| Detection: | LC-MS/MS, positive ESI mode | |
| Flow Rate: | 1.0 mL/min | |
| Mobile Phase: | A: Acetonitrile and ammonium formate (25 mM, pH 6.3) 90:10 v/v | |
| | B: Acetonitrile and ammonium formate (25 mM, pH 6.3) 80:20 v/v | |
| Temperature: | 50 °C | |
| Sample: | 20 ppb of each, metanephrine, normetanephrine, dopamine, epinephrine and norepinephrine in mobile phase. | |



| Time (min) | A (%) | B (%) | Flow Rate (mL/min) |
|---|---|---|---|
| 0.00 | 100 | 0 | 0.40 |
| 7.00 | 20 | 80 | 0.40 |
| 8.00 | 20 | 80 | 0.80 |
| 8.01 | 100 | 0 | 0.80 |
| 10.0 | 100 | 0 | 0.80 |

### Chromatographic Data

| No. | Compound | Retention Time (min) | m/z | |
|---|---|---|---|---|
| 1 | Void volume | 0.5 | – | |
| 2 | Metanephrine | 1.6 | 198.1→165.0 | 198.1→148.0 |
| 3 | Normetanephrine | 2.5 | 184.1→149.1 | 184.1→134.1 |
| 4 | Dopamine | 4.0 | 154.0→137.1 | 154.0→119.1 |
| 5 | Epinephrine | 4.3 | 184.0→151.0 | 184.0→135.1 |
| 6 | Norepinephrine | 6.7 | 170.0→152.1 | 154.0→135.2 |

# Ethyl Glucuronide and Ethylsulfate
## – alcohol abuse biomarkers

Ethyl glucuronide (EtG) is a metabolite of ethyl alcohol which is formed in the body by glucuronidation following exposure to ethanol, such as by drinking alcoholic beverages. The usefulness of ETG as a recent alcohol consumption biomarker has been studied widely. A disadvantage of the test is that because EtG can be detected in samples at very low levels, it can also be positive after exposure to alcohol from non-beverage sources, or incidental exposure, which can lead to innocent positives. It has been found that EtG can only be formed after alcohol ingestion and has been found not to be formed endogenously. Therefore, the presence of EtG is definitive evidence of anti-mortem alcohol intake. However, negative results of EtG should be interpreted with caution as false negative results may be obtained. There is a time lag between alcohol present in blood and EtG which may lead to false negative results if death happened shortly after alcohol consumption.

Recently, the stability of EtG has been brought into question which was found to be degraded by bacteria. Therefore, ethylsulfate (ETS) has been introduced as a complementary marker with EtG due to its stable pattern and resistance to bacterial infection; the presence of EtG and ETS provides strong evidence of recent alcohol consumption. An alternative marker for ethanol intake is phosphatidylethanols (PEth), a group of phospholipids formed only in the presence of ethanol via the action of phospholipase D.

EtG and ETS are though promising biomarkers because they are phase two ethanol metabolites and their excretion profiles have been studied and documented. Also, their standards and internal standards are available commercially and can be detected using LC-MS/MS.

The following method was aimed at developing and validating an LC-ESI-ion trap-MS/MS method for identification and quantification of EtG and ETS as ethanol biomarkers from urine samples.*
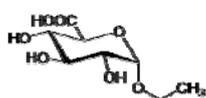
Ethyl glucuronide (EtG)          Ethyl sulfate (ETS)
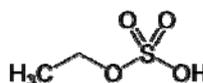
# Ethyl Glucuronide and Ethyl sulfate
## SeQuant® ZIC®-HILIC

Recommended column:
SeQuant® ZIC®-HILIC (3µm, 100Å) PEEK 150 × 2.1 mm                    (1.50442.0001)
SeQuant® ZIC®-HILIC (guard fitting) PEEK 14 × 1.0 mm                    (1.50434.0001)

Recommended solvents and reagents:

Acetonitrile:    hypergrade for LC-MS LiChrosolv®                    (1.00029)

Water:           Water for chromatography LiChrosolv®                    (1.15333)
                 or freshly purified water from Milli-Q® water purification system

Ammonium acetate (HPLC grade) or in-situ prepared buffer from ammonia and acetic acid

Ammonia solution 28-30% for analysis EMSURE® ACS, Reag. Ph Eur       (1.05423)
Acetic acid 96% for analysis EMSURE®                                 (1.00062)
Formic acid 98-100% for analysis EMSURE® ACS, Reag. Ph Eur           (1.00264)

**Mobile phase:**

| Time (min) | A (%) | B (%) | Flow Rate (mL/min) |
|---|---|---|---|
| 0.00 | 90 | 10 | 0.20 |
| 3.00 | 90 | 10 | 0.20 |
| 3.01 | 90 | 10 | 0.40 |
| 4.00 | 70 | 30 | 0.40 |
| 7.00 | 70 | 30 | 0.20 |
| 12.00 | 50 | 50 | 0.20 |
| 12.01 | 90 | 10 | 0.20 |
| 20.00 | 90 | 10 | 0.20 |

### HPLC Sample Preparation
Following addition of pentadeuterated internal standards for ETG and ETS, 200 µl of acetonitrile was added to 0.1 ml of urine and centrifuged at 10000 rpm. The supernatant was then evaporated before reconstituting with 100 µL of initial mobile phase prior to LC-MS/MS analysis.
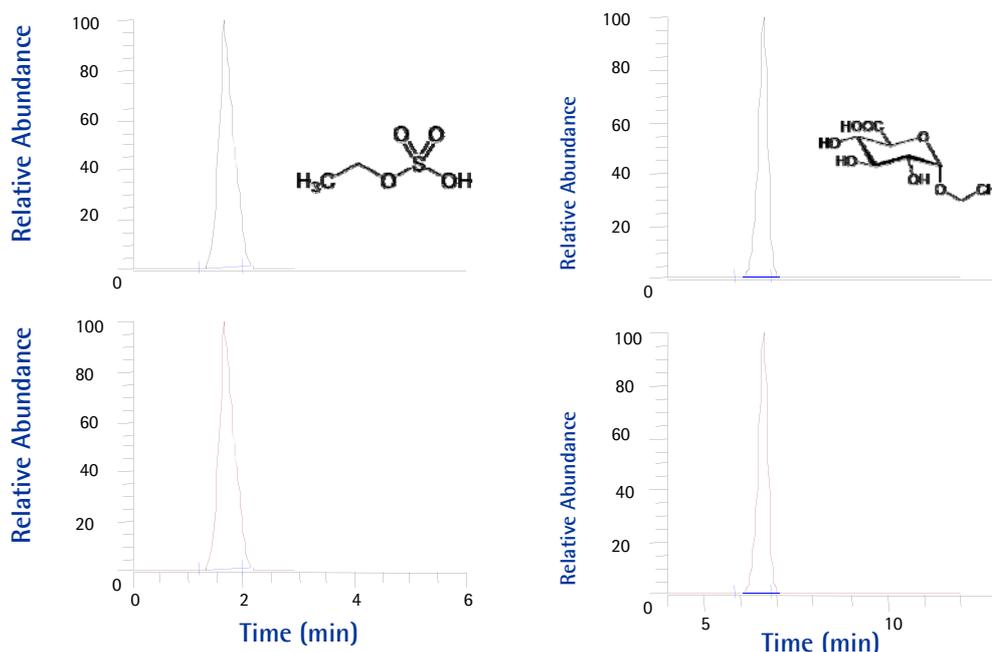
# Ethyl Glucuronide and Ethyl sulfate in Urine
## SeQuant® ZIC®-HILIC

## Chromatographic Conditions

| | | |
|---|---|---|
| Column: | SeQuant® ZIC®-HILIC (3µm, 100Å) PEEK 150 × 2.1 mm | (1.50442.0001) |
| Injection: | 5 µL in mobile phase | |
| Detection: | LC-ESI ion trap MS/MS | |
| Flow Rate: | See table | |
| Gradient | See table | |
| Mobile Phase: | A: Acetonitrile<br>B: Ammonium acetate 5mM, pH 6.8 | |
| Temperature: | 25 °C (column oven) and 4 °C (autosampler) | |
| Sample: | Urine samples treated according to sample preparation protocol. | |

### Quantitation of ES and EtG in Urine
### Lower limit of Quantitation = 1 µg/L (1 ppt)



## Chromatographic Data

| No. | Compound | Retention Time (min) | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|
| 1 | Void volume | 1.3 | – | |
| 2 | Ethylsulfate | 1.7 | 125.5 | 97.5 |
| 3 | Ethyl glucuronide (EtG) | 6.7 | 221.5 | 103, 113 |

# Cortisol
## – the most important corticosteroid

Cortisol is the main glucocorticoid in humans. It is synthesized in the adrenal cortex and follows ACTH's diurnal variation causing the cortisol concentration in plasma to vary during the day, with the highest concentration in the morning and lowest concentration around midnight. Cortisol is released in response to stress and a low level of blood glucocorticoids. Its primary functions are to increase blood sugar; suppress the immune system; and aid in fat, protein and carbohydrate metabolism. It also decreases bone formation. Cortisol is also used for diagnosing Cushing's syndrome. In plasma cortisol is transported bound. The free fraction represents about 10% of the total content of cortisol. The main indication for the determination of serum cortisol is suspicion of adrenocortical hyperfunction. One of the earliest signs of overproduction of cortisol is an absence of diurnal variation in serum cortisol levels. Salivary cortisol is a measure of the levels of free cortisol, but urine samples are also commonly taken from patients for clinical analysis.
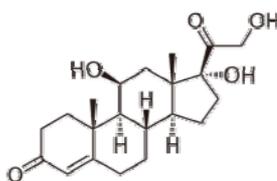


Cortisol

On the following pages, we show a new method for analysis of cortisol using reversed phase LC-MS/MS were patient samples were analysed along with standards and control samples.

# Cortisol in Saliva and Urine
## Purospher® STAR RP-18 endcapped

Recommended column:
Purospher® STAR RP-18 endcapped (2µm) Hibar® HR 100-2.1 mm          (1.50648.0001)

Recommended solvents and reagents:

Methanol:      hypergrade for LC-MS LiChrosolv®          (1.06035)

Water:        Water for chromatography LiChrosolv®          (1.15333)
              or freshly purified water from Milli-Q® water purification system

Formic acid 98-100% for analysis EMSURE® ACS, Reag. Ph Eur          (1.00264)

Mobile phase:  A: 0.1% formic acid in Milli-Q water
               B: 0.1% formic acid in methanol

| Time (min) | A (%) | B (%) | Flow Rate (mL/min) |
|---|---|---|---|
| 0.00 | 70 | 30 | 0.40 |
| 0.50 | 50 | 50 | 0.40 |
| 4.80 | 50 | 50 | 0.40 |
| 4.81 | 0 | 100 | 0.60 |
| 5.80 | 0 | 100 | 0.60 |
| 5.81 | 70 | 30 | 0.40 |
| 7.00 | 70 | 30 | 0.40 |

### HPLC Sample Preparation

Saliva and Urine samples:
Centrifuged saliva or urine samples. Add internal standard (Cortisol-D4).
(IS solution preparation (50 nM): 1.0 mL IS-standard (Cortisol-D4 1µM) in 20 mL milli-Q water)
The samples are thereafter prepared using supported liquid extraction (SLE) where methyl tert-butyl ether is used as eluent. Eluted sample is taken to dryness with nitrogen and reconstituted in initial mobile phase composition prior to injection on LC-MS/MS system.

### Quantitation of Cortisol in saliva and urine
Linear range: 0.25 – 300 nM (nmol/L).
Lower limit of Quantitation = 0.25 nM

# Cortisol in Saliva and Urine
## Purospher® STAR RP-18endcapped

## Chromatographic Conditions

| | |
|---|---|
| Column: | Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 100-2.1 mm (1.50648.0001) |
| Injection: | 10 µL |
| Detection: | LC-ESI MS/MS; MRM transitions: m/z 363.1/121.1 (cortisol) and m/z 367.1/121.1 (Cortisol-D4) |
| Flow Rate: | 0.4 mL/min |
| Gradient | See table |
| Mobile Phase: | A: 0.1% formic acid in Milli-Q water |
| | B: 0.1% formic acid in methanol |
| Temperature: | 50 °C |
| Sample: | Urine samples treated according to sample preparation protocol. |
| Backpressure: | 360 bar (5184 psi) at start of gradient |

### Quantification of cortisol (1.0 nM standard) using cortisol-D4 as I.S.
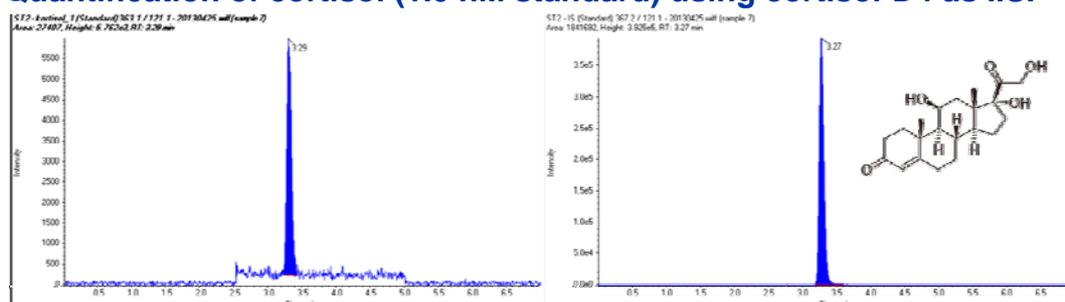


### Analysis of cortisol in patient samples (urine)
### - Calculated concentration: 16 nM
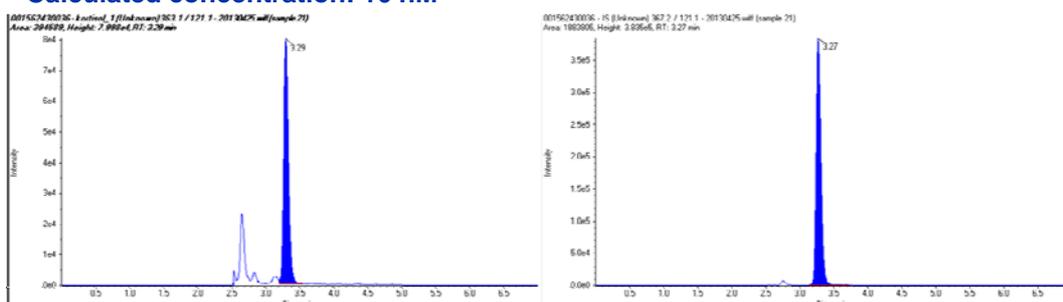


### Chromatographic Data

| No. | Compound | Retention Time (min) | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|
| 1 | Void volume | 1.3 | – | |
| 2 | Cortisol-D4 | 3.27 | 363.1 | 121.1 |
| 3 | Cortisol | 3.29 | 367.1 | 121.1 |

# Vitamin D2 and D3 in Serum/Plasma

Vitamin D is a group of fat-soluble secosteroids. In humans, the most important compounds are vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). These compounds can be ingested from the diet, from supplements but also through sun exposure of the skin. Vitamin D is not an essential dietary vitamin since a substance is only classified as an essential vitamin when it cannot be synthesized in sufficient quantities by an organism, and must be obtained from diet.

In the liver, vitamin D3 is converted to 25-hydroxyvitamin D3, whereas vitamin D2 is converted to 25-hydroxyvitamin D2. These are the two specific vitamin D metabolites that are measured in serum and plasma to determine a person's vitamin D status. Analysis of vitamin D's (25-OH D2 and D3) have emerged as a very important clinical method and currently several different patient conditions can be linked to low concentrations of vitamin D's.
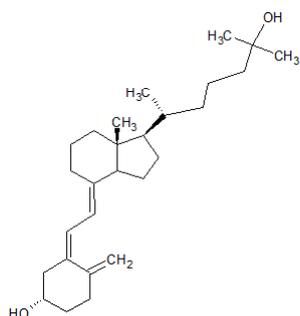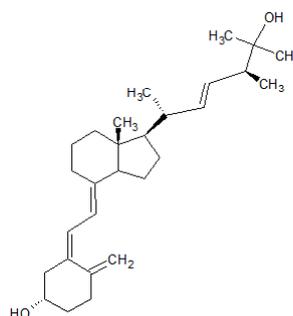


25-Hydroxyvitamin D3                    25-Hydroxyvitamin D2

We present here a method for analysis of aforementioned molecules using reversed phase LC-MS/MS. Serum and plasma samples from patients were analysed along with standards and control samples. Samples were prepared via simple protein precipitation using acetonitrile containing 1% formic acid and the two internal standards 2H3-25-OH vitamin D2/D3. It is not possible to completely eliminate the endogenous levels of 25-OH Vitamin D2/D3 in human serum/plasma wherefore deuteriated 25-OH Vitamin D2/D3 are needed. These deuteriated internal standards each have six hydrogen atoms replaced with deuterium; 2H6-25-OH vitamin D2/D3. Standard curves are created from standard samples where the ratio of the deuteriated 25-OH vitamin D2/D3 (2H6-25-OH vitamin D2/D3) and the two internal standards 2H3-25-OH vitamin D2/D3 plotted against the ratio of the concentrations of the same.

# Vitamin D2 and D3 in Serum/Plasma
## Purospher® STAR RP-18 endcapped

Recommended column:
Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 100-2.1 mm           (1.50648.0001)

Recommended solvents and reagents:

Methanol:      hypergrade for LC-MS LiChrosolv®                              (1.06035)

Water:         Water for chromatography LiChrosolv®                         (1.15333)
               or freshly purified water from Milli-Q® water purification system

Formic acid 98-100% for analysis EMSURE® ACS, Reag. Ph Eur           (1.00264)

Mobile phase:  A: Milli-Q water
               B: Methanol

| Time (min) | A (%) | B (%) | Flow Rate (mL/min) |
|---|---|---|---|
| 0.00 | 15 | 85 | 0.40 |
| 4.50 | 15 | 85 | 0.40 |
| 4.51 | 0 | 100 | 0.50 |
| 5.53 | 0 | 100 | 0.50 |
| 5.55 | 15 | 85 | 0.40 |
| 6.50 | 15 | 85 | 0.40 |

**Quantitation of Vitamin 25-hydroxy-D2 and D3 in plasma/serum**
Linear range: 11 – 370 nM (nmol/L).

# Vitamin D2 and D3 in Serum/Plasma
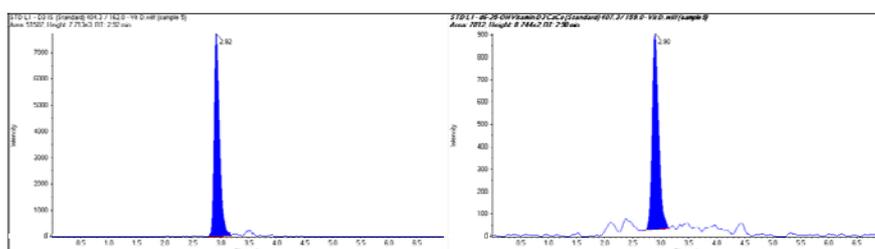## Purospher® STAR RP-18endcapped

### Chromatographic Conditions

| | |
|---|---|
| Column: | Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 100-2.1 mm    (1.50648.0001) |
| Injection: | 10 µL |
| Detection: | LC-MS/MS APCI; MRM transitions: m/z 419.3/355.1, 416.3/358.1 (vitamin D2) and m/z 407.3/159.0 and 404.3/162.0 (vitamin D3) |
| Flow Rate: | See table |
| Gradient | See table |
| Mobile Phase: | A: Milli-Q water<br>B: Methanol |
| Temperature: | 50 °C |
| Sample: | Patient urine samples. |
| Backpressure: | 360 bar (5184 psi) at start of gradient |

**Lowest standard for 25-hydroxyvitamin D2, 12.9 nmol/L, (right chromatogram)**
**Internal standard D3-25-hydroxyvitamin D2, 50 nmol/L, (left chromatogram)**
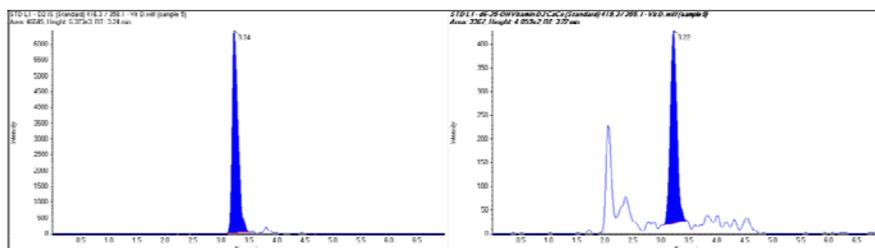


**Lowest standard for 25-hydroxyvitamin D3, 11.3 nmol/L, (right chromatogram)**
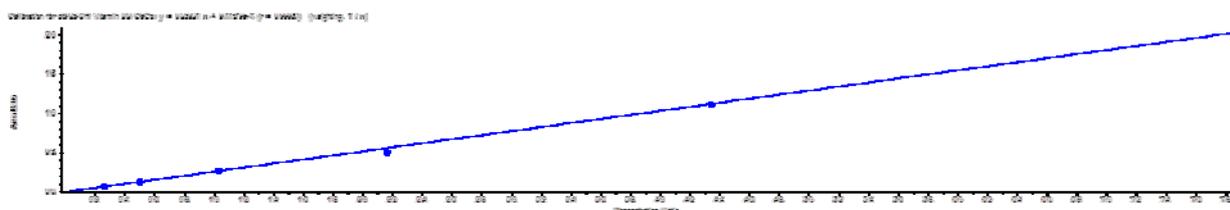**Internal standard D3-25-hydroxyvitamin D3, 50 nmol/L, (left chromatogram)**



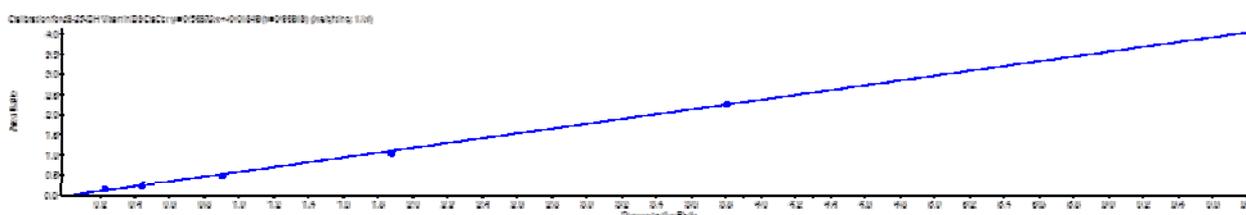### Chromatographic Data

| No. | Compound | Retention Time (min) | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|
| 1 | Void volume | 0.5 | – | |
| 2 | 25-OH vitamin D3 | 2.9 | 404.3 | 162.0 |
| 3 | 25-OH vitamin D2 | 3.2 | 416.3 | 358.1 |

# Vitamin D2 and D3 in Serum/Plasma
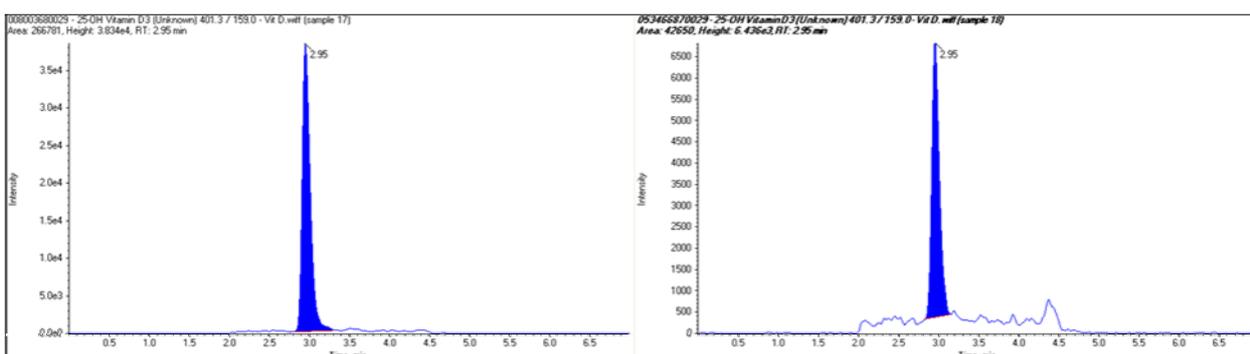## Purospher® STAR RP–18endcapped



**Calibration curve for 25-hydroxyvitamin D2 (12.9–393 nmol/L), r = 0.9985**



**Calibration curve for 25-hydroxyvitamin D3 (11.3–341 nmol/L), r = 0.9992**

## Patient samples



**Analysis of two patient samples containing 25-hydroxyvitamin D3 (55.7 and 10.0 nmol/L). No 25-hydroxyvitamin D2 was found in these samples.**

# Exogenous Compounds

Most drugs (i.e. active pharmaceutical ingredients - API's) are small molecules, although some drugs can be proteins, e.g. insulin. APIs have traditionally followed the Lipinski rule of five and been relatively hydrophobic molecules. The Lipinski rule states that a compound is more likely to be membrane permeable and easily absorbed by the body if it matches the following criteria:

-       Its molecular weight is less than 500.

-       The compound's lipophilicity, expressed as a quantity known as logP (the logarithm of the partition coefficient between water and 1-octanol), is < 5.

-       The number of groups in the molecule able to donate hydrogen atoms to hydrogen bonds (usually the sum of hydroxyl and amine groups in a drug molecule) is < 5.

-       The number of groups that can accept hydrogen atoms to form hydrogen bonds (estimated by the sum of oxygen and nitrogen atoms) is < 10.

The rules apply only to absorption by passive diffusion of compounds through cell membranes; compounds that are actively transported through cell membranes by transporter proteins are exceptions to the rule. Small molecules are more likely to be absorbed, although some of them are only absorbed after oral administration if given as prodrugs. One advantage small molecule drugs (SMDs) have over "large molecule" biologics is that many SMDs can be taken orally whereas biological drugs generally require injection or another type of administration.

Reversed phase HPLC has been the backbone of pharmaceutical analysis for over 25 years, and the technique has been particularly successful for APIs. Herein we exemplify this with bioanalytical methods for two drugs; **tramadol** and **buprenorphine**.

Roughly 10% of marketed drugs do not however follow Lipinski's rule, i.e. many chemotherapeutic agents (anti-cancer drugs) since they are highly hydrophilic. Here we exemplify this with a bioanalytical method for a hydrophilic drug; **metformin** (diabetes treatment). For this drug the technique of choice is HILIC-MS in positive ESI mode.

# Metformin
## – Type 2 Diabetes medication

Metformin (sold as Glucophage) is an oral antidiabetic drug. It is the first-line treatment of type 2 diabetes, in particular, in overweight and obese people and those with normal kidney function. Metformin is the only antidiabetic drug that, convincingly, prevent the cardiovascular complications of diabetes. It helps reduce LDL cholesterol and triglyceride levels, and is not associated with weight gain. Metformin is believed to be the most widely prescribed antidiabetic drug in the world; in the United States alone, more than 48 million prescriptions were filled in 2010 for its generic formulations. Metformin is also used in the treatment of polycystic ovary syndrome, and has been investigated for other diseases where insulin resistance may be an important factor. Metformin works by suppressing glucose production by the liver.

Previous analytical methods typically involve separations on reversed phase columns with ion pair reagents, or ion exchange columns. Both these approaches are unsuitable for mass spectrometry detection (MS). HILIC is on the other hand very suitable for connection to MS and gives a simplified sample preparation for plasma and other biomatrices. HILIC is also suitable for the metformin impurity assay which by recent guidelines from FDA now involves melamine analysis. We present here a rapid bioanalytical method using HILIC-MS combined with simple protein crash sample preparation that allow a high sample-throughput, yet sensitive and with excellent recoveries. An external calibration curve were constructed in the range 0.05–1.0 ppm. The new method was, furthermore, also tested for plasma analysis against an alternative HILIC column having a lower amount of bonded stationary phase which also turned out to be more unstable, leading to higher background and thus lower sensitivity and accuracy.
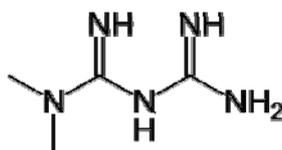


Metformin

# Metformin in Plasma
## SeQuant® ZIC®-HILIC

Recommended column:

SeQuant® ZIC®-HILIC (5µm, 200Å) PEEK 100 × 2.1 mm                    (1.50452.0001)

Recommended solvents and reagents

Acetonitrile:   hypergrade for LC-MS LiChrosolv®                    (1.00029)

Water:          Water for chromatography LiChrosolv®               (1.15333)
                or freshly purified water from Milli-Q® water purification system

Ammonium acetate (HPLC grade) or in-situ prepared buffer from ammonia and acetic acid

Ammonia solution 28-30% for analysis EMSURE® ACS,Reag. Ph Eur      (1.05423)
Acetic acid 96% for analysis EMSURE®                              (1.00062)

### HPLC Sample Preparation
Sample preparation by dilution of plasma/serum in acetonitrile (1:4), followed by centrifugation at 6400 rpm (15 °C) and direct injection of 1 µL supernatant in the LC/MS instrument.

# Metformin in Plasma
## SeQuant® ZIC®-HILIC

### Chromatographic Conditions

| | |
|---|---|
| Column: | SeQuant® ZIC®-HILIC (5µm, 200Å) PEEK 100 × 2.1 mm         (1.50452.0001) |
| Injection: | 1 µL of supernatant from protein precipitation (acetonitrile and Milli-Q water; 80:20 v/v) |
| Detection: | LC–MS, positive ESI mode, SIM; m/z 130 |
| Flow Rate: | 0.5 mL/min |
| Mobile Phase: | Acetonitrile and 10 mM ammonium acetate, pH 6.8 (85:15 v/v). Total ionic strength: 1.5 mM |
| Temperature: | 30 °C |
| Sample: | Patient plasma sample treated according to sample preparation protocol. |

| Parameter | ZIC®-HILIC | Other HILIC |
|---|---|---|
| Retention Time | 2.1 min | 1.7 min |
| Relative Signal | 100 | 21 |
| Relative Background | 1 | 3.2 |
| Linearity | 0.9999 | 0.9974 |
| Recovery | 108% | 140% |
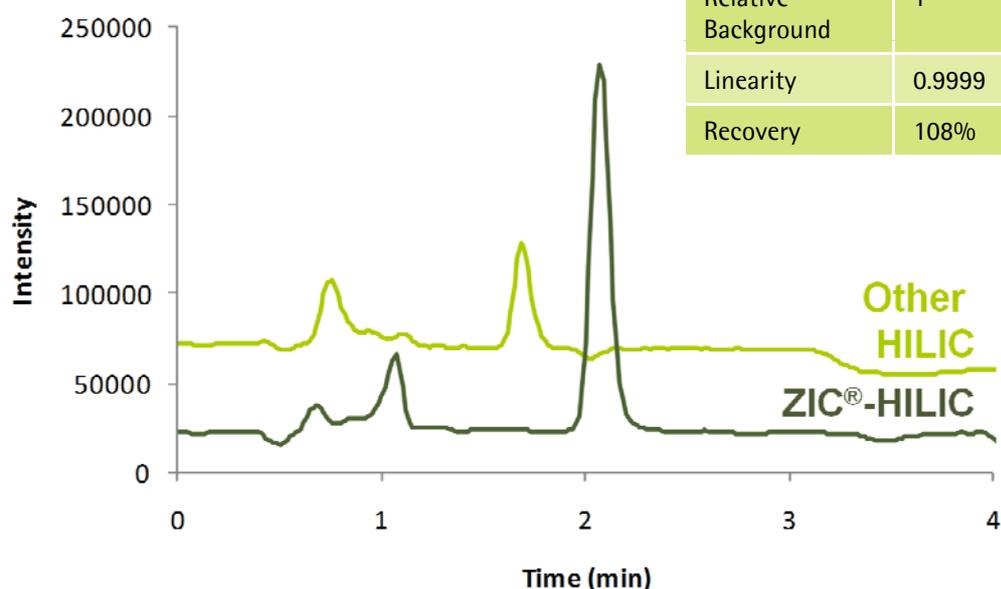


### Chromatographic Data

| No. | Compound | Retention Time (min) | m/z |
|---|---|---|---|
| 1 | Void volume | 0.4 | – |
| 2 | Metformin | 2.1 | 130 |

# Buprenorphine in Urine
## – Maintenance medication for opioid addiction

Buprenorphine is a synthetic opioid used for pain treatment and maintenance medication for opioid addiction. Buprenorphine is metabolized to nor-buprenorphine involving enzyme CYP3A4 in the liver. Both the parent compound and its metabolite conjugate to glucuronoides. Maximum plasma concentrations are reached within 30 minutes to three hours after administration. On the following pages, we show a method for analysis of buprenorphine and norbuprenorphine using reversed phase LC-MS/MS aided by isotope internal standards. Patient samples (urine) were analysed along with standards and control samples.
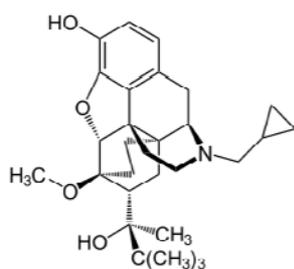


Buprenorphine
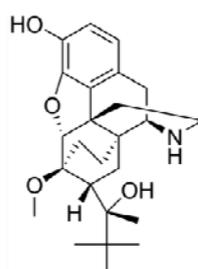


Norbuprenorphine

# Buprenorphine in Urine
## Purospher® STAR RP-18 endcapped

Recommended column:
Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 50-2.1 mm          (1.50646.0001)

Recommended solvents and reagents:

Acetonitrile:   hypergrade for LC-MS LiChrosolv®          (1.00029)

Water:          Water for chromatography LiChrosolv®          (1.15333)
                or freshly purified water from Milli-Q® water purification system

Formic acid 98-100% for analysis EMSURE® ACS, Reag. Ph Eur          (1.00264)

Mobile phase: A: 0.1% formic acid in Milli-Q water
              B: 0.1% formic acid in acetonitrile

| Time (min) | A (%) | B (%) |
|---|---|---|
| 0.00 | 90 | 10 |
| 0.25 | 90 | 10 |
| 2.00 | 10 | 90 |
| 2.10 | 90 | 10 |
| 3.00 | 90 | 10 |

## Sample preparation:
Take 1 ml of urine sample to 10 mL glass tube, add 50 µL internal standard followed by 1 ml 0.1 M acetate buffer pH. Thereafter mix samples thoroughly and place in centrifuge (4000 rpm) for 5 minutes. Take a mixed-mode solid phase extraction cartridge (6 mL) and condition with 2 ml methanol followed by 2 ml of 0.1 M acetate buffer, pH 4. Apply samples, standards and internal controls on solid phase extraction (SPE) cartridges and let the samples slowly run through the cartridges by gravity. Wash the cartridge with 2 mL of 0.05 M HCl followed by 2 mL of a solution containing methanol/water (50:50 v/v), and allow the fluids to flow through the cartridges. Dry the cartridge with full vacuum for 4 minutes. Elute SPE tubes with 2 ml solution of dichloromethane/isopropanol/2% ammonia. Take the eluate and from each sample and evaporate all solvent. Add 200 µL of mobile phase and make sure all surface is wetted to re-constitute the sample completely. Transfer sample to autosampler vials and run analysis on LC-MS/MS system.

### Quantitation of buprenorphine and norbuprenorphine in urine
Linear range: 1 – 100 ng/ml for buprenorphine.
Linear range: 5 – 1000 ng/ml for norbuprenorphine

# Buprenorphine in Urine
## Purospher® STAR RP-18endcapped

### Chromatographic Conditions

| | | |
|---|---|---|
| Column: | Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 50-2.1 mm | (1.50646.0001) |
| Injection: | 10 µL | |
| Detection: | LC-MS/MS ESI; MRM transitions: 468.6/55.2 (Bup) and m/z 414.6/83.2 (Norbuprenorphine) | |
| Flow Rate: | 0.7 mL/min | |
| Gradient | See table | |
| Mobile Phase: | A: 0.1% formic acid in Milli-Q water | |
| | B: 0.1% formic acid in acetonitrile | |
| Temperature: | 40 °C | |
| Sample: | Urine samples treated according to sample preparation protocol. | |
| Backpressure: | 230 bar (3312 psi) at start of gradient | |

| Time (min) | A (%) | B (%) |
|---|---|---|
| 0.00 | 90 | 10 |
| 0.25 | 90 | 10 |
| 2.00 | 10 | 90 |
| 2.10 | 90 | 10 |
| 3.00 | 90 | 10 |
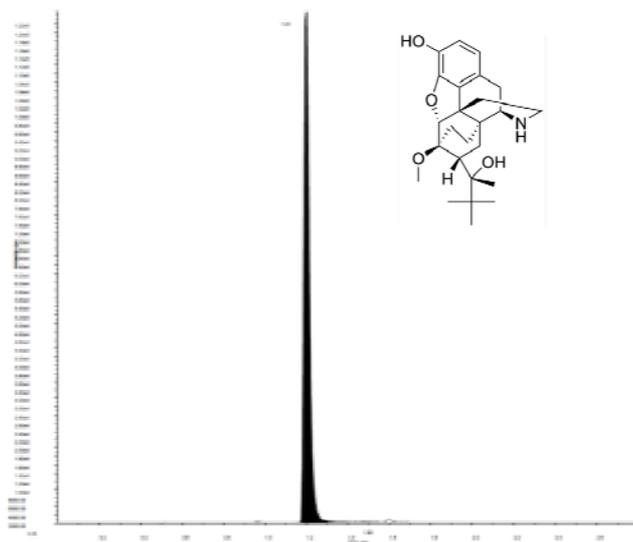


Norbuprenorphine concentration: 642 ng/ml
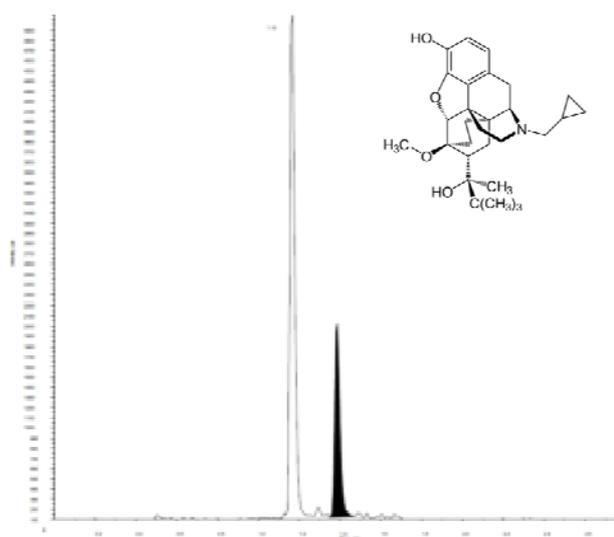


Buprenorphine concentration: 2.16 ng/ml

### Chromatographic Data

| No. | Compound | Retention Time (min) | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|
| 1 | Void volume | 0.2 | – | |
| 2 | Norbuprenorphine | 1.2 | 414.6 | 83.2 |
| 3 | Buprenorphine | 1.4 | 468.6 | 55.2 |

# Tramadol in Urine
## – Analgesics

Tramadol is a weak μ-opioid receptor agonist and inhibitor of serotonin/noradrenalin re-uptake used for pain treatment and other indications such as treatment for restless leg syndrome and fibromyalgia. Tramadol has a structural similarity to the natural opiate codeine. Tramadol is reported to have a lower risk of developing dependence than codeine. Tramadol is a racemate, where the (+) form inhibit the reuptake of serotonin and the (–) form inhibit norepinephrine. In addition, tramadol is extensively metabolized, and where a total of 24 metabolites have been identified. The active metabolite O-desmethyl tramadol has a higher affinity for opioid receptors than the parent compound.

Urine samples from patients were analysed in both methods along with standards and control samples.
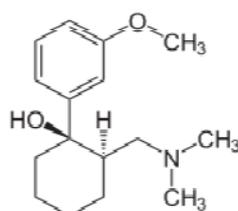


Tramadol

# Tramadol in Urine
## Purospher® STAR RP-18 endcapped

Recommended column:
Purospher® STAR RP-18endcapped (2 μm) Hibar® HR 50-2.1 mm         (1.50646.0001)

Recommended solvents and reagents:
Acetonitrile:   hypergrade for LC-MS LiChrosolv®         (1.00029)

Water:        Water for chromatography LiChrosolv®         (1.15333)
                or freshly purified water from Milli-Q® water purification system

Formic acid 98-100% for analysis EMSURE® ACS, Reag. Ph Eur     (1.00264)

Mobile phase: A: 0.1% formic acid in Milli-Q water
               B: 0.1% formic acid in acetonitrile

| Time (min) | A (%) | B (%) | Flow Rate (mL/min) |
|---|---|---|---|
| 0.00 | 95 | 5 | 0.40 |
| 0.20 | 95 | 5 | 0.40 |
| 2.00 | 50 | 50 | 0.40 |
| 2.50 | 10 | 90 | 0.40 |
| 2.80 | 10 | 90 | 0.40 |
| 3.00 | 95 | 5 | 0.50 |
| 4.50 | 95 | 5 | 0.50 |

Sample preparation:
Take 0.2 ml of each sample into a 5 mL glass tube with screw cap. Add 25 μL internal standard followed by 1.0 ml 0.1 M acetate buffer pH 4. Use vortex mixer for a few seconds to homogenize the samples. Centrifuge, if necessary, at 4000 rpm for 5 minutes. Take a mixed-mode solid phase extraction cartridge (6 mL) and condition with 2 ml methanol followed by 2 ml of 0.1 M acetate buffer, pH 4. Apply samples, standards and internal controls on solid phase extraction (SPE) cartridges and let the samples slowly run through the cartridges by gravity. Wash the cartridge with 2 mL of 0.05 M HCl followed by 2 mL of a solution containing methanol/water (50:50 v/v), and allow the fluids to flow through the cartridges. Dry the cartridge with full vacuum for 5 minutes. Elute SPE tubes with 2 ml solution of dichloromethane/isopropanol/2% ammonia. Take the eluate from each sample and evaporate all solvent to dryness. Add 4.0 mL of mobile phase and make sure all surface is wetted to re-constitute the sample completely. Transfer 2.0 mL sample to autosampler vials and run analysis on LC-MS/MS system.

### Quantitation of Tramadol in urine
Linear range: 25 – 1500 ng/mL

# Tramadol in Urine
## Purospher® STAR RP-18endcapped

### Chromatographic Conditions

| | |
|---|---|
| Column: | Purospher® STAR RP-18 endcapped (2 µm) Hibar® HR 50-2.1 mm       (1.50646.0001) |
| Injection: | 5 µL |
| Detection: | LC-MS/MS ESI; MRM transitions: m/z 264/58 (Tramadol) and m/z 250/44 |
| Flow Rate: | 0.4 mL/min |
| Gradient | See table |
| Mobile Phase: | A: 0.1% formic acid in Milli-Q water<br>B: 0.1% formic acid in acetonitrile |
| Temperature: | 40 ℃ |
| Sample: | Urine samples treated according to sample preparation protocol. |
| Backpressure: | 170 bar (2448 psi) at start of gradient |
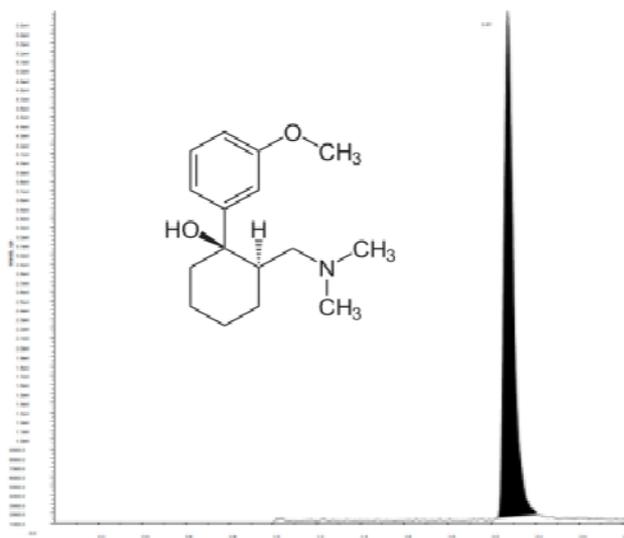


Tramadol concentration: 23.2 ng/mL
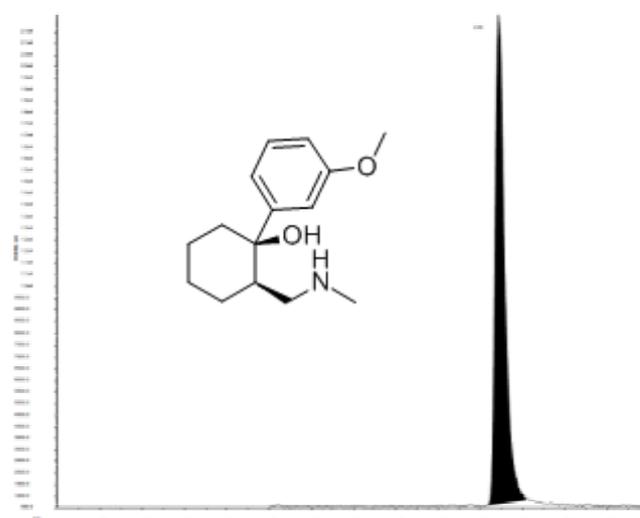


N-desmethyl-tramadol concentration: 21.2 ng/mL

### Chromatographic Data

| No. | Compound | Retention Time (min) | Precursor ion (m/z) | Product ions (m/z) |
|---|---|---|---|---|
| 1 | Void volume | 0.2 | – | |
| 2 | Tramadol | 2.07 | 264.0 | 58.0 |
| 3 | N-desmethyl-tramadol | 2.09 | 254.0 | 44.0 |

# Recommended Reagents and Chemicals

| Product | Ordering Number |
|---|---|
| Acetonitrile hypergrade for LC–MS LiChrosolv® | 1.00029 |
| Methanol hypergrade for LC–MS LiChrosolv® | 1.06035 |
| Ammonium acetate | 1.01116 |
| Acetic acid 96% for analysis EMSURE® | 1.00062 |
| Formic acid 98–100% for analysis EMSURE® ACS, Reag. Ph Eur | 1.00264 |
| Ammonia solution 28–30% for analysis EMSURE® ACS, Reag. Ph Eur | 1.05423 |
| Water for chromatography* | 1.15333 |
| * Or use a Milli-Q Integral Water Purification System | |