Evaluation of Cassette Analysis in Pharmacokinetic Studies

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Abstract

In the lead optimisation of drug candidates, the first discovery pharmacokinetic (PK) *in vivo* study is of great importance to provide an initial assessment of the drug PK parameters. Data from *in vivo* PK studies is generally used to give feedback to chemists to improve the properties of the lead compound series, and also to calculate the initial doses in further effect studies. As the throughput in lead optimisation in drug discovery is constantly increasing, methods for high throughput bioanalysis and sample reduction are of great interest. This study presents an evaluation and a method for cassette analysis (compounds incubated separately followed by combining each time point for multiple compounds) of discovery drug compounds from PK *in vivo* studies in rat using high performance liquid chromatography/ mass spectrometry (HPLC-MS/MS) and ultra performance liquid chromatography/ mass spectrometry (UPLC-MS/MS) for quantification. Strategies for sample reduction, fast chromatography and challenges in ionization suppression using different formulation solutions are addressed.

The number of DMPK (drug metabolism and pharmacokinetics) studies in the drug industry has increased vastly over the last years. Only at AstraZeneca R&D Södertälje, the number of PK studies performed has increased more than threefold the last five years. Therefore, the need to develop time saving methods has also increased. It is of great interest to the drug industry to reduce sample analysis time, increase throughput and maintain data quality by using methods, which are reproducible and easily implemented.

Liquid chromatography/ mass spectrometry (LC-MS/MS) is widely used for quantitative analysis. This is usually a time- and resource-consuming process. The need to rapidly identify lead compounds requires as short intervals between the sample submission and the data reporting as possible.

Cassette analysis decrease the number of samples to analyze, and therefore reduces the time for analysis compared to the traditional discrete methods. But there can be limitations. A pooled analysis may result in a low limit of quantification (LOQ) and/or limitations in data parameters obtained. The LOQ can be a problem because of the dilution of the samples in the cassette analysis. But because of the more sensitive mass spectrometers that have been developed the recent years, this is of less concern than earlier.

In the present study, a cassette analysis method was developed and evaluated. The method should be able to analyze samples from three *in vivo* studies at once. Six drug compounds were chosen as reference compounds. Two cassette groups (n = 3) were established, containing basic and acidic reference compounds respectively.
The compounds were analysed on different LC-MS/MS systems, and for all analytes, MS/MS methods and chromatography methods were developed. Further, there were made standard curves with different experiments. Spiked plasma simulating the PK profile of the reference compounds and samples from *in vivo* animal studies at AstraZeneca were used for the assessment. Equal volumes of three plasma samples corresponding to each time point of three individually dosed rats were pooled and further prepared with protein precipitation and analysed with HPLC-MS/MS or UPLC-MS/MS. The matrix effect of different formulations, in terms of ionization suppression was also examined. Finally a validation experiment was performed to be able to evaluate the previous results in the study.
The results showed that there were no large differences between the discrete/single and cassette/pooled samples. However, the results showed some variations, and not all the samples fulfilled the acceptance criterion of ± 25 %. This difference is not related to the LC-technique, but rather to the variability in the analytical method and variations in the sample preparations. The basis for this assumption is verified by the validation experiment at the end of this study.
The loss of sensitivity due to extra dilution in the sample preparation step is only of concern when working with very small concentrations. Some formulations can give ion suppression e.g. PEG 400 in this study. Therefore, when using PEG 400, an ion suppression check should be performed.
[1,7]

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Abbreviations and concepts

ACN	Acetonitrile
AZ	AstraZeneca
CE	Collision energy
CMC	Carboxymethylcellulose
CV	Cone voltage
DMA	Dimethyl acetamid
DMPK	Drug metabolism and pharmacokinetics
DMSO	Dimethylsulfoxide
ESI	Electrospray ionization
ESP+	Electrospray in positive ionization mode
ESP-	Electrospray in negative ionization mode
F	Flow rate
GC	Gas chromatography
HPMC	Hydroxypropyl methylcellulose
HPLC	High-performance liquid chromatography
Hz	Hertz (cycles per second)
in vitro	Experiment performed in test tubes
in vivo	Experiment performed in living organisms
IS	Internal standard
IV	Intravenous dosing route
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
Lipoid S100®	Phospholipid formulation used for drugs with poor water solubility for parenteral application
LOD	Limit of detection
LOQ	Limit of quantification
M	Mol/litre
MCC	Microcrystalline cellulose
MeOH	Methanol

MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Mass spectrometry/mass spectrometry (Tandem mass spectrometry)
m/z	Mass to charge ratio
N_2	Nitrogen
PEG	Polyethylene glycol
PO	Oral dosing route
Q	Quadrupole
QC	Quality control
QuanLynx	Quantification data program of the software MassLynx 4.0 and 4.1
QuanOptimize	Optimization data program of the software MassLynx 4.0 and 4.1
R&D	Research and development
S/N	Signal to noise ratio
Tween80®	Polyoxyethylene sorbitanmonostearate
UPLC	Ultra Performance Liquid Chromatography
v/v	Volume to volume

Sammendrag

Screeningfasen for oppdagelse av nye legemiddelkandidater i farmakokinetiske *in vivo* studier er et viktig steg for å anslå initielle farmakokinetiske (PK) parametre. Data fra disse studiene benyttes til optimering av PK parametre fra kjemiske serier, og for å kalkulere initielle doseringsverdier i videre *in vivo* studier. På grunn av den stadig økende mengden av data, og det medfølgende behovet for økt throughput i den analytiske metoden, er det et konstant behov for nye raskere metoder og for reduksjon av antall prøver som skal analyseres. Hensikten med denne studien var å utvikle og evaluere metoder for kassettanalyse/ pooling av prøver fra tre standardiserte PK *in vivo* studier. Strategier som ble undersøkt nærmere var steg i prøveopparbeidelsen, sensitivitet, ionesuppressjon/ enhancement, forskjeller mellom diskrete/single og kassett/poolede analyser og validering av resultater fra analysene.

I denne studien ble det benyttet seks refransesubstanser. Substansene ble undersøkt hver for seg, og også i to kassettgrupper (n = 3) med henholdsvis kun basiske og kun sure forbindelser sammen. Warfarin ble benyttet som internstandard (IS) ettersom den lar seg detektere i både positiv og negativ mode. Referansesubstansene ble benyttet for å undersøke hvordan pooling av prøver påvirker nøyaktighet, presisjon, LOQ og PK-parametre. Blank plasma fra rotte ble tilsatt ulike mengder referansesubstanser (spiked plasma) for å lage standardkurver og simulerte PK profiler. Prøver fra *in vivo* dyrestudier ved AstraZeneca ble også undersøkt. Like volumer fra tre plasmaprøver med korresponderende tidspunkter i PK-kurvene fra individuelt doserte dyr ble poolet sammen. Matrix effekter på grunn av ulike doseringsformuleringer som for eksempel cyclodextrine eller PEG 400 ble også undersøkt med hensyn på ionesuppressjon. Både for å se på forskjeller mellom ulike analysesystem, og på grunn av kapasitetsproblemer ble prøvene analysert på ulike HPLC-MS systemer, og også på UPLC-MS.

Resultatene fra denne studien viser at det ikke er store forskjeller mellom prøver som er analysert som diskret/singel eller som kassett/poolet. Ved å benytte kassettanalyse fremfor diskrete analyser øker throughput betraktelig. Man vil også spare analysetid og kostnader knyttet til dette.

Det finnes imidlertid enkelte variasjoner som avviker fra aksept kriteriene på ± 25%. Dette synes derimot ikke å skyldes ulikheter mellom diskret og kassett, men heller på grunn av variasjoner i analysemetode/ spredning innenfor analysesystem. Grunnlaget for denne antagelsen kommer frem i validering-/ spredningsforsøk som også viser noe variasjon. Dermed vil det kunne forekomme enkelte variasjoner uansett om man analyserer som diskret eller kassett.

På grunn av ekstra fortynning i prøveopparbeidelsen ved kassettanalyse taper man litt sensitivitet, men dette har kun betydning ved svært lave konsentrasjoner. Enkelte formuleringer som PEG 400, som ble benyttet i denne studien kan gi ionesuppressjon.

1. Introduction

The common mission of major pharmaceutical companies such as AstraZeneca is to develop new efficient medicines to improve people's health. DMPK screens at early stages in the development are important when potential new medicines are investigated. The increased speed with which scientists can profile new drug candidates will shorten the time needed to develop a new medicine. In order to implement these screens in shorter time periods, the use of higher throughput assays are based on different strategies and approaches such as fast chromatography, direct injection, parallel MS methods and various sample reduction methods where the cassette approach belongs.

In the drug industry, DMPK studies are performed in order to obtain useful information on the properties of potential drug candidates. The number of these studies has increased vastly over the last years.

DMPK studies can be divided into for steps: sample preparation, analysis, quantification and reporting. The analysis step is a limiting factor since it is relatively time consuming, and with the ever-increasing number of samples to analyze, this limitation will increase.

On the other hand, the development of faster and more efficient mass spectrometers not only decreases the analyze time, but it also increases the quality and the quantity. The use of autosamplers, robot methods, double- and even triple column systems also provides faster analyze times. But in the drug industry there will always be a desire to perform more analyses in shorter time periods. Therefore, the opportunity to analyze samples from several studies at once, which the cassette mode gives, will be of great value in the future [5].

1.1 Aim of the study

The aim of this study was to develop and evaluate methods involving cassette analysis that would generate reliable results. The final objective was to develop a cassette method that could be used for analysis of biological samples from three different pharmacokinetic studies at one time.

2. Theory

2.1 Liquid Chromatography

High-performance liquid chromatography (HPLC) is the most used method in determining and analysis of medicines in pharmaceutical and biological material. The method involves a liquid, the mobile phase that is compressed with a pump through an injector and a column filled with a material that gives high-resolution separations. The separations is taken up by a detector that gives an electronic response which is adapted by a computer system giving arise to a chromatogram.

Liquid chromatography is a physical separation method that acts through selective distribution between a liquid and a solid phase. The solid-/stationary phase is based on silica with hydrophobic adsorbents bounded to silanol groups. The mechanism of separation is based on the difference between the analyte distribution in relation to the mobile and stationary phase.

The column is together with the mobile phase, separating the compounds in the sample. An ideal column should separate the compounds in as short time as possible, and give as small diffusion as possible of the compounds when being transported by the mobile phase.

There are several different detectors available for liquid chromatography. Some examples are the UV-detector, the fluorescence detector or the electrochemical detector. A mass spectrometer is widely used as a detector to provide both qualitative and quantitative information.

In HPLC, there are a great number of variables like the columns particle size, the composition of the stationary phase and the mobile phase, the flow rate and the properties of the analytes that affects the separation [8,9].

2.2 Mass spectrometry

As a result of the ability to combine the mass spectrometer with other chromatographic techniques, such as liquid- and gas chromatography, the usage of mass spectrometry has increased vastly over the last years. A mass spectrometer is using the differences in the relationship between mass to charge to separate ions in gas phase. The information obtained from a mass spectrometer is used for both quantitative and qualitative analyses.

The mass spectrometer instrument can be divided into four main regions.

Source region: the samples are introduced into the ionization source. Ions are generated from the molecules by inducing a loss or gain of charge. After ionization, the molecule ion usually has enough residual internal energy to break into fragments.

Transfer region: the ions are transported through the radio frequency lens that delivers them in a tightly focused beam to the separator.

Analyzer region: in the analyzer region, the ions are separated according to their mass/ charge ratio.

Detector: at the detector, the signal is amplified and detected. A photomultiplier dynode is usually used as the detector. As the ions strike the dynode, an emission of electrons are resulting. The electrons then strike a phosphorus screen that releases photons that are detected by the photomultiplier [7,8,9].

2.2.1 Electrospray

Electrospray ionisation (ESI) is a widely used ionisation technique when a mass spectrometer is used as a detector in HPLC. The sample is provided into the mass spectrometer fluid stream, which passes through a capillary tube. At the end of this capillary, there is a strong electric field, which enable the fluid to be transformed into small droplets. To the ion source, it is also added a hot nitrogen gas, causing the droplets to evaporate. Then the electric field increases, and the ions move towards the droplets surface. The mutual repulsion between like charges on this surface becomes so great that it exceeds the forces of the surface tension, causing the ions to leave the droplets through a cone into the mass analyzer [7,9].

2.2.2 Tandem mass spectrometry (MS/MS)

A tandem mass spectrometer consists of two mass separators (quadrupoles) and a collision cell. The mixture of ions from the ion source is separated by the first quadrupole (Q1) where a precursor (parent ion) is selected. The ion(s) of interest are then introduced to the collision cell (Q2), where they are exposed to a collision gas, causing them to break into fragments (daughter ions). The selected fragment(s) are then analysed in the following quadrupole (Q3) in order to obtain a daughter ion spectrum [7,8].

2.2.3 Ultra Performance Liquid Chromatography (UPLC)

The throughput and resolving power of liquid chromatography has increased considerably the past decades. The development of the UPLC system has been an important contribution to this evolution. Based on 1.7 and 1.8 µm particles and instrumentation, the system delivers increased levels of resolution speed and sensitivity. Because the column is packed with smaller particles, the instrumentation is capable of higher pressure operations, which have led to reduced system volumes, faster autosamplers and detectors with higher data capture rates. But, also –to obtain the benefits of smaller particles in the column, other measures need to be considered. Pumps capable of delivering solvent smoothly and reproducibly at the higher pressure, and the detector sampling rate must be high enough to capture enough data points across the peaks. It is also desirable with injection valves designed to work at higher pressure, fast injection cycles and narrow capillaries [8, 12].

2.3 Cassette analysis

To increase speed and throughput when using HPLC-MS/MS systems, the major time saving strategies include sample reduction and cassette analysis. Figure 1 summarizes the strategies and approaches that have been employed for increasing the throughput of DMPK studies.

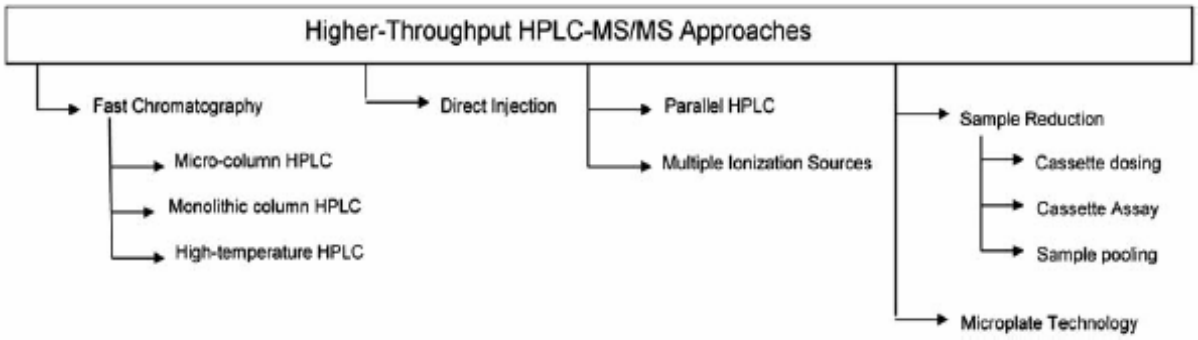


Figure 1 High throughput technologies and strategies using HPLC-MS/MS [1]

As shown in the figure, cassette dosing and cassette assay provides sample reduction, which gives higher throughput and speed, which will save time, instrumentation, and personnel and hence costs.

It is important to distinguish between the two approaches cassette dosing and cassette assay (cassette analysis). Cassette dosing involves dosing the test-animal with a mixture of multiple compounds, whereas cassette analysis involves pooling the different compounds together prior to the sample preparation and the analysis. In the present study, we will not go further into the cassette dosing approach, but concentrate on cassette analysis of *in vivo* pharmacokinetic studies. The cassette analysis approach is a widespread method in the drug industry, mainly to save time and costs, but is not widely used for *in vivo* studies. AstraZeneca R&D Södertälje has earlier not used cassette analysis for *in vivo* studies because of the number of issues to consider. Loss of sensitivity due to the pooling and hence diluting of the samples, which can involve higher limits of quantification (LOQ). There are also matrix effects, mainly ion suppression or ion enhancement that need to be considered. Pooling samples from different studies can involve several different formulation solutions in the same pooled sample. Endogenous compounds e.g. phospholipids can bias the analytical results, and of course the molecular weight of the analytes and their metabolites should not interfere with each other [1].

2.4 Matrix effects/ ion suppression

An analytical sample consists of the analyte and the rest of the sample, which is the matrix. If the matrix affects the analyte and hence the analytical result in some way, there is a matrix effect. The mainly matrix effects to consider are ion suppression or ion enhancement. Matrix effects resulting in either ion suppression or ion enhancement of analyte response has become one of the most common cause of failure or errors in bioanalysis, and when not understood, it can lead to errors in the calculation of PK parameters in animal models.

Matrix effects can cause stability issues for biological assays using LC-MS/MS. Major sources of matrix effects includes a few classes of endogenous phospholipids which are present in biological matrices such as plasma and serum.

In a typical discovery PK study, drug candidates are administered to rats via the intravenous (IV) and/or the oral (PO) route. A solution formulation is required for the IV route, and is also preferable for the PO route in order to enhance absorption. Common formulation solutions are cyclodextrine, gluconic acid, meglumine and PEG.

To avoid interference between the phospholipids, the formulations and the analytes, the chromatography methods need to be modified such that the analytes of interest do not co-eluate with the regions displaying significant matrix effects. This can be done by optimizing the chromatographic conditions (e.g., using a longer HPLC gradient) to give sufficient separation between formulations and analytes in the HPLC step. [4, 10].

2.5 Validation

Selective and sensitive analytical methods for the quantitative evaluation of analytes are necessary for a successful conduct of pharmacology studies. Bioanalytical method validation includes procedures that demonstrate the usefulness of particular methods used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum or urine. Validation involves documenting, through the use of specific laboratory experiments, that the performances of the method are reliable for the analytical applications.

It is necessary to perform a validation experiment to be able to evaluate other results in the study, to give evidence of adequate assay performance in the original lab/ method, and thus additionally prove reliability of data.

A validation experiment can be performed by analysis of several samples at different concentration levels (quality controls, QC) over a specific time period. Acceptance criterions of percentage deviation, and how many samples that allow failing the criterions need to be implemented in the method [13, 14].

3. Methods and experimental

3.1 Chemicals and solvents

Warfarin was used as internal standard (IS) and supplied by compound management, AstraZeneca (Södertälje, Sweden). The reference compounds (diazepam, diclofenac, imipramine, naproxen, propranolol and rofecoxib) and the formulations (cyclodextrine, gluconic acid, meglumine, DMA, PEG 400, HPMC + Tween80® and MCC/NaCMC + Lipoid S100®) were also supplied by compound management AstraZeneca. Acetonitrile, methanol, acetic acid and ammonium acetate were purchased from E. Merck (Darmstadt, Germany). The Milli-Q water used for preparing solvents and solutions was obtained using a Reagent Grade Milli-Q Plus water purification system from Millipore Corporation (Bedford, USA). Dimethylsulfoxide (DMSO) was purchased from Sigma (St Louis, USA). Blank rat plasma was supplied from Animal Care, AstraZeneca R&D (Södertälje, Sweden). The rat plasma from the animal studies containing test compounds was supplied from DMPK, AstraZeneca R&D (Södertälje, Sweden).

3.2 Apparatus

The compounds were analysed on different LC-MS/MS systems, both because we wanted to evaluate the differences between systems, and because of capacity limitations.
These systems were:

1. Quattro Ultima, 2-column system using H_2O/ MeOH gradient

2. Quattro Ultima/Quattro Premier, 1 column system using H_2O/ acetonitrile gradient

3. Quattro Premier Acquity UPLC, 1 column system using H_2O/ acetonitrile gradient (Analysis performed both at AstraZeneca in Södertälje and at Waters corporation in Sollentuna)

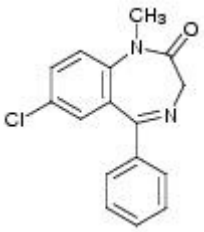
Figure 2 UPLC-MS/MS, Waters Acquity Quattro Premier XE system

Figure 3 HPLC-MS/MS, CTC auto sampler, Rheos 2000 HPLC pump, Waters Quattro Premier XE

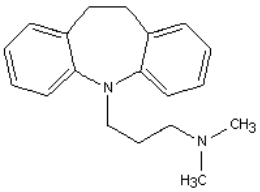
The UPLC system (Figure 2) used a C-18 column, Acquity UPLC BEH C-18, 2.1x30 mm with particle size 1.7µm, acetonitrile gradient, 2-80%, and flow rate 0.6 ml/min. The time between injections was approximately 1.6 minutes.

The HPLC system (Figure 3) used a Hypurity C-18 column, 2.1x30 mm with particle size 5.0 µm, acetonitrile gradient, 2-80%, and flow rate 0.4 ml/min. The time between injections were approximately 3.5 minutes. When using the 2-column system with MeOH gradient 15-85%, the flow rate was 0.35 – 0.4 ml/min. The time between injections varied from 4 – 8 minutes.

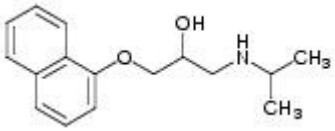
3.3 Reference compounds



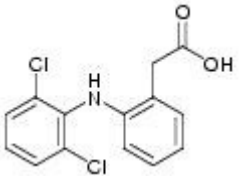
Diazepam, MW 284,75



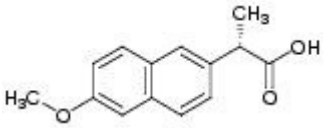
Imipramine, MW 280,41



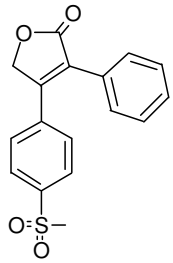
Propranolol, MW 295,81



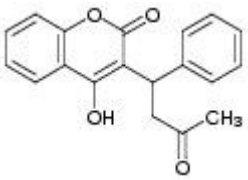
Diclofenac, MW 296,15



Naproxen, MW 230,26



Rofecoxib, MW 314,36



Warfarin (IS), MW 308,34

Figure 4 The six reference compounds and the internal standard (warfarin) used for analysis

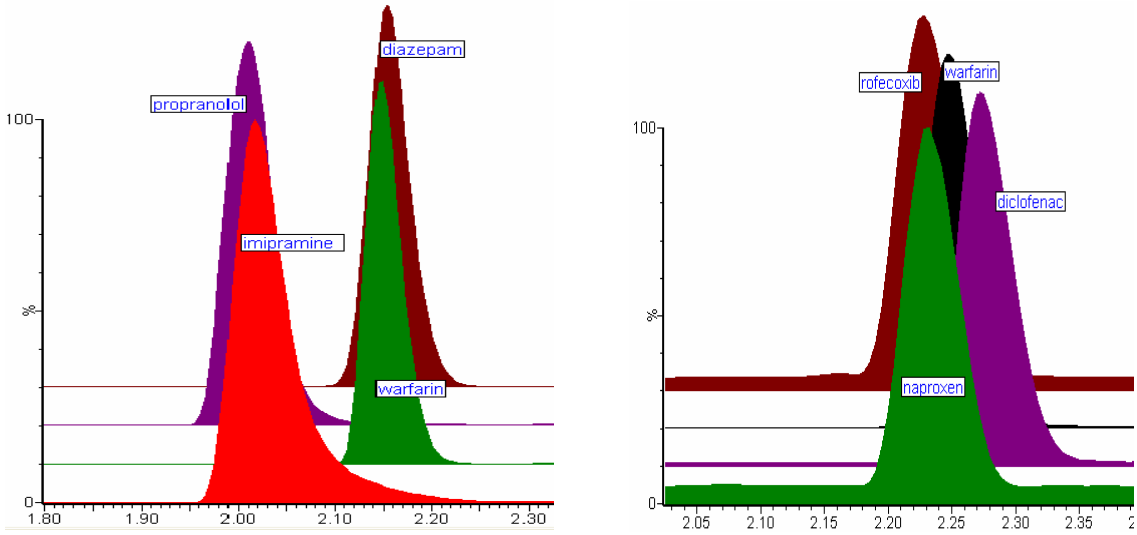


Figure 5 HPLC chromatograms, pooled compounds, 3.5 minutes between injections, 1 column system, Hypurity C-18 column

Compound	Parent (m/z)	Daughter (m/z)	CV (V)	CE (eV)	Ion mode
Diazepam	284.95	193.12	46	28	ES+
Imipramine	281.09	85.91	19	16	ES+
Propranolol	260.09	115.95	35	16	ES+
Warfarin (IS)	308.88	163.1	18	16	ES+
Diclofenac	293.85	250.1	19	12	ES-
Naproxen	229.11	169.1	19	34	ES-
Rofecoxib	312.97	284.94	37	22	ES-
Warfarin (IS)	306.91	161.02	18	20	ES-

Table 3.3-1 MS methods/ MRM-Scan transitions for the reference compounds

All compounds were dissolved in DMSO, and had a concentration of 10 mM (stock solutions).

3.4 Formulation solutions

Dosing formulations are commonly used in pharmacokinetic (PK) studies during the early drug discovery stage. Examples of widely used formulations are cyclodextrine, gluconic acid, meglumine, methylcellulose, Tween 80 and polyethylene glycol (PEG). These formulations are dosed to test animals like rats together with other compounds, usually one or more drug candidates. The dosing vehicles are usually dosed through the intravenous (IV) or the oral (PO) route. A dosing formulation is required for the

IV route, and is also preferable for the PO route in order to enhance the absorption by dissolving the test compounds/ drug candidates.

Like other compounds in the sample beside the analyte, the formulation can cause matrix effects. When pooling several *in vivo* compounds together, there can also be several different formulations in the same sample to analyze, causing further issues, especially concerning ion suppression or ion enhancement [4].

3.5 Phospholipids

Major sources of matrix effects include a few classes of endogenous phospholipids and lysophospholipids that naturally occur in biological matrices such as plasma and serum. Phospholipid interference is a major component of matrix effect in bioanalysis. To reduce this matrix effect, it is necessary to account for the phospholipid impact on the analytes during the method development. Chromatography methods can then be modified such that the analytes of interest do not co-elute with regions displaying significant matrix effects from the phospholipids [10].

3.6 Pharmacokinetic profiles

In this study, plasma samples were spiked with six drug compounds respectively where concentrations are corresponding to literature pharmacokinetic (PK) profiles. The PK concentrations of these compounds at eight time-points for the PO route are shown in table 3.6-1.

compound	Time (h)							
	0.25	0.5	0.75	1	1.5	2.5	6	24
Naproxen (µmol/L)	35	43	43	43	45	45	40	3
Diclofenac (µmol/L)	3	2	1.5	1	0.8	0.65	0.4	0.01
Rofecoxib (µmol/L)	0.07	0.15	0.27	0.3	0.45	0.6	0.45	0.07

compound	Time (h)							
	0.25	0.5	0.75	1	1.5	2.5	6	24
Imipramine (µmol/L)	0.36	0.27	0.43	0.27	0.27	0.23	0.15	0.07
Diazepam (µmol/L)	0.65	0.75	0.7	0.65	0.45	0.22	0.025	0.005
Propanolol (µmol/L)	0.28	0.16	0.1	0.06	0.04	0.02	0.008	0

Table 3.6-1 Literature PK concentrations at eight time points for the oral route [15,16,17,18]*

*The literature PK (PO) values for Naproxen and Rofecoxib are taken from AstraZeneca in-house studies.

The literature PK concentrations for the IV route are shown in table 3.6-2. The concentrations for the 24 hour time point for the compounds diclofenac, rofecoxib, diazepam and propranolol are to low to measure/ quantify.

compound	Time (h)							
	0.03	0.1	0.3	0.6	1	3	6	24
Naproxen (µmol/L)	35	30	25	21	20	16	10	1
Diclofenac (µmol/L)	16	8	2.5	0.8	0.35	0.2	0.06	
Rofecoxib (µmol/L)	5.50	4.40	2.80	1.00	0.50	0.08	0.04	

compound	Time (h)							
	0.03	0.1	0.3	0.6	1	3	6	24
Imipramine (µmol/L)	2.2	2.1	2	1.9	1.7	1	0.5	0.01
Diazepam (µmol/L)	4.2	3.7	2.5	1.5	1	0.25	0.025	
Propanolol (µmol/L)	10.5	8.5	6	4.5	3.5	1	0.25	

Table 3.6-2 Literature PK concentrations at eight time points for the IV route [15,16,17,18]*

*The literature PK (IV) values for Naproxen and Rofecoxib are taken from AstraZeneca in-house studies.

3.7 LC-MS/MS analysis

Before injection to the analysis system, either HPLC-MS/MS or UPLC-MS/MS, a sample preparation is necessary. In this step, preparation of requested concentrations are made, and proteins from the plasma are removed by protein precipitation.

3.7.1 Preparation of stock solutions

The reference compounds (diazepam, diclofenac, imipramine, naproxen, propranolol and rofecoxib) were weighed in separately from solid compounds to obtain a concentration of 10 mM of each compound when dissolved in DMSO. The stock solutions were stored in dark at room temperature.

3.7.2 Preparation of standards

The stock solutions of the reference compounds had a concentration of 10 mM. Standard curves with eight points were produced from the standard stock solution by serial dilution, using ACN:H_2O (50:50) as dilutor. The concentrations of the working standard solution were approximately: 50, 100, 338, 1125, 3750, 12 500, 33 333 and 100 000 nM. The standards were diluted (1:10) with blank rat plasma in new tubes. From the plasma-diluted standards, 25 µL was transferred to a deep well plate. The concentrations received were: 5, 10, 34, 113, 375, 1250, 3333 and 10 000 nM. The working standards were precipitated with a deproteinising solvent, 150 µL ice cold ACN with 200 nM Warfarin. The deep well plate was vortexed for 5 minutes and centrifuged for 20 minutes, using 4000 rpm at a temperature of 4°C. After centrifugation, 120 µL of the supernatant was transferred to a new deep well plate, and 300 µL buffer (2 % ACN in 10 mM acetic acid) was added to the supernatant. The standards were injected on the LC-MS/MS system. To be able to quantify samples with higher concentrations than 10 000 nM, e.g. in the PK curves, it was necessary to also prepare standard curves with higher concentration ranges, although, the sample preparation was the same.

3.7.3 Preparation of samples

Analytes in spiked plasma samples were made by spiking blank rat plasma with compounds with preferred concentrations. Stock solutions were stored at a concentration of 10 mM, then diluted with ACN: H_2O (50:50). These sample solutions were then diluted with blank plasma in new tubes (1:10).

For single compounds, 25 µL of the plasma solution was transferred to a deep well plate, then precipitated with 150 µL ice cold ACN containing 200 nM Warfarin. The deep well plate was vortexed for 5 minutes and centrifuged for 20 minutes. After centrifugation, 120 µL of the supernatant was transferred to a new deep well plate, and 300 µL buffer (2 % ACN in 10 mM acetic acid) was added to the supernatant. The samples were injected on the LC-MS/MS system.

For pooled compounds (n =3) 75 µL (25µL x 3) of the plasma solution was transferred to a deep well plate, then precipitated with 200 µL ice cold ACN containing 200 nM Warfarin. The next steps were the same as for the single compounds.

Figure 6 illustrates the variation in the sample preparation step between single and pooled samples.

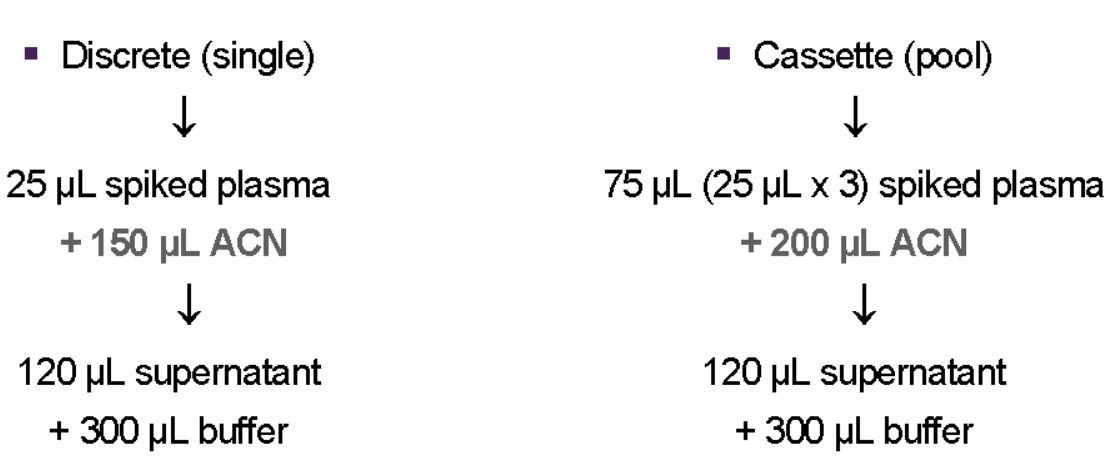


Figure 6 Differences in sample preparation between single and pooled samples

As shown later in this study, the amount of ACN used for protein precipitation for pooled samples was varied before the use of 200 µL was implemented as a standard procedure. When using 200 µL ACN in the cassette method, this correspond to a dilution that is 1,6 times higher than for the single samples.

3.7.4 Preparation of samples from AstraZeneca *in vivo* PK animal studies

At DMPK AstraZeneca R&D Södertälje, Sweden, a great number of animal studies are implemented. The most common test animal at this site is rat. In this study, plasma samples from three *in vivo* PK rat studies are being used for analysis. The plasma contains test compounds from AstraZeneca, and due to industrial restrictions, these compounds are referred to as AZ1, AZ2 and AZ3.

The plasma samples are taken from the test animals at different time points to give the PK profile of the test compounds. In the sample preparation step, 25 µL plasma was precipitated with 150 µL ice cold ACN in the same way as for the spiked plasma samples. For the pooled samples (25 µL x 3), 200 µL ACN was used for precipitation. The next steps were also the same as for the spiked samples.

4. Results and discussion

4.1 Introducing experiments/ Method development

The first period at AstraZeneca was used for method development, finding MS-methods and chromatography methods for the reference compounds. There were prepared standard curves for all the compounds, and then they were pooled together. The three basic/positive compounds (diazepam, imipramine and propranolol) and the three acidic/negative compounds (diclofenac, naproxen and rofecoxib) were pooled together in two cassette groups (n = 3) respectively, using warfarin as internal standard in both cassette groups. The "basic cassette" was analysed in positive ESI mode, whilst the "acidic cassette" was analysed in negative ESI mode.

The single standards were as mentioned earlier made by transferring 25 µL of each standard concentration in spiked plasma to a deep well plate, then protein precipitated with 150 µL ice cold ACN. After vortexing and centrifugation, 120 µL of the supernatant was transferred to a deep well plate, and 300 µL buffer was added. The pooled standards were made by pooling 25 µL x 3 (25 µL of each standard concentration) together in the same vial. To precipitate, 450 µL ice cold ACN was added, and then vortexed and centrifuged. 120 µL of the supernatant was transferred to a deep well plate, and 300 µL buffer was added before analysis. A second pooling method was also performed by pooling 10 µL x 3 together, precipitated with 150 µL ice cold ACN, 120 µL of the supernatant was transferred to a deep well plate, and 300 µL buffer was added.
These samples were analysed on two different MS systems, MS PREMIER using an acetonitrile gradient and MS ULTIMA using a methanol ammonium acetate gradient. There were eight standard points, concentration range 5 – 10000 nmol/ml in spiked plasma (5, 16.7, 50, 166.7, 500 1666.7, 5000 and 10 000 nmol/ml).

The experiments showed, as expected a loss of sensitivity with the pooled samples because of extra diluting when pooling them together. Table 4.1-1 shows a summary of the results in limits of quantification (LOQ).

Single vs. Pooled

1: 25 µL plasma std + 150 µL ACN
2: 25 µL plasma std x 3 + 450 µL ACN
3: 10 µL plasma std x 3 + 150 µL ACN

8 std points, range 5 - 10000 nmol/L

	Acetonitrile gradient MS:PREMIER				MeOH amm.acetat gradient MS:ULTIMA		
	Single	**Pool 3x25**	**Pool 3x10**		**Single**	**Pool 3x25**	**Pool 3x10**
Diazepam	5	16.7	5		16.7	16.7	16.7
Imipramine	5	50	16.7		5	16.7	16.7
Propranolol	5	50	16.7		5	16.7	16.7
Diclofenac	5	5	5		16.7	16.7	16.7
Naproxen	5	16.7	16.7		5	16.7	16.7
Rofecoxib	5	16.7	50		16.7	16.7	16.7

Standard conc.: 5, 16.7, 50, 166.7, 500, 1666.7, 5000, 10000

Table 4.1-1 LOQ for standard curves, comparing single vs. pooled samples

4.2 Formulation experiments

The formulation solutions used in this experiment were Meglumine (used for diclofenac and naproxen), Gluconic acid (used for imipramine and propranolol) and Cyclodextrine (used for diazepam and rofecoxib).
The formulations were prepared by spiking 5 % directly in blank plasma.

4.2.1 Pre-experiment using imipramine and gluconic acid

Standard curve for imipramine in blank plasma was obtained. The standard concentrations were 5, 10, 34, 113, 375, 1250, 3333, and 10 000 nmol/ml. Quality controls (QC) were made of the concentrations 113 nmol/ml (low control), 1250 nmol/ml (medium control) and 10 000 nmol/ml (high control). There were six of each controls taken from the same plasma vial, but placed in 6 different wells on the deep well plate. The controls contained formulation (gluconic acid), whereas the standard curve did not.

This experiment was performed at Waters Corporation in Sollentuna, Sweden, using a Quattro Premier mass spectrometer with an UPLC. Table 4.2.1-1 shows the summarized result from this experiment. The results from the standard curve show

some variation, but are overall quite good. The percentage deviation are varying from –18,25 % to 36,01%. In order to go further with the results at AstraZeneca, this variation generally should not exceed ± 25%, and this limit were also set as the general acceptance criterion for deviations between single and pooled samples in this study. The results from the controls falls within this range with a good margin, percentage deviation varying from –8,42% (low control), -7,27% (high control) to 13,3% (medium control).

ID	Type	Theoretical values [nM]	Area	Measured values [nM]	%Dev
imipramine1	Standard	5.06	356	6.68	32.05
imipramine2	Standard	10.13	993	8.28	-18.25
imipramine3	Standard	33.75	2654	21.57	-36.1
imipramine4	Standard	112.5	7460	92.39	-17.85
imipramine5	Standard	375	23429.5	362.19	-3.4
imipramine6	Standard	1250	87829.5	1355.74	8.45
imipramine7	Standard	3333.33	191853.5	3273.32	-1.8
imipramine8	Standard	10000	583780.5	10004.21	0
low control	QC	112.5	9338	103.03	-8.42
medium control	QC	1250	96851.83	1416.21	13.3
high control	QC	10000	505360.5	9271.7	-7.27

Table 4.2.1-1 Imipramine standard curve with quality controls (QC) containing formulation

4.2.2 Pre-experiment, pooling diazepam, diclofenac and propranolol

This was an experiment to detect the limits of quantification (LOQ) by pooling both basic and acidic compounds, and to compare the difference in LOQ of three single compounds vs. the same compounds pooled together. The compounds pooled together were diazepam and propranolol, which are basic and diclofenac, which is acidic. These compounds were pooled together with formulations, cyclodextrine for diazepam, meglumine for diclofenac, and gluconic acid for propranolol. Both standard curves and controls contained formulations, and because of the pooling, each pooled sample would contain all three formulations.

As expected, the LOQ were higher when the compounds were pooled together. The LOQ was also higher when pooling both basic and acidic compounds together compared with pooling only basic or acidic compounds. Now, the LOQ for the pooled

compounds was in the range between 30 – 100 nM/ml. (When pooling only basic or acidic compounds, this range was between 5-50 nM/ml).
But still, the results were acceptable with a accuracy within ±25% for all concentration levels, these results are summarized in table 4.2.2-1.

	Discrete/ Single	**Cassette/ Pool**
Diazepam	98% - 116%	85% - 125%
Diclofenac	100% - 115%	90% - 114%
Propranolol	95% - 108%	94% - 118%

Table 4.2.2-1 Discrete vs. cassette accuracy (%), concentration levels 34 nM – 3330 nM, positive/negative switching mode

4.3 Sample preparation development -LOQ

There is an increase of LOQ due to the diluting in the sample preparation step when pooling compounds together. In standard single sample preparation, 25 µL spiked plasma is precipitated with 150 µL ice cold ACN, after precipitation, 120 µL of the supernatant is mixed with 300 µL buffer before analysis. When pooling, 25 µL of each compound in spiked plasma, n =3 (25 µL x3) are pooled, then precipitated with 450 µL ice cold ACN. Then again, 120 µL of the supernatant is mixed with 300 µL buffer prior to the analysis. This corresponds to a dilution that is 3 times higher for the pooled samples than for the single samples.
To reduce the loss of sensitivity, the volumes in the sample preparation needed to be changed.

4.3.1 Reducing the amount of acetonitrile used for protein precipitation

This experiment was performed with the two cassette groups, the acidic and the basic cassette.
Together with the standard sample preparation method when pooling, two new methods using less ACN were performed:

A) standard AZ method: **450 µL ACN** (25 µL x3 spiked plasma + 450 µL ACN → 120 µL supernatant + 300 µL buffer)

B) alternative 1: **300 µL ACN** (25 µL x3 spiked plasma + 300 µL ACN → 120 µL supernatant + 300 µL buffer)

C) alternative 2: **200 µL ACN** (25 µL x3 spiked plasma + 200 µL ACN → 120 µL supernatant + 300 µL buffer)

These experiments were performed both with and without formulation solutions (5% cyclodextrine, gluconic acid and meglumine in plasma).
The samples were analysed on the two-column system Quattro Ultima using two different methods (total duration of each analysis 4.25 min and 7.50 min respectively). The parameters for these methods are shown in table 4.3.1-1.

Column 1	Column 2
Loading Pump	**Eluting Pump**
Isocratic	Binary
A: 15% meOH in 10 mM amm.acetat	**A**: 15% meOH in 10 mM amm.acetat
B: meOH	**B**: 85% meOH in 10 mM amm.acetat

Time between injections: 4.25 min

	Start	Sec	Flow	Comp	SD	CD	Valve D	Flow	Grad	% B	Comments
0/1	0.00	60	1.00	A	Load	→	N/A	0.40	Step	20.0	Load Sample and Discard Plasma
2	1.00	60	1.00	A	Elute	→	N/A	0.40	Ramp	100.0	Elute sample to detector
3	2.00	90	1.00	A	Elute	→	N/A	0.40	Ramp	100.0	Elute sample to detector
4	3.50	30	1.00	B	Load	→	N/A	0.40	Step	20.0	Clean column + re-equilibrate
5	4.00	15	1.00	A	Load	→	N/A	0.40	Step	20.0	Clean column + re-equilibrate

Time between injections 7.50 min

	Start	Sec	Flow	Comp	SD	CD	Valve D	Flow	Grad	% B	Comments
0/1	0.00	120	1.00	A	Load	→	N/A	0.35	Step	20.0	Load Sample and Discard Plasma
2	2.00	60	1.00	A	Elute	→	N/A	0.35	Ramp	90.0	Elute sample to detector
3	3.00	180	1.00	A	Elute	→	N/A	0.35	Ramp	90.0	Elute sample to detector
4	6.00	60	1.00	B	Load	→	N/A	0.35	Step	20.0	Clean column + re-equilibrate
5	7.00	30	1.00	A	Load	→	N/A	0.35	Step	20.0	Clean column + re-equilibrate

Table 4.3.1-1 Gradient parameters, Quattro Ultima 2 column system using a MeOH-gradient

This was an important experiment for further method development. The result showed that the response (area) was higher when adding less ACN in the sample preparation, and hence the LOQ was better (lower).
The area of the chromatography peaks for the internal standard (warfarin) decreased as expected when adding less ACN. (The ACN used in the experiment for protein precipitation always contained 200 nm warfarin).
Adding formulations to the samples gave little impact on the results compared to the samples without formulation, but there were some variations.
The percentage deviations between the total area for the reference compounds with and without formulation are summarized in table 4.3.1-2, 4.3.1-3 and 4.3.1-4.

450 µL ACN:

Compound	4.25 minute method	7.50 minute method
Diazepam	-1%	-7%
Imipramine	13%	12%
Propranolol	0%	-8%
Diclofenac	26%	8%
Naproxen	27%	14%
Rofecoxib	16%	9%

Table 4.3.1-2 Deviation from standard curves without formulation

300 µL ACN:

Compound	4.25 minute method	7.50 minute method
Diazepam	1%	12%
Imipramine	-43%	72%
Propranolol	1%	13%
Diclofenac	-10%	-7%
Naproxen	8%	-16%
Rofecoxib	-24%	-13%

Table 4.3.1-3 Deviation from standard curves without formulation

200 µL ACN:

Compound	4.25 minute method	7.50 minute method
Diazepam	6%	20%
Imipramine	25%	78%
Propranolol	8%	7%
Diclofenac	-1%	18%
Naproxen	-28%	-6%
Rofecoxib	-51%	-5%

Table 4.3.1-4 Deviation from standard curves without formulation

As shown in table 4.3.1-2, 4.3.1-3 and 4.3.1-4, which shows the percentage difference when adding formulation, the impact is quite small for most of the substances, imipramine though shows a greater variation. The deviations diversifies in both positive and negative manner, it is therefore difficult to say if the formulations give suppression or enhancement. But overall, the deviations are small, they do not appear to be systematic, and the variations seem to be occasional.
Other results from this part of the study can be found in Appendix A.

Figure 7 illustrates the summarized area of the pooled standard curves for the compound propranolol comparing the amount of ACN added in the protein precipitation. As shown in the figure, the response/ area increased when adding less ACN in the sample preparation.

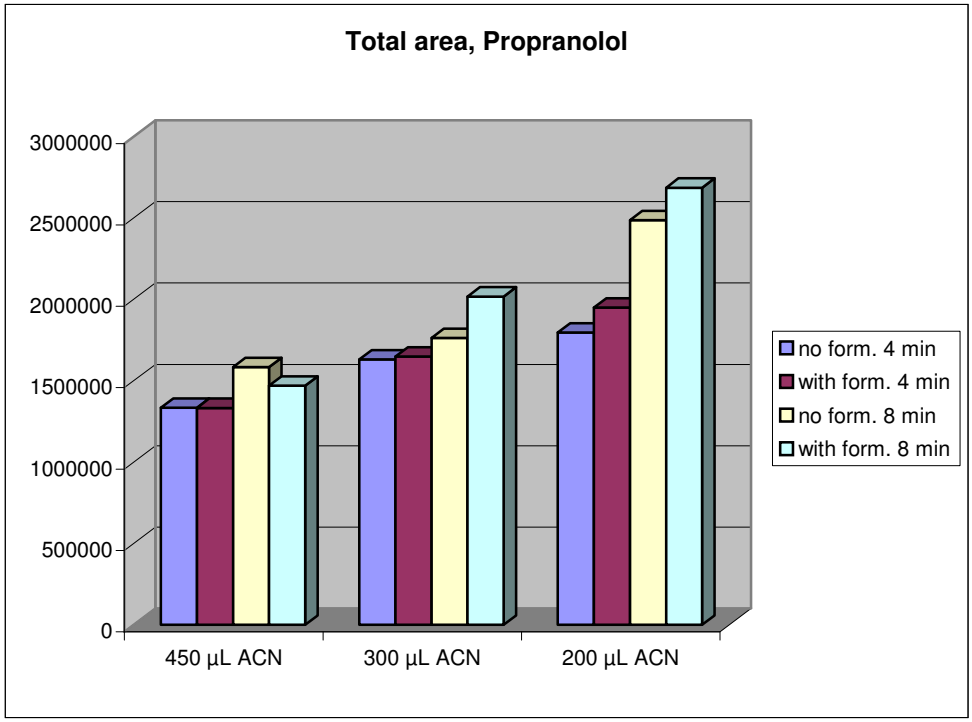


Figure 7 Summarized area of propranolol with different amounts of ACN added in the sample preparation

Table 4.3.1-5 shows the LOQ for all the reference compounds in the 4.25 min method both with and without formulation also considering the amount of ACN added in the sample preparation.

Standard concentrations: 5-10-34-113-375-1250-3333-10 000 (nmol/L)

LOQ [nM]	**No formulation**			**With formulation**		
Amount of ACN	**450µL**	**300µL**	**200µL**	**450µL**	**300µL**	**200µL**
Diazepam	5	5	5	5	5	5
Imipramine	5	5	5	5	5	5
Propranolol	5	5	5	5	5	5
Diclofenac	5	5	5	34	5	5
Naproxen	34	10	5	34	5	5
Rofecoxib	34	34	5	34	5	5

Table 4.3.1-5 LOQ (nmol/L) for the reference compounds with and without formulation, different amounts of ACN added in the sample preparation

As shown in the table, the lowest standard concentration (5 nmol/L) can be detected for all the compounds both with and without formulation when adding the lowest amount of ACN (200 µL) in the sample preparation. Even though there are some small variations, the formulations did not seem to affect the results. This experiment involved that 200 µL ACN was set to be used in the sample preparation of pooled samples.

4.4 Pharmakokinetic profiles

Pharmacokinetics (PK) is the study of rate processes such as absorption, distribution and excretion of a drug and the multiple relationships that affects the drug. PK describes the quantitative relationship between administrated doses and dosing regimens and the observed plasma levels of a drug. The drug can be administered to the test animal in several ways, in this study we will go further into the PK profiles from the oral (PO) and intravenous (IV) route [7].

4.4.1 Pre-check, PK profiles of the basic reference compounds, PO route

In this experiment, plasma samples were spiked with the basic reference compounds (diazepam, imipramine and propranolol) corresponding to the concentrations of literature PK values as described in methods and experimental. The samples were analysed both in discrete/single and cassette/pooled mode.

Eight time points for the literature PK values were chosen, administered via the oral route. Standard sample preparation was followed, 25µL spiked plasma was precipitated with 150 µL ice cold ACN, 120 µL of the supernatant was mixed with 300 µL buffer (mobile phase) before analysis. For the pooled samples, 75 µL (25 µL x 3) spiked plasma was precipitated with 200 µL ice cold ACN, 120 µL of the supernatant was mixed with 300 µL buffer.

MS instrumentation used for this experiment was the two-column system Quattro Ultima, using a 4.25 min method, similar to the method used for reducing the amount of ACN in the sample preparation.

The results are summarized in table 4.4.1-1 showing the measured concentrations for both single and pooled samples compared with the literature values.

Diazepam:

Time (h)	Literature conc.[nM]	Measured conc.[nM] Single	Measured conc.[nM] Pool
0.25	650	709	725
0.5	750	804	835
0.75	700	760	724
1	650	718	686
1.5	450	442	397
2.5	220	418	351
6	25	48	45
24	5	7	9

Imipramine:

Time (h)	Literature conc.[nM]	Measured conc.[nM] Single	Measured conc.[nM] Pool
0.25	360	403	453
0.5	270	329	332
0.75	430	535	535
1	270	297	341
1.5	270	313	347
2.5	230	284	270
6	150	185	160
24	70	95	95

Propranolol:

Time (h)	Literature conc.[nM]	Measured conc.[nM] Single	Measured conc.[nM] Pool
0.25	280	374	432
0.5	160	204	254
0.75	100	125	170
1	60	83	109
1.5	40	51	69
2.5	20	26	27
6	8	12	12
24	0	0	0

Table 4.4.1-1 PK concentrations showing literature, single and pooled values for the basic reference compounds, PO route

There are some variations in the measured concentrations compared to the literature concentration values, all over, the literature values are lower than the measured values for both single and pooled compounds. Although, the deviations between single and pooled samples are small. This is illustrated in figure 8 and 9 where the literature, single and pooled values are compared for diazepam and imipramine.

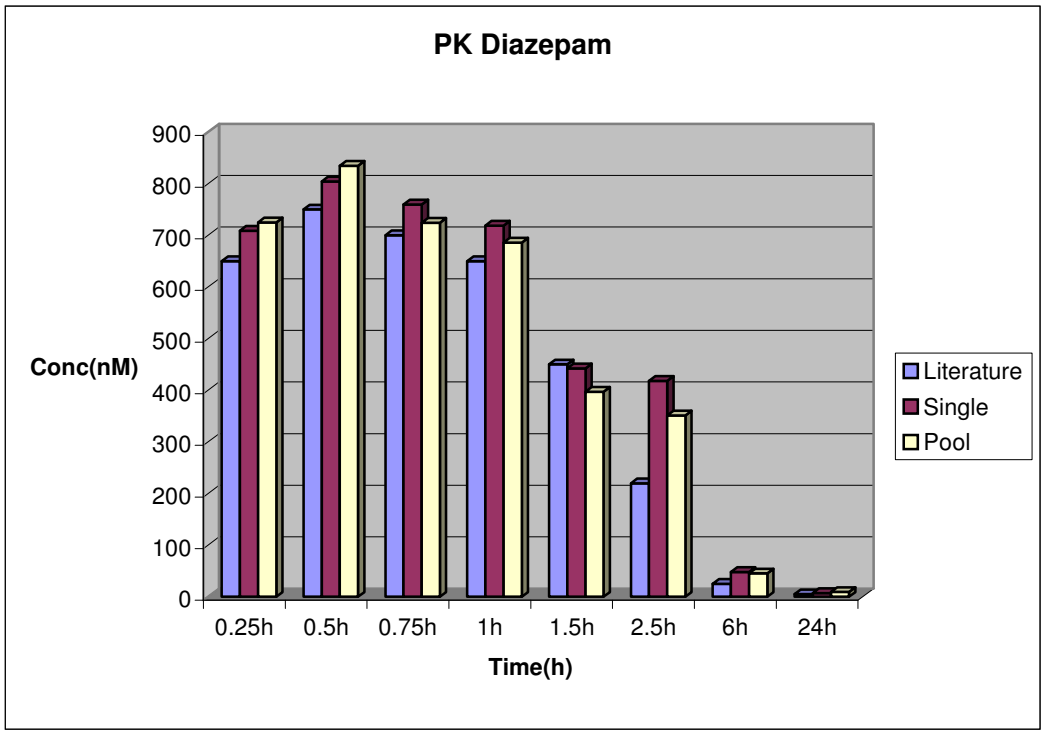


Figure 8 Comparing diazepam PK concentrations for literature, single and pooled values, PO route

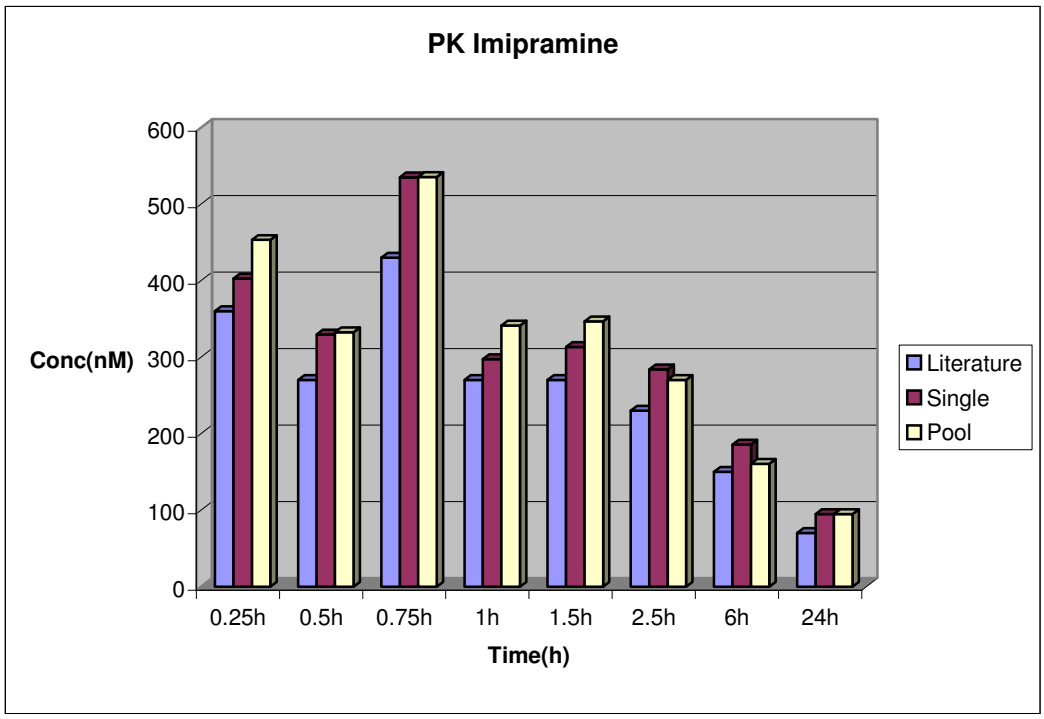


Figure 9 Comparing imipramine PK concentrations for literature, single and pooled values, PO route

The results of the deviation between single and pooled samples are summarized in table 4.4.1-2 showing the percentage deviation from the single samples.

Time (h)	Diazepam	Imipramine	Propranolol
0.25	2%	11%	13%
0.5	4%	1%	20%
0.75	-5%	0%	27%
1	-5%	13%	23%
1.5	-11%	10%	26%
2.5	-19%	-5%	2%
6	-6%	-16%	-5%
24	24%	0%	0%

Table 4.4.1-2 PK profiles, pooled concentrations values, deviation from single concentration values, PO route

The results show only small variations between the single and pooled measured values. The variations for diazepam vary from –11% to 24%, for imipramine –16% to 13%, and for propranolol –5% to 27%.

4.4.2 PK profiles of the acidic reference compounds, PO route

As in the previous experiment with the basic compounds, the plasma samples were now spiked with the acidic reference compounds (diclofenac, naproxen and rofecoxib) corresponding to the concentrations of literature PK values as described in methods and experimental. The samples were analysed both in discrete/single and cassette/pooled mode.

MS instrumentation used for this experiment was a one-column system Quattro Ultima, using a 4 min method with an acetonitrile gradient.

The results are summarized in table 4.4.2-1 showing the measured concentrations for both single and pooled samples compared with the literature values.

Diclofenac:

Time (h)	Literature conc.[nM]	Measured conc.[nM] Single	Measured conc.[nM] Pool
0.25	3000	3751	3392
0.50	2000	2518	2616
0.75	1500	1998	1830
1.00	1000	1119	1097
1.50	800	954	946
2.50	650	737	605
6.00	400	415	461
24.00	10	41	25

Naproxen:

Time (h)	Literature conc.[nM]	Measured conc.[nM] Single	Measured conc.[nM] Pool
0.25	35000	28174	26882
0.50	43000	34490	34386
0.75	43000	30069	32582
1.00	43000	36473	38203
1.50	45000	39000	38063
2.50	45000	38872	36385
6.00	40000	35631	39091
24.00	3000	3149	2866

Rofecoxib:

Time (h)	Literature conc.[nM]	Measured conc.[nM] Single	Measured conc.[nM] Pool
0.25	70	62	34
0.50	150	106	191
0.75	270	194	233
1.00	300	268	284
1.50	450	390	345
2.50	600	502	438
6.00	450	403	428
24.00	70	50	49

Table 4.4.2-1 PK concentrations showing literature, single and pooled values for the acidic reference compounds, PO route

As for diazepam, imipramine and propranolol, the literature concentration values were a little lower than the measured single and pooled concentration values for diclofenac, but for naproxen and rofecoxib, it was the other way around. This is illustrated in figure 10 and 11.

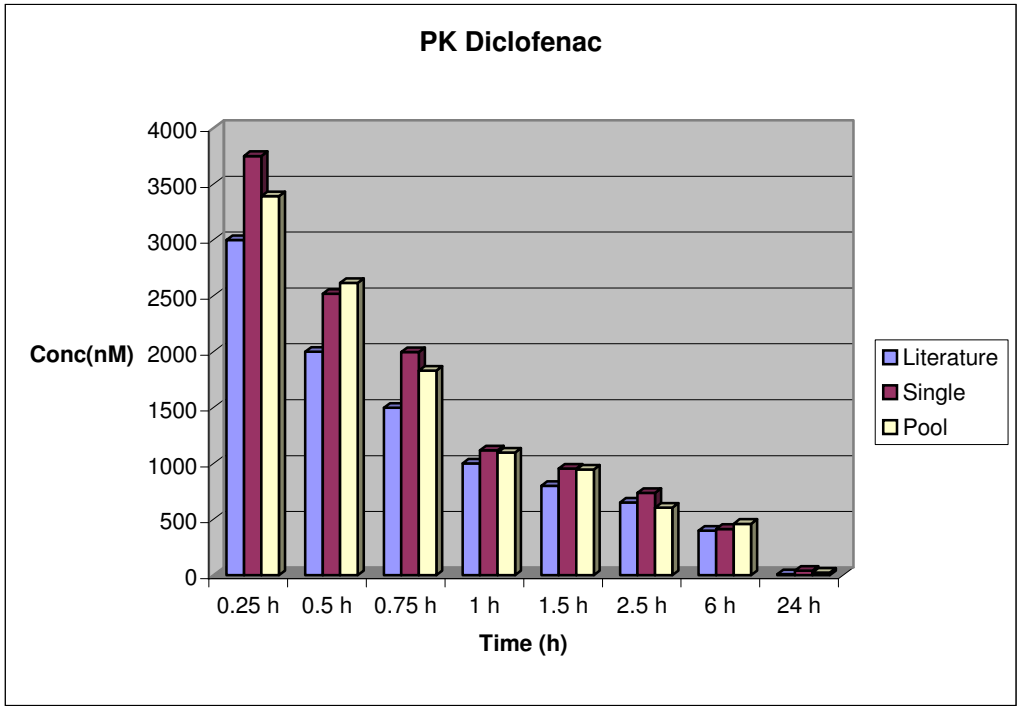


Figure 10 Comparing diclofenac PK concentrations for literature, single and pooled values, PO route

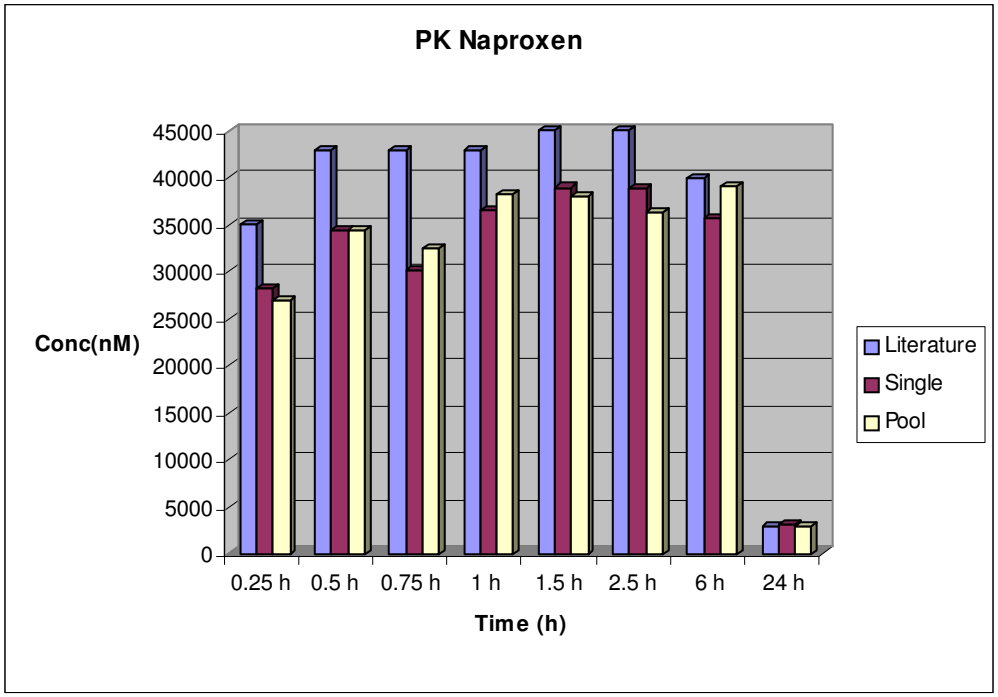


Figure 11 Comparing naproxen PK concentrations for literature, single and pooled values, PO route

Although, the difference between the literature and the single/pooled values were not very large, what is interesting are the relative small difference between the single and the pooled concentration values.

The deviations between the single and pooled samples for the acidic cassette are listed in table 4.4.2-2. As shown in the table, three values exceed the ±25% acceptance criterion, but all the other values are good, so the reason for this is probably occasional variations.

Time (h)	**Diclofenac**	**Naproxen**	**Rofecoxib**
0.25	-11%	-5%	-82%
0.5	4%	0%	45%
0.75	-9%	8%	17%
1	-2%	5%	6%
1.5	-1%	-2%	-13%
2.5	-22%	-7%	-15%
6	10%	9%	6%
24	-64%	-10%	-2%

Table 4.4.2-2 PK profiles, pooled concentrations values, deviation from single concentration values, PO route

4.4.3 PK curves, PO route, compilation

The PK parameters and the PK curves for all the reference compounds are shown in table 4.3.1-1 to table 4.4.1-12 and in figure 13 – 18. The figures illustrates the estimated PO route for three trials analysed both on UPLC and HPLC in both discrete and cassette mode.

Figure 12 explains the PK parameters used in the following tables and figures.

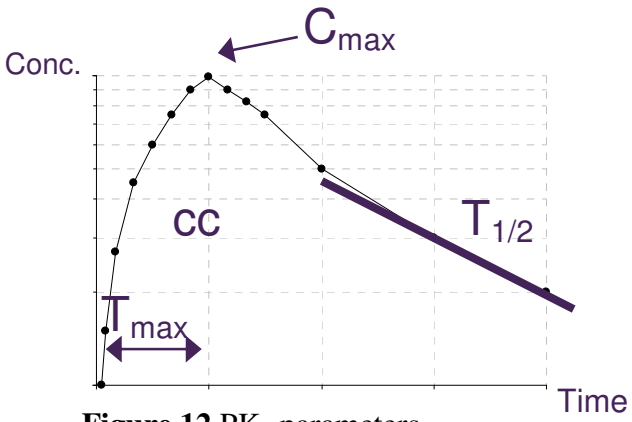


Figure 12 PK- parameters

C_{max} is the highest concentration in the PK curve, $T_{1/2}$ is the biological half time. T_{max} is the time point where the concentration is highest (C_{max}) and AUC_{last} refers to the "area under curve", the area under the PK curve plotted in the diagram.

When looking at the PK parameters, the reader should have in mind, that for the PO route, the important parameters are C_{max}, $T_{1/2}$ and AUC, whilst for the IV route, the most important PK parameters are volume of distribution (V) and clearance (CL).

Diazepam:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	AUCextr	V_F (L/kg)	CL_F (L_kg)
Literature		4.7	750	0.5	1737	1766	1.7	38.3	5.7
Trial 1	Single	4.6	784	0.5	1808	1842	1.9	36.1	5.4
	Pooled	5.0	728	0.5	2005	2060	2.7	34.9	4.9
Trial 2	Single	4.3	804	0.5	2356	2394	1.6	25.7	4.2
	Pooled	4.9	835	0.5	2225	2282	2.5	30.7	4.4
Trial 3	Single	4.4	979	0.5	2355	2388	1.4	26.6	4.2
	Pooled	4.6	1067	0.5	2532	2573	1.6	25.9	3.9

Table 4.4.3-1 PK parameters for diazepam, PO route

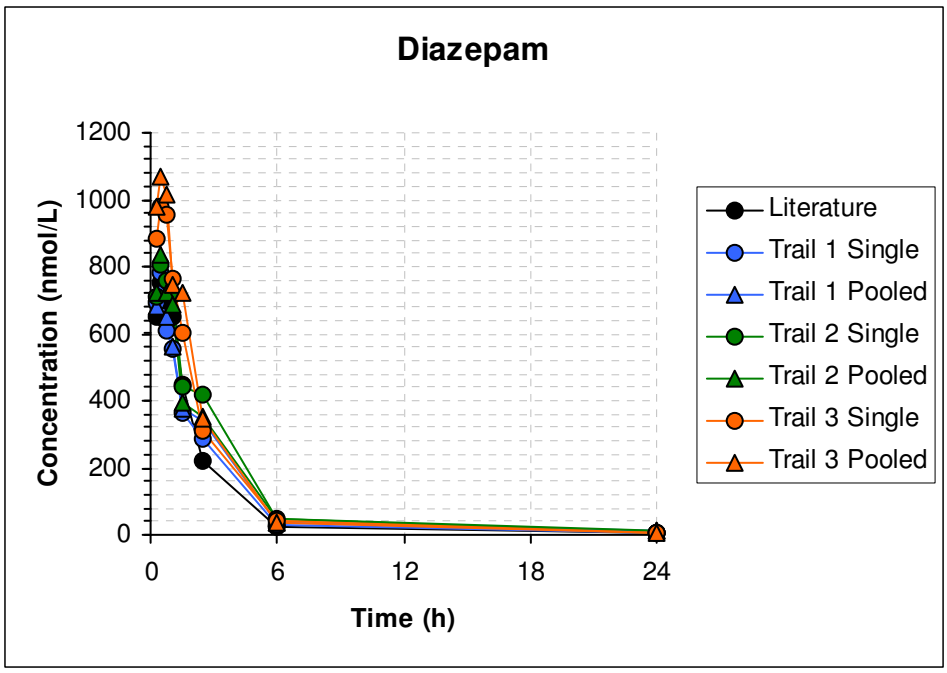


Figure 13 PK curves for diazepam, PO route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V_F (L/kg)	CL_F (L_kg)
Trial 1	8%	7%	0%	11%	12%	3%	11%
Trial 2	14%	4%	0%	6%	5%	20%	5%
Trial 3	5%	9%	0%	8%	8%	2%	7%

Table 4.4.3-2 PK parameters for diazepam, deviation from single, PO route

Diazepam show very small variations between single and pooled samples, all the PK parameters fulfilled the ± 25% acceptance criterion.

Imipramine:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	AUCextr	V_F (L/kg)	CL_F (L_kg)
Literature		13.6	430	0.75	3226	4566	29.4	214.4	11.0
Trial 1	Single	15.4	500	0.75	3620	5503	34.2	201.9	9.1
	Pooled	14.5	371	0.75	2877	4163	30.9	250.5	12.0
Trial 2	Single	14.9	535	0.75	4041	6025	32.9	178.1	8.3
	Pooled	13.4	648	0.75	4356	6112	28.7	158.6	8.2
Trial 3	Single	14.0	468	0.75	3723	5333	30.2	189.2	9.4
	Pooled	13.5	405	0.75	3476	4920	29.4	198.6	10.2

Table 4.4.3-3 PK parameters for imipramine, PO route

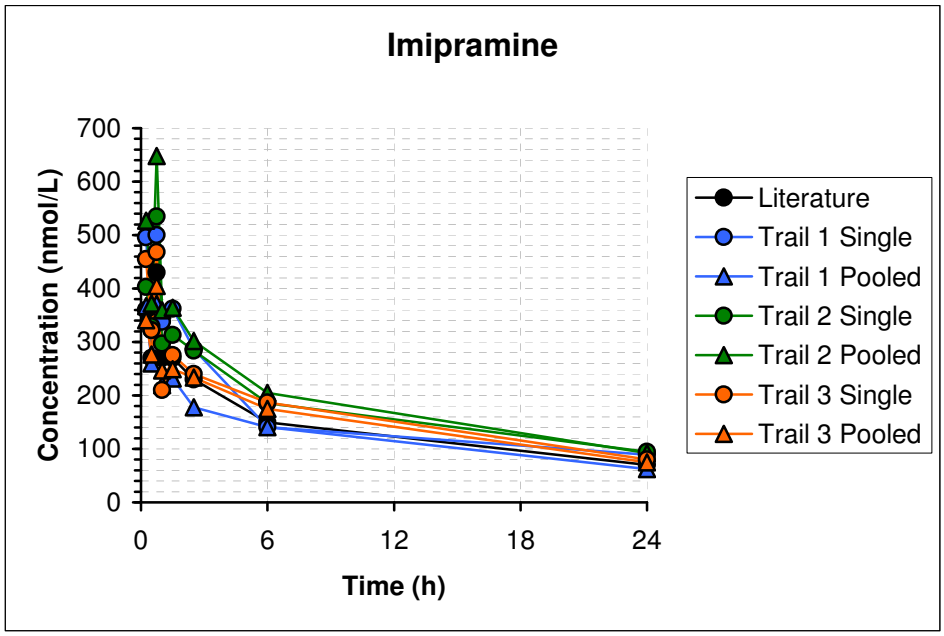


Figure 14 PK curves for imipramine, PO route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V_F (L/kg)	CL_F (L_kg)
Trial 1	6%	26%	0%	21%	24%	24%	32%
Trial 2	10%	21%	0%	8%	1%	11%	1%
Trial 3	3%	13%	0%	7%	8%	5%	8%

Table 4.4.3-4 PK parameters for imipramine, deviation from single, PO route

For imipramine, there seem to be a little variation between single and pooled samples looking at the PK curves. But, looking at the PK parameters, the deviations between single and pooled samples are overall quite small, varying from 1% to 32%.

Propranolol:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	AUCextr	V_F (L/kg)	CL_F (L_kg)
Literature		2.1	280	0.25	239	262	8.8	263.5	87.6
Trial 1	Single	0.8	248	0.25	191	209	8.2	125.6	110.3
	Pooled	2.2	222	0.25	198	220	9.9	337.3	104.7
Trial 2	Single	1.8	343	0.25	300	321	6.5	181.8	71.6
	Pooled	2.0	432	0.25	377	407	7.5	159.1	56.5
Trial 3	Single	2.5	301	0.25	242	273	11.3	308.6	84.3
	Pooled	2.5	169	0.5	209	242	13.7	349.8	95.1

Table 4.4.3-5 PK parameters for propranolol, PO route

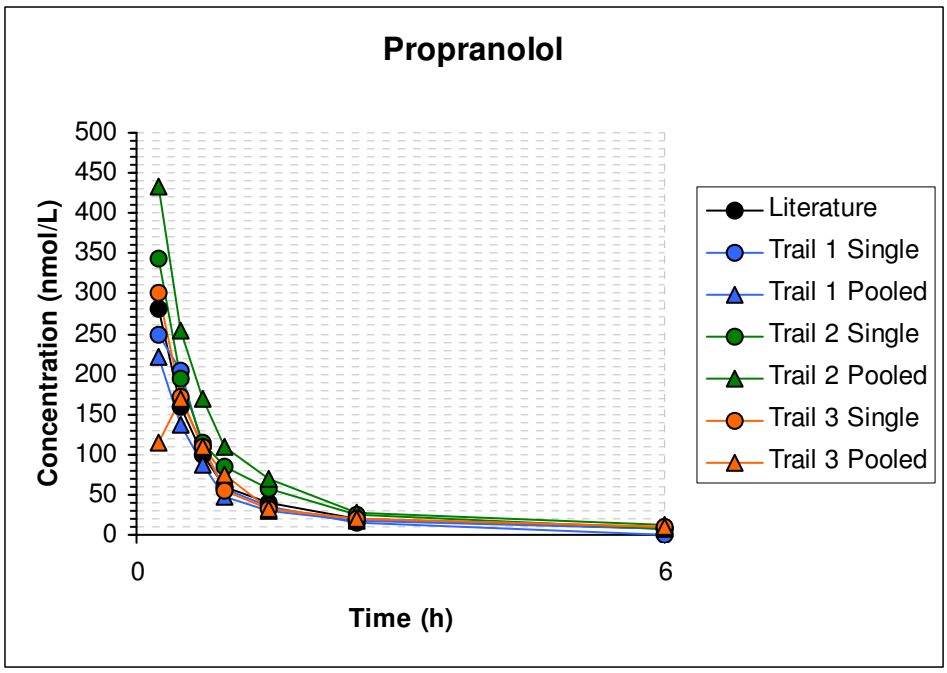


Figure 15 PK curves for propranolol, PO route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V_F (L/kg)	CL_F (L_kg)
Trial 1	183%	10%	0%	3%	5%	168%	5%
Trial 2	11%	26%	0%	25%	27%	13%	21%
Trial 3	0%	44%	100%	14%	11%	13%	13%

Table 4.4.3-6 PK parameters for propranolol, deviation from single, PO route

For propranolol, there are some variations between single and pooled samples as shown in the PK curves and in the PK parameters. The deviations do not appear to be systematic, but rather occasional, because looking at trial 2 isolated, it is quite acceptable.

Diclofenac:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	AUCextr	V_F (L/kg)	CL_F (L_kg)
Literature		3.5	3000	0.25	6610	6662	0.8	7.6	1.5
Trial 1	Single	5.2	3751	0.25	8412	8719	3.5	8.7	1.1
	Pooled	4.5	3392	0.25	7898	8066	2.1	8.1	1.2
Trial 2	Single	3.5	2334	0.25	5485	5527	0.8	9.1	1.8
	Pooled	3.6	3430	0.25	7245	7308	0.9	7.1	1.4
Trial 3	Single	3.3	3151	0.25	6493	6531	0.6	7.4	1.5
	Pooled	3.7	2592	0.25	5791	5846	0.9	9.1	1.7

Table 4.4.3-7 PK parameters for diclofenac, PO route

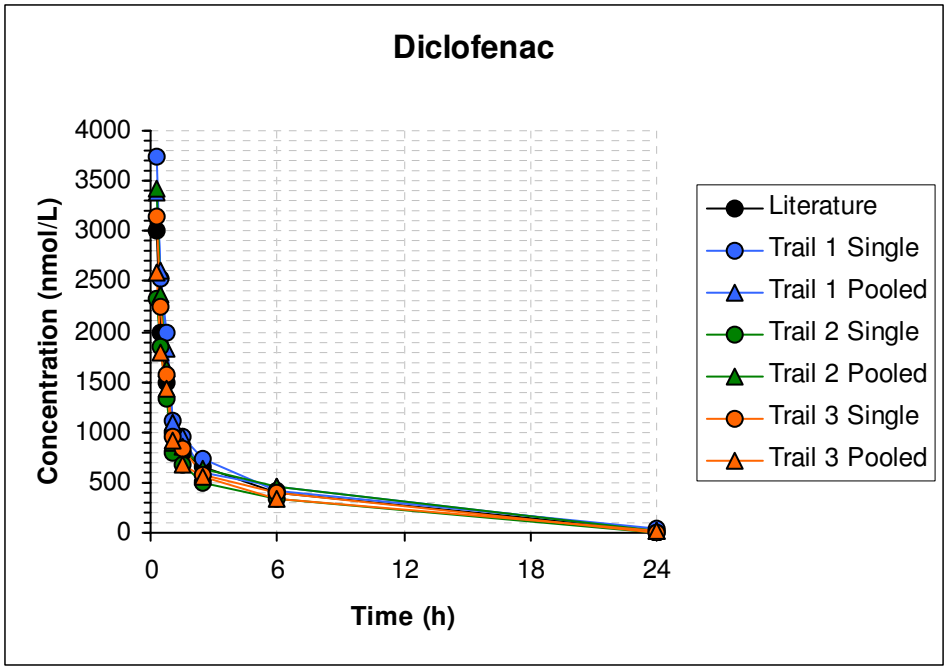


Figure 16 PK curves for diclofenac, PO route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V_F (L/kg)	CL_F (L_kg)
Trial 1	13%	10%	0%	6%	7%	6%	8%
Trial 2	3%	47%	0%	32%	32%	22%	24%
Trial 3	10%	18%	0%	11%	10%	23%	12%

Table 4.4.3-8 PK parameters for diclofenac, deviation from single, PO route

The PK curves for diclofenac show very little variation between single and pooled samples. In trial 2, some of the PK parameters exceeds the acceptance criterion, the reason for this is the heavy slope in the beginning of the PK curve, where small variations affects the PK parameters in a major degree.

Naproxen:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	AUCextr	V_F (L/kg)	CL_F (L_kg)
Literature		5.3	45000	1.5	508320	531781	4.4	0.1	0.0
Trial 1	Single	5.6	39000	1.5	456823	483269	5.5	0.2	0.0
	Pooled	5.5	39091	6	466150	489774	4.8	0.2	0.0
Trial 2	Single	6.5	26336	1.5	325806	354023	8.0	0.3	0.0
	Pooled	5.8	29426	2.5	342796	363796	5.8	0.2	0.0
Trial 3	Single	6.7	27925	2.5	346813	379965	8.7	0.3	0.0
	Pooled	5.6	28836	0.75	342747	362493	5.4	0.2	0.0

Table 4.4.3-9 PK parameters for naproxen, PO route

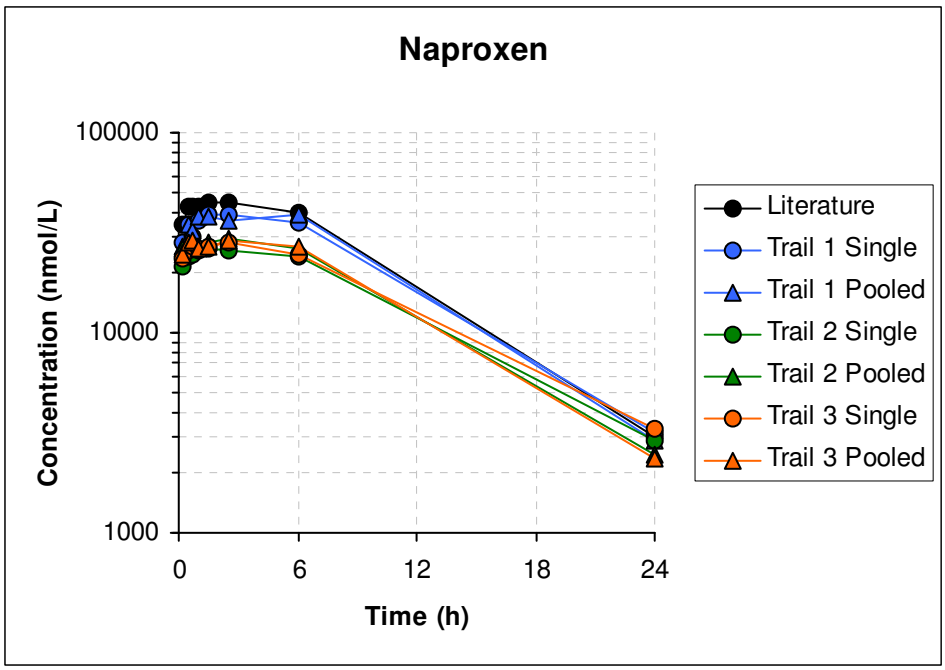


Figure 17 PK curves for naproxen, PO route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V_F (L/kg)	CL_F (L_kg)
Trial 1	3%	0%	300%	2%	1%	5%	1%
Trial 2	12%	12%	67%	5%	3%	14%	2%
Trial 3	16%	3%	70%	1%	5%	12%	5%

Table 4.4.3-10 PK parameters for naproxen, deviation from single, PO route

The differences between single and pooled samples for naproxen are all over small. The reason for the great variations for the Tmax is because this parameter comes very early in the PK curve, and the values are very small. Therefore, small variations in the time for Tmax will give a great influence on the deviation between single and pooled samples. As mentioned earlier, Tmax is not an important parameter in the PO route.

Rofecoxib:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	AUCextr	V_F (L/kg)	CL_F (L_kg)
Literature		6.9	600	2.5	6373	7066	9.9	14.0	1.4
Trial 1	Single	6.3	502	2.5	5357	5811	7.9	15.6	1.7
	Pooled	6.4	437.5	2.5	5356	5807	8.0	15.9	1.7
Trial 2	Single	7.3	730	2.5	6728	7642	11.7	13.8	1.3
	Pooled	8.4	531	2.5	6254	7360	15.0	16.5	1.4
Trial 3	Single	5.7	807	2.5	7532	8030	6.2	10.2	1.2
	Pooled	7.3	536	2.5	6119	6893	11.3	15.4	1.4

Table 4.4.3-11 PK parameters for rofecoxib, PO route

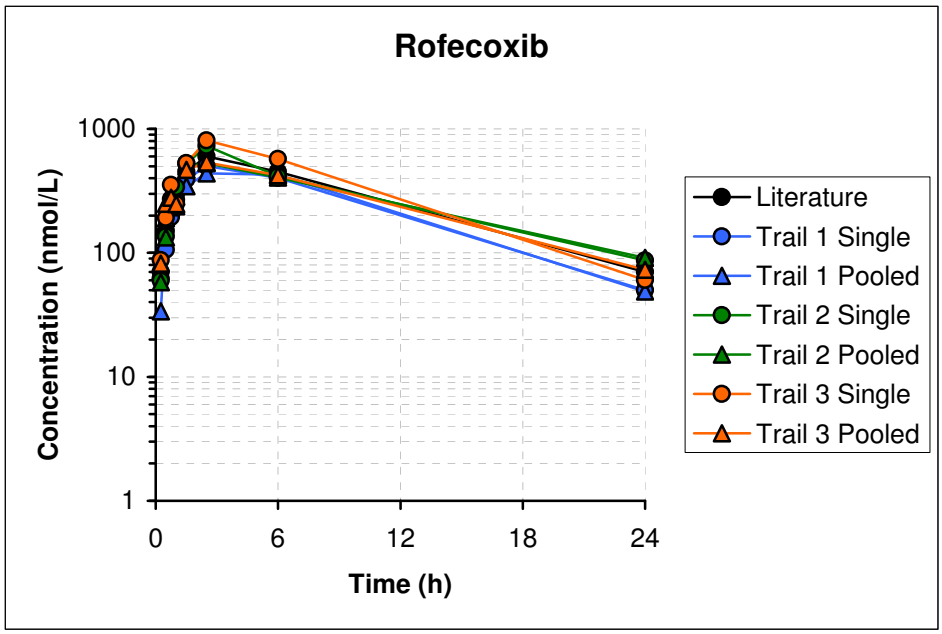


Figure 18 PK curves for rofecoxib, PO route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V_F (L/kg)	CL_F (L_kg)
Trial 1	2%	13%	0%	0%	0%	2%	0%
Trial 2	16%	27%	0%	7%	4%	20%	4%
Trial 3	29%	34%	0%	19%	14%	50%	16%

Table 4.4.3-12 PK parameters for rofecoxib, deviation from single, PO route

Like most of the other reference compounds, there are only small deviations between single and pooled samples for rofecoxib.

In this study, one of the main goals was that the deviation between single and pooled samples should not exceed ±25 %. However, for some reference compounds, occasional deviations >25% are observed, but this does not appear to be systematic.

4.5 AstraZeneca *in vivo* animal studies

Three AstraZeneca Animal (rat) studies were chosen to study PK profiles further. These studies consisted of one compound in each study, totally three compounds. Each compound had been dosed to a rat; three rats were given the same compound, which means that there were totally nine series. In each study, the AZ compound were dosed to three rats via the PO route, three rats via the IV route, and also to three rats as an infusion. In this part of the study, we will only go further into the PO route. The dose given to the test animals were 10 µmol/kg for the PO route in all studies.

Blood samples were taken from the rats after dosing at these time intervals:

15 min – 30 min – 45 min – 60 min – 1,5 h – 2,5 h – 6 h and 24 h after dosing.

The three AstraZeneca compounds are in this report referred to as AZ1, AZ2 and AZ3. The molecular weights of these compounds are respectively 351.32, 360.46 and 474.26. Table 4.5-1 is showing the MS methods/ MRM-scan transitions for the compounds.

Compound	Parent (m/z)	Daughter (m/z)	CV (V)	CE (eV)	Ion mode
1) AZ1	352.01	173.00	46	22	ES+
2) AZ2	361.13	219.94	37	34	ES+
3) AZ3	473.98	190.03	46	28	ES+
Warfarin (IS)	308.88	163.1	18	16	ES+

Table 4.5-1 MS methods/ MRM-scan transitions for the AstraZeneca compounds

4.5.1 Standard curves

In the first experiment, standard curves for the three compounds were made, also as a cassette. The standard concentrations were 5, 10, 34, 112.5, 375, 1250, 3333 and 10000 nM. The samples were analysed on the mass spectrometer Quattro Ultima, a 2-column system using a meOH gradient. Total duration time for one analysis was app. 4 minutes.

The results were satisfying; figure 19 shows a typical response- and residual graph of the standard curve of compound AZ2. Figure 20 shows the chromatogram for the same compound together with the chromatogram for the internal standard, warfarin. The retention time for AZ2 was 3.01 min and 2,62 min for warfarin.

The maximum deviation from the theoretical values was –7% for this compound. For the compounds AZ1 and AZ3, the maximum deviation were respectively 4,6% and 14,3%. The lowest standard concentration value (5 nM) for the compound AZ1 was below the LOQ, and had to be excluded from the standard curve. For the pooled standard curve, the maximum deviation from the theoretical values were 9,3%, 11,8% and 11,8% respectively for the compounds AZ1, AZ2 and AZ3.

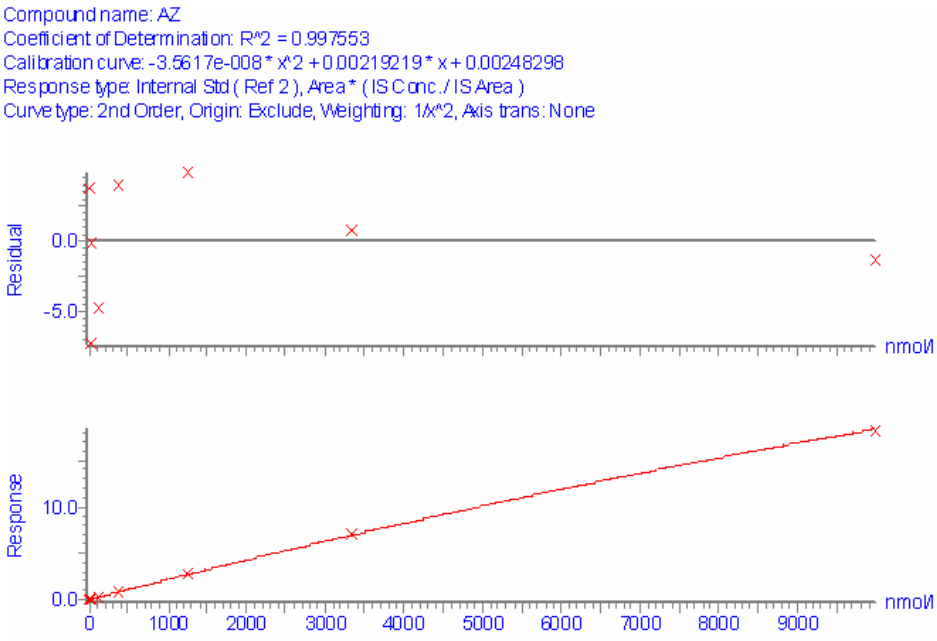


Figure 19 Standard curve, compound AZ2, the residual graph shows the deviation for each measured concentration value from the theoretical values (%)

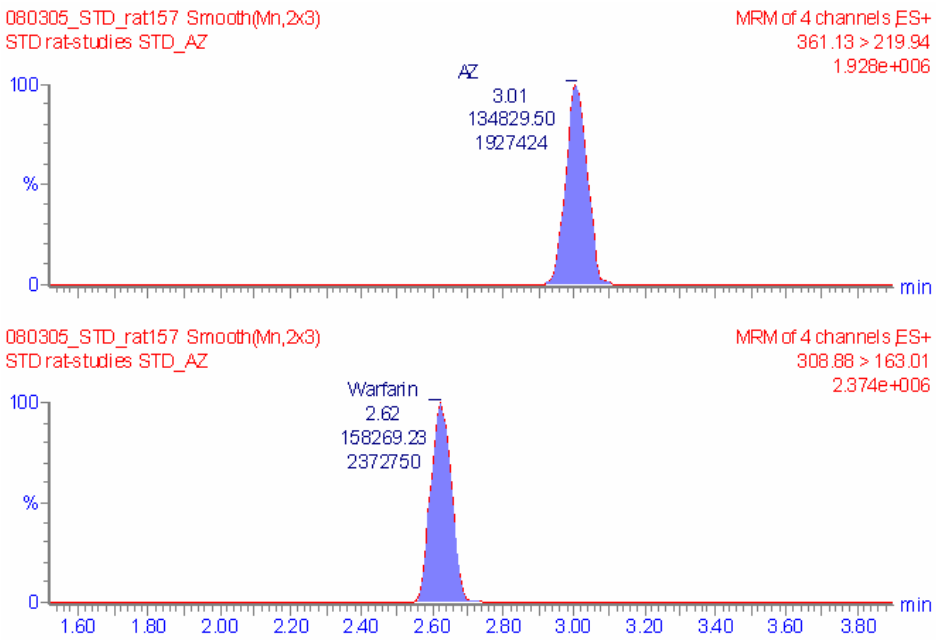


Figure 20 HPLC chromatogram for compound AZ2 with IS (warfarin) using a Hypurity C-18 column

The comparison of the results between single and pooled samples is shown in table 4.5.1-1. The percentage deviation is listed at the right of each table; the values are marked with a green colour because they all are satisfying and fall within the acceptance criterion of ±25 % deviation between single and pooled samples. The deviation is in fact 13 % or better for all values.

AZ1

Theory	Single	Pool	
5	NV	NV	NV *
10	10.1	9.8	-3%
34	32.5	37.2	13%
112	113.5	106.7	-6%
375	369.3	362	-2%
1250	1307.3	1312	0%
3333	3292.2	3165	-4%
10000	9994.6	10126.4	1%

*These values were below LOQ

AZ2

Theory	Single	Pool	
5	5.2	4.9	-6%
10	9.3	10.7	13%
34	34	33.1	-3%
112	106.6	103.3	-3%
375	389.9	366.4	-6%
1250	1311	1397.7	6%
3333	3358.2	3254.9	-3%
10000	9867.5	9905	0%

AZ3

Theory	Single	Pool	
5	5.1	5.3	4%
10	9.5	9	-6%
34	33.8	33.1	-2%
112	105.7	110.2	4%
375	351.4	356.8	2%
1250	1428.5	1397	-2%
3333	3429.3	3496	2%
10000	9719.1	9671.6	0%

Table 4.5.1-1 Comparison of single and pooled standard curve values for the compounds AZ1, AZ2 and AZ3

4.5.2 Single vs. pooled, PO route

Formulations used in the AZ studies were 5% dimethyl acetamide (DMA) in 20% cyclodextrine in water for AZ1 and AZ2. For AZ3, the formulation solution used were 5% DMA in 95% cyclodextrine.

Although, the most interesting from this experiment is the difference between single and pooled samples. These results are listed in table 4.5.2-1 and 4.5.2-2, showing the percentage deviation between single and pooled samples of the AZ compounds in serial1 (single 1 and pool 1) and serial 2 (single 2 and pool 2) respectively.

Deviation from single 1

	AZ1-rat 4	AZ1-rat 5	AZ1-rat 6
Time(h)	**Pool1**	**Pool1**	**Pool1**
15min	-7%	15%	NV
30min	-2%	23%	21%
45min	-1%	20%	26%
1h	3%	10%	27%
1.5h	13%	7%	38%
2.5h	16%	1%	33%
6h	18%	6%	33%
24h	0%	0%	0%

	AZ2-rat 4	AZ2-rat 5	AZ2-rat 6
Time(h)	**Pool1**	**Pool1**	**Pool1**
15min	-31%	0%	1%
30min	-38%	9%	-7%
45min	-40%	-19%	-13%
1h	-8%	0%	0%
1.5h	-8%	-28%	-13%
2.5h	-9%	-13%	-2%
6h	-18%	-20%	0%
24h	0%	0%	0%

	AZ3-rat 4	AZ3-rat 5	AZ3-rat 6
Time(h)	**Pool1**	**Pool1**	**Pool1**
15min	-4%	-4%	19%
30min	-18%	17%	7%
45min	-28%	1%	13%
1h	-24%	2%	10%
1.5h	1%	-8%	12%
2.5h	-6%	-1%	14%
6h	-1%	11%	1%
24h	0%	0%	0%

Table 4.5.2-1 Percentage deviation between single and pooled AZ compounds (AZ1, AZ2 and AZ3) in the first run

Deviation from single 2

	AZ1-rat 4	AZ1-rat 5	AZ1-rat 6
Time(h)	Pool2	Pool2	Pool2
15min	5%	5%	NV
30min	7%	4%	18%
45min	9%	5%	13%
1h	16%	1%	14%
1.5h	10%	5%	33%
2.5h	0%	11%	13%
6h	3%	3%	2%
24h	0%	0%	0%

	AZ2-rat 4	AZ2-rat 5	AZ2-rat 6
Time(h)	Pool2	Pool2	Pool2
15min	-17%	0%	5%
30min	-29%	-19%	-19%
45min	-17%	-48%	-33%
1h	-27%	0%	0%
1.5h	-26%	-35%	-8%
2.5h	-40%	-24%	10%
6h	-30%	-18%	-6%
24h	0%	0%	0%

	AZ3-rat 4	AZ3-rat 5	AZ3-rat 6
Time(h)	Pool2	Pool2	Pool2
15min	-7%	2%	11%
30min	-8%	4%	8%
45min	-10%	-21%	11%
1h	5%	4%	1%
1.5h	-13%	7%	-13%
2.5h	-14%	-7%	-7%
6h	-16%	15%	-1%
24h	0%	0%	0%

Table 4.5.2-2 Percentage deviation between single and pooled AZ compounds (AZ1, AZ2 and AZ3) in the second run

The results are overall quite satisfying, but there are some variations between the single and pooled samples. For the compound AZ1, the pooled values are overall higher than for the single values. But for the compound AZ2, the concentration values are overall lower than for the single concentration values, and as for the last compound, AZ3 there are more variations in the way the pooled values differs from the single values. For animal 4 (rat 4), the values are overall lower for the pooled values than for the single values, for animal 5, there are higher values for pooled samples, and for animal 6, the values are for the most higher for the pooled samples than for the single samples.

Also, looking at the differences between the two runs, it seems that the values drop some in the second run. This results in both “better” and “worse” values for the pooled samples in the second run compared with the single values, since there are differences in both ways. In other words, when pooled values that are higher than the single values drops some, they get closer to the single values and hence, they are

"better", but when pooled values that are lower than the single values drops even more, they get further away from the single values, and hence, they become worse. Considering that there are some variations in the analysis system (differences between runs), the results between the single and pooled samples are acceptable, most of the values fall within the limit of ±25 % deviation.

4.5.3 PK parameters, compilation, single vs. pooled, PO route

From the values of each time point in the PK profile, there were some variations between the single and pooled samples, even though most of the values fulfilled the acceptance criterion. But, if we look at the most important PK parameters for the PO route (AUC, C_{max} and $T_{1/2}$), the deviations between single and pooled samples are very small. For instance, the average deviations between single and pooled samples for the substance AZ1 were 2% for AUC, 9% for C_{max} and –2% for $T_{1/2}$. This is illustrated in table 4.5.3-1 and figure 21– 23, comparing the deviations between PK parameters for single and pooled samples.

AUC		**AZ1**	**AZ2**	**AZ3**
average	Discrete	925.7	154.8	1703.8
	Cassette	949.2	141.2	2077.3
stdev	Discrete	25.5	26.3	373.9
	Cassette	113.3	33.3	693.8

Cmax		**AZ1**	**AZ2**	**AZ3**
average	Discrete	380.7	57.7	643.7
	Cassette	418.7	55.7	709.3
stdev	Discrete	2.5	16.2	100.4
	Cassette	50.1	17.7	84.5

$T_{1/2}$ (h)		**AZ1**	**AZ2**	**AZ3**
average	Discrete	3.9	4.0	3.3
	Cassette	3.8	4.1	3.2
stdev	Discrete	1.1	0.9	1.7
	Cassette	0.7	1.3	1.4

Table 4.5.3-1 Average PK parameters for the AZ substances AZ1, AZ2 and AZ3, both discrete and cassette mode

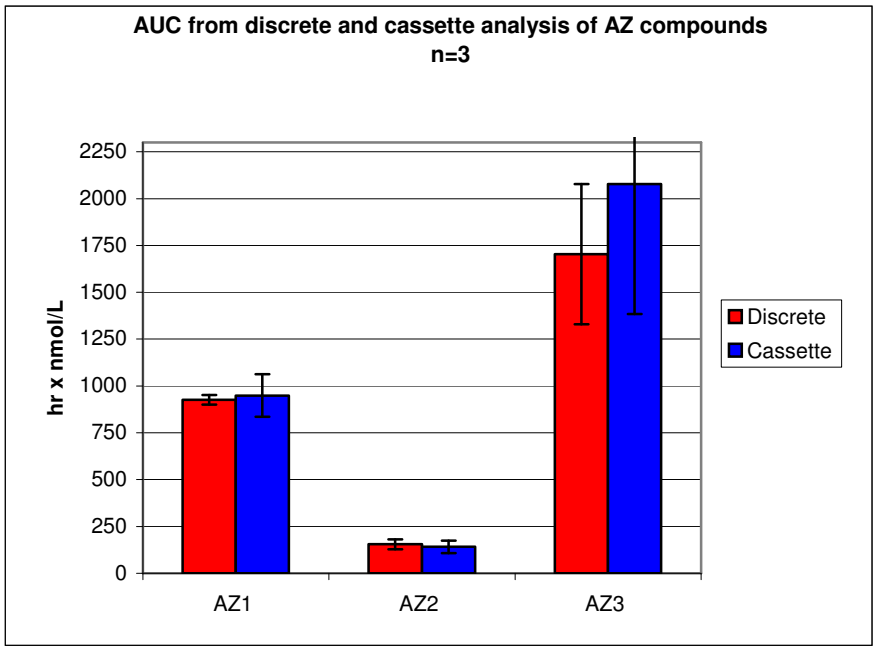


Figure 21 Comparing AUC between single and pooled samples for the AZ compounds AZ1, AZ2 and AZ3

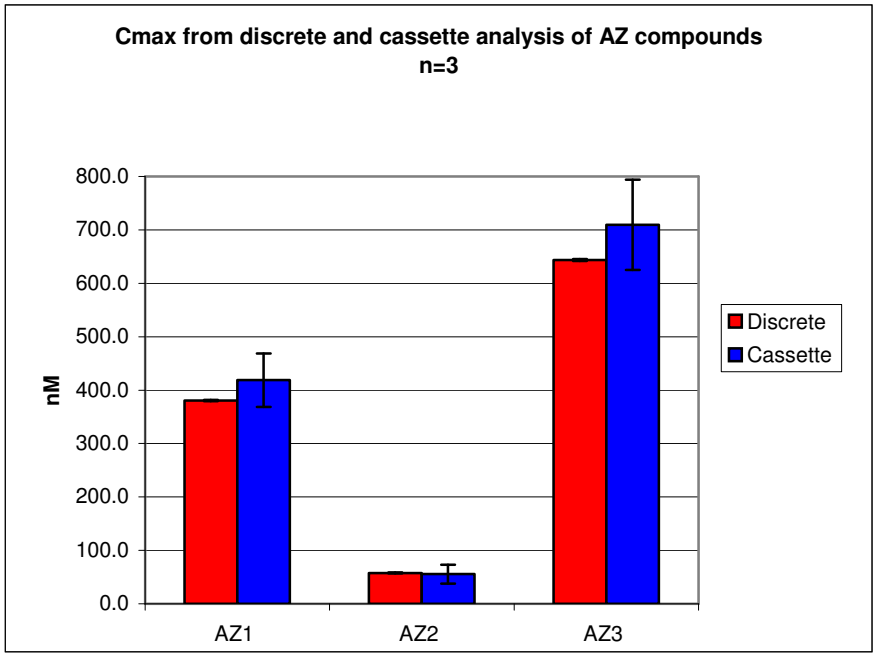


Figure 22 Comparing C_{max} between single and pooled samples for the AZ compounds AZ1, AZ2 and AZ3

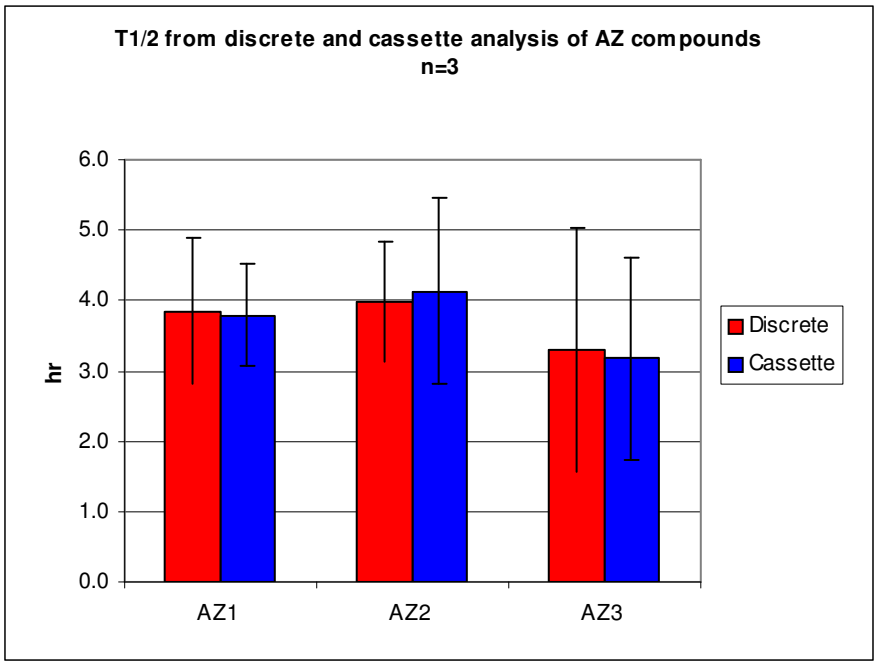


Figure 23 Comparing $T_{1/2}$ between single and pooled samples for the AZ compounds AZ1, AZ2 and AZ3

As shown in figure 21 – 23, the deviations between single and pooled samples for the AZ compounds are very small. All the deviations pass the acceptance criterion of ±25% . The largest deviation is the AUC parameter for compound AZ3, where the deviation between single and pooled samples is 18 %.

4.5.4 Deviations between runs, PO route

The plasma samples from the three rat studies were analysed together with spiked plasma standard curves. The samples were analysed both in discrete- and cassette mode. There were three serials in each study (animal 4, animal 5 and animal 6). Animal 4 from the one study were pooled together with animal 4 from the other studies, animal 5 from each study were pooled together, and also animal 6 from each study were placed in the same cassette group. These PK profiles from the test compounds were dosed to the test animals via the PO route.

The samples were analysed on the same LC-MS system as the standard curves, Quattro Ultima, 1 column system using a meOH gradient. The same analysis plate were analysed twice with approximately a 24-hour interval between the two runs (serial 1 and serial 2).

First, a comparison between the two runs was made (serial 1 serial 2). The deviations between measured concentrations of the single series are listed in table 4.5.4-1. The results were satisfying with a maximum deviation at 19 %. Overall it seemed that the second run (single 2) gave a slightly higher concentration response even though the values varied both ways. One explanation to this could be that some of the sample could have evaporated when stored in the sample organizer. Though, this should give a systematic higher concentration in the second run, but that is not an occasion here.

Deviation from single 1

	AZ1-rat 4	AZ1-rat 5	AZ1-rat 6
Time(h)	**Single2**	**Single2**	**Single2**
15min	0%	7%	18%
30min	4%	5%	0%
45min	6%	3%	6%
1h	-9%	-1%	5%
1.5h	2%	5%	-6%
2.5h	5%	-18%	5%
6h	6%	-1%	18%
24h	0%	0%	0%

	AZ2-rat 4	AZ2-rat 5	AZ2-rat 6
Time(h)	**Single2**	**Single2**	**Single2**
15min	-12%	0%	4%
30min	-4%	14%	6%
45min	-3%	16%	16%
1h	7%	0%	0%
1.5h	3%	9%	4%
2.5h	11%	13%	-14%
6h	0%	8%	0%
24h	0%	0%	0%

	AZ3-rat 4	AZ3-rat 5	AZ3-rat 6
Time(h)	**Single2**	**Single2**	**Single2**
15min	-1%	-12%	5%
30min	-7%	-1%	-8%
45min	-13%	9%	-3%
1h	-15%	-3%	1%
1.5h	1%	-8%	18%
2.5h	-10%	4%	19%
6h	7%	-9%	0%
24h	0%	0%	0%

Table 4.5.4-1 The percentage deviation between the two runs of single AZ compounds (AZ1, AZ2 and AZ3)

The deviations between the pooled samples are listed in table 4.5.4-2.

Deviation from pool 1

	AZ1-rat 4	AZ1-rat 5	AZ1-rat 6
Time(h)	**Pool2**	**Pool2**	**Pool2**
15min	11%	0%	-2%
30min	12%	-12%	-4%
45min	15%	-4%	-11%
1h	6%	0%	-12%
1.5h	-1%	4%	-15%
2.5h	-13%	4%	-23%
6h	-11%	9%	-20%
24h	0%	0%	0%

	AZ2-rat 4	AZ2-rat 5	AZ2-rat 6
Time(h)	**Pool2**	**Pool2**	**Pool2**
15min	0%	0%	-4%
30min	3%	-12%	-7%
45min	14%	-4%	-5%
1h	-9%	0%	-9%
1.5h	-13%	4%	-6%
2.5h	-15%	4%	-1%
6h	-10%	9%	-2%
24h	0%	0%	0%

	AZ3-rat 4	AZ3-rat 5	AZ3-rat 6
Time(h)	**Pool2**	**Pool2**	**Pool2**
15min	-4%	-5%	-4%
30min	2%	-16%	-7%
45min	3%	-11%	-5%
1h	12%	-2%	-9%
1.5h	-13%	7%	-6%
2.5h	-18%	-2%	-1%
6h	-7%	-5%	-2%
24h	0%	0%	0%

Table 4.5.4-2 Percentage deviation between the two runs of pooled AZ compounds (AZ1, AZ2 and AZ3)

As for the single samples, the results for the pooled samples were also satisfying with a maximum deviation between the two runs at –23 %. An interesting difference from the single samples is that the values for the second run of the pooled samples tend to be lower than for the first run. This is not comparable with the theory from the single samples, that some of the sample could have evaporated when stored in the sample organizer. Most likely, the deviations are occasional as they go both ways, and overall, the deviations between single and pooled samples are small.

4.6 Pharmacokinetic profiles, IV route

As for the PK profiles of the PO route, plasma samples were spiked with the reference compounds corresponding to the concentrations of literature PK values as described in methods and experimental. The samples were analysed both in single and cassette/pooled mode.

Seven time points for the literature PK values were chosen, administered via the intravenous route (the concentration values for the last time point, 24h were not included because of the low concentrations). Standard sample preparation was followed, 25µL spiked plasma was precipitated with 150 µL ice cold ACN, 120 µL of the supernatant was mixed with 300 µL buffer (mobile phase), before analysis. For the pooled samples, 75 µL (25 µL x 3) spiked plasma was precipitated with 200 µL ice cold ACN, 120 µL of the supernatant was mixed with 300 µL buffer.

MS instrumentation used for this experiment was both the UPLC system, and the 1-column HPLC system, both using acetonitrile gradients.

4.6.1 Single vs. pooled, IV route, results from UPLC

In table 4.6.1-1 and 4.6.1-2, the results for the PK profiles, IV route analysed on UPLC are shown. The figure shows the literature values for seven time points together with the measured concentration values for single and pooled samples, and also the deviation from literature values and the deviation between single and pooled samples.

	Diclofenac, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	16000	15611	14640	-2%	-9%	-7%
0.1	8000	8465	6948	5%	-15%	-22%
0.3	2500	2706	2687	8%	7%	-1%
0.6	800	868	879	8%	9%	1%
1	350	354	356	1%	2%	0%
3	200	207	254	3%	21%	19%
6	60	63	49	4%	-21%	-27%

	Naproxen, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	35000	36197	33502	3%	-4%	-8%
0.1	30000	35870	32324	16%	7%	-11%
0.3	25000	29658	28606	16%	13%	-4%
0.6	21000	25382	22468	17%	7%	-13%
1	20000	22083	20990	9%	5%	-5%
3	16000	19690	19611	19%	18%	0%
6	10000	12404	11846	19%	16%	-5%

	Rofecoxib, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	5500	5951	5178	8%	-6%	-15%
0.1	4400	4457	4280	1%	-3%	-4%
0.3	2800	3296	2476	15%	-13%	-33%
0.6	1000	1231	991	19%	-1%	-24%
1	500	597	527	16%	5%	-13%
3	80	46	72	-73%	-11%	36%
6	40	51	NV	22%	NV	NV

Table 4.6.1-1 Results for the acidic reference compounds analysed on UPLC

	Diazepam, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	4200	3996	4922	-5%	15%	19%
0.1	3700	3408	3568	-9%	-4%	4%
0.3	2500	2252	2564	-11%	2%	12%
0.6	1500	1318	1490	-14%	-1%	12%
1	1000	918	1129	-9%	11%	19%
3	250	215	265	-16%	6%	19%
6	25	23	33	-7%	24%	29%

	Imipramine, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	2200	1989	2252	-11%	2%	12%
0.1	2100	2038	2032	-3%	-3%	0%
0.3	2000	1864	1789	-7%	-12%	-4%
0.6	1900	1755	1519	-8%	-25%	-16%
1	1700	1621	1690	-5%	-1%	4%
3	1000	963	895	-4%	-12%	-8%
6	500	452	395	-11%	-27%	-14%

	Propranolol, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	10500	9285	9691	-13%	-8%	4%
0.1	8500	7948	7367	-7%	-15%	-8%
0.3	6000	5113	4788	-17%	-25%	-7%
0.6	4500	3862	2605	-17%	-73%	-48%
1	3500	3432	2964	-2%	-18%	-16%
3	1000	935	694	-7%	-44%	-35%
6	250	215	187	-16%	-34%	-15%

Table 4.6.1-2 Results for the basic reference compounds analysed on UPLC

The results from the UPLC analysis are overall quite good with small deviations between single and pooled samples. However, there are a few values that exceeds the acceptance criterion of ± 25%, but the deviations are not large, and the deviations are for single time points and will not affect the whole PK-curve distinctly as shown in the following compilation. If we look at the deviation between the single and pooled samples for all the reference compounds, only 6 out of 41 values exceeds the acceptance criterion. As we will see from the validation experiment later in this study, these differences are not related to the LC-technique, but rather to variations in the analytical method and scattering in the analytical system.

4.6.2 Single vs. pooled, IV route, results from HPLC

In table 4.6.2-1 and 4.6.2-2, the results for the PK profiles, IV route analysed on HPLC are shown. The figure shows the literature values for seven time points together with the measured concentration values for single and pooled samples, and also the deviation from literature values and the deviation between single and pooled samples.

	Diclofenac, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	16000	16036	15760	0%	-2%	-2%
0.1	8000	7918	7122	-1%	-12%	-11%
0.3	2500	2775	2562	10%	2%	-8%
0.6	800	838	830	5%	4%	-1%
1	350	364	347	4%	-1%	-5%
3	200	247	170	19%	-18%	-45%
6	60	70	84	14%	29%	17%

	Naproxen, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	35000	38450	40479	9%	14%	5%
0.1	30000	34713	34414	14%	13%	-1%
0.3	25000	25969	30020	4%	17%	13%
0.6	21000	25502	24189	18%	13%	-5%
1	20000	22508	22865	11%	13%	2%
3	16000	19100	20937	16%	24%	9%
6	10000	12101	13333	17%	25%	9%

	Rofecoxib, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	5500	5416	4751	-2%	-16%	-14%
0.1	4400	4480	4190	2%	-5%	-7%
0.3	2800	2894	2994	3%	6%	3%
0.6	1000	1066	1209	6%	17%	12%
1	500	499	591	0%	15%	16%
3	80	52	63	-54%	-27%	17%
6	40	31	26	-29%	-54%	-19%

Table 4.6.2-1 Results for the acidic reference compounds analysed on HPLC

	Diazepam, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	4200	5932	6435	29%	35%	8%
0.1	3700	4683	4644	21%	20%	-1%
0.3	2500	3056	3096	18%	19%	1%
0.6	1500	1745	1786	14%	16%	2%
1	1000	1193	1274	16%	22%	6%
3	250	262	308	4%	19%	15%
6	25	31	35	20%	28%	9%

	Imipramine, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	2200	1991	1650.3	-10%	-33%	-21%
0.1	2100	1997	1530.4	-5%	-37%	-30%
0.3	2000	1956	1473.6	-2%	-36%	-33%
0.6	1900	1932	1555	2%	-22%	-24%
1	1700	1493	1252.3	-14%	-36%	-19%
3	1000	988	791.7	-1%	-26%	-25%
6	500	498	372.1	0%	-34%	-34%

	Propranolol, conc. [nM]			Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.03	10500	8052	9361	-30%	-12%	14%
0.1	8500	5993	6758	-42%	-26%	11%
0.3	6000	4781	4938	-26%	-22%	3%
0.6	4500	3463	4082	-30%	-10%	15%
1	3500	2566	3200	-36%	-9%	20%
3	1000	844	868	-19%	-15%	3%
6	250	207	230	-21%	-9%	10%

Table 4.6.2-2 Results for the basic reference compounds analysed on HPLC

The results from the HPLC analysis are also quite satisfying comparing the differences between single and pooled samples. The deviations between single and pooled sample are small, and only 4 out of 42 samples exceeds the acceptance criterion. As for the UPLC analysis, these deviations must rather be linked to variations in the analytical system than to the LC-technique. Comparing the UPLC and the HPLC results, they are quite similar, but the advantage using UPLC over HPLC is a significant reduction in analysis time.

4.6.3 PK curves, IV route, compilation

The PK parameters and the PK curves for all the reference compounds are listed in table 4.6.3-1 to table 4.6.3-12 and in figure 24 - 29. The figures illustrates the estimated IV route for four trials analysed both on UPLC and HPLC in both discrete

and cassette mode. The tables also show the deviation between single and pooled PK-parameters.

Diazepam:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast (hr*nmol/L)	AUCinf_pred (hr*nmol/L)	AUCextr (%)	V (L/kg)	CL (L/kg)
Literature		0.9	4200	0.03	3789	3822	0.9	1.0	0.79
Trial 1 HPLC	Single	1.0	5932	0.03	4535	4577	0.9	0.9	0.66
	Pooled	1.0	6435	0.03	4810	4857	1.0	0.9	0.62
Trial 2 UPLC	Single	0.9	3996	0.03	3422	3454	0.9	1.2	0.87
	Pooled	1.0	4922	0.03	4040	4086	1.1	1.0	0.73
Trial 3 UPLC	Single	0.9	3869	0.03	3400	3433	1.0	1.2	0.87
	Pooled	1.0	5024	0.03	4296	4340	1.0	1.0	0.69
Trial 4 UPLC	Single	0.9	3951	0.03	3369	3399	0.9	1.2	0.88
	Pool	0.9	4428	0.03	3377	3400	0.7	1.1	0.88

Table 4.6.3-1 PK parameters for diazepam, IV route

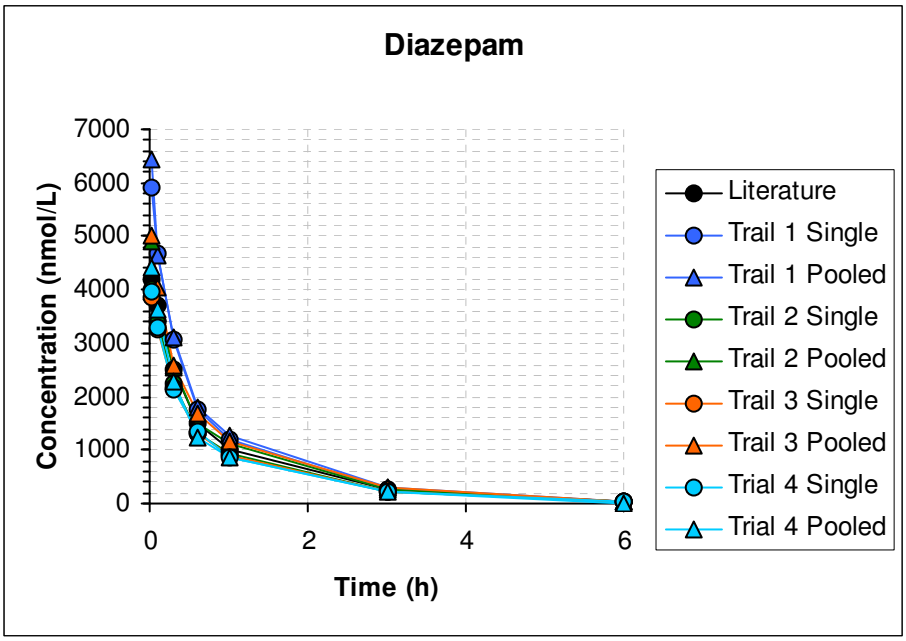


Figure 24 PK curves for diazepam, IV route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V (L/kg)	CL (L/kg)
Trial 1	0%	8%	0%	6%	6%	6%	6%
Trial 2	4%	23%	0%	18%	18%	12%	16%
Trial 3	1%	30%	0%	26%	26%	20%	21%
Trial 4	4%	12%	0%	0%	0%	4%	0%

Table 4.6.3-2 PK parameters for diazepam, deviation from single, IV route

Diazepam shows small variations between single and pooled samples, the maximum deviation for Cmax is 30%. But the most important parameters for the IV route, volume of distribution (V) and clearance (CL) show only small deviations.

Imipramine:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast (hr*nmol/L)	AUCinf_pred (hr*nmol/L)	AUCextr (%)	V (L/kg)	CL (L/kg)
Literature		2.8	2200	0.03	6882	8887	22.6	1.4	0.34
Trial 1 HPLC	Single	3.1	1996.8	0.1	6572	8845	25.7	1.5	0.34
	Pooled	2.8	1650.3	0.03	5705	7301	21.9	1.6	0.41
Trial 2 UPLC	Single	2.7	2038	0.1	6516	8284	21.4	1.4	0.36
	Pooled	2.4	2252	0.03	6261	7607	17.7	1.4	0.39
Trial 3 UPLC	Single	2.7	2198	0.03	6811	8618	21.0	1.4	0.35
	Pooled	2.5	2273	0.03	6178	7553	18.2	1.4	0.40
Trial 4 UPLC	Single	2.7	2089	0.03	6564	8358	21.5	1.4	0.36
	Pool	2.6	2159	0.03	5528	6898	19.9	1.6	0.44

Table 4.6.3-3 PK parameters for imipramine, IV route

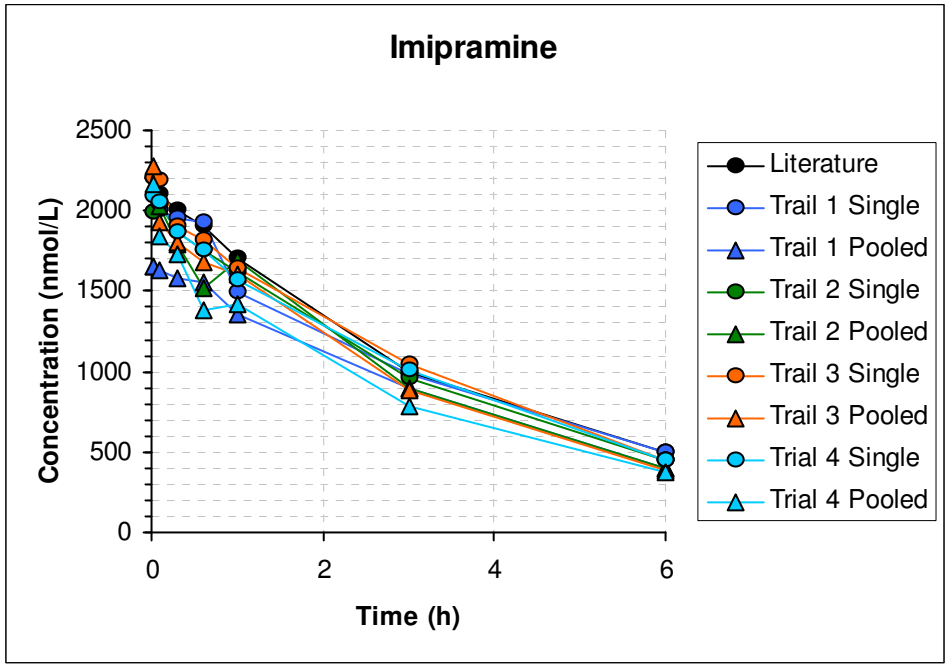


Figure 25 PK curves for imipramine, IV route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V (L/kg)	CL (L/kg)
Trial 1	12%	17%	70%	13%	17%	7%	21%
Trial 2	12%	11%	70%	4%	8%	4%	9%
Trial 3	10%	3%	0%	9%	12%	3%	14%
Trial 4	5%	3%	0%	16%	17%	15%	21%

Table 4.6.3-4 PK parameters for imipramine, deviation from single, IV route

All over, imipramine shows very small variations between single and pooled samples. Almost all the PK parameters fulfilled the acceptance criterion, the reason for why the deviation for Tmax is so great for trial 1 and 2 is because of the small values, and because Tmax is located at the beginning of the PK curve.

Propranolol:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast (hr*nmol/L)	AUCinf_pred (hr*nmol/L)	AUCextr (%)	V (L/kg)	CL (L/kg)
Literature		1.3	10500	0.03	11995	12431	3.5	0.45	0.24
Trial 1 HPLC	Single	1.4	8052	0.03	9255	9656	4.2	0.62	0.31
	Pooled	1.3	9361	0.03	10560	10945	3.5	0.50	0.27
Trial 2 UPLC	Single	1.3	9038	0.03	10689	11045	3.2	0.49	0.27
	Pooled	1.2	9364	0.03	9720	10006	2.9	0.52	0.30
Trial 3 UPLC	Single	1.3	9285	0.03	11094	11458	3.2	0.47	0.26
	Pooled	1.2	9691	0.03	9924	10229	3.0	0.52	0.29
Trial 4 UPLC	Single	1.3	9589	0.03	11117	11524	3.5	0.49	0.26
	Pool	1.3	9790	0.03	10011	10353	3.3	0.54	0.29

Table 4.6.3-5 PK parameters for propranolol, IV route

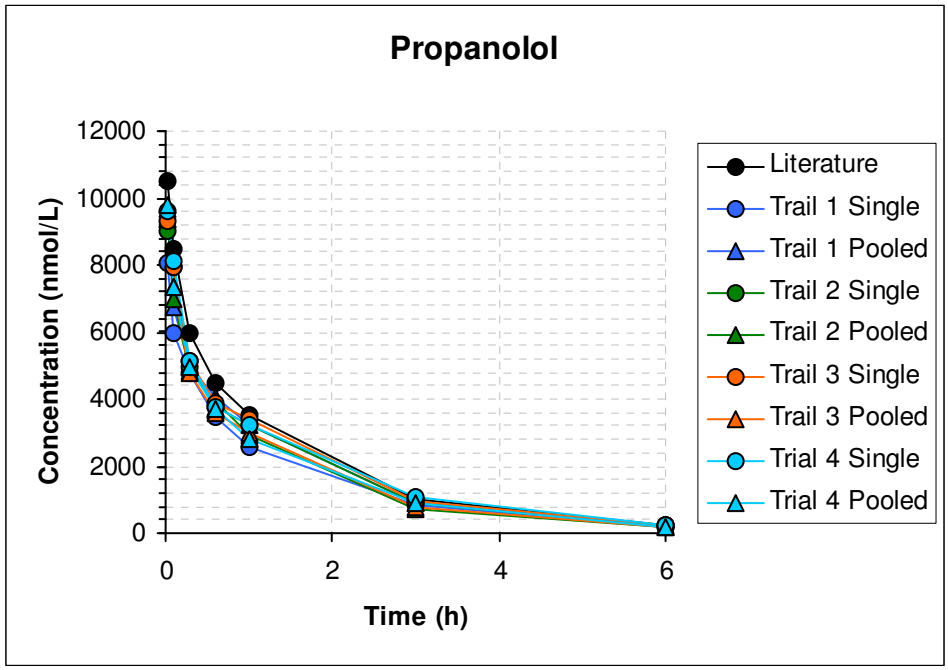


Figure 26 PK curves for propranolol, IV route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V (L/kg)	CL (L/kg)
Trial 1	7%	16%	0%	14%	13%	19%	12%
Trial 2	4%	4%	0%	9%	9%	6%	10%
Trial 3	2%	4%	0%	11%	11%	10%	12%
Trial 4	1%	2%	0%	10%	10%	10%	12%

Table 4.6.3-6 PK parameters for propranolol, deviation from single, IV route

As shown in the figures for propranolol, there are no large discrepancies between the runs, and all the PK parameters fulfilled the acceptance criterion.

Diclofenac:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast (hr*nmol/L)	AUCinf_pred (hr*nmol/L)	AUCextr (%)	V (L/kg)	CL (L/kg)
Literature		1.9	16000	0.03	4118	4293	4.1	2.0	0.70
Trial 1 HPLC	Single	2.1	16036	0.03	4343	4566	4.9	2.0	0.66
	Pooled	2.5	15760	0.03	4030	4324	6.8	2.5	0.69
Trial 2 HPLC	Single	1.9	16030	0.03	4386	4585	4.3	1.8	0.65
	Pooled	2.3	16652	0.03	4076	4315	5.5	2.3	0.70
Trial 3 UPLC	Single	2.0	15611	0.03	4245	4432	4.2	1.9	0.68
	Pooled	1.5	14640	0.03	4089	4199	2.6	1.5	0.71
Trial 4 UPLC	Single	1.7	19619	0.03	5053	5187	2.6	1.4	0.58
	Pool	1.7	15232	0.03	4029	4161	3.2	1.8	0.72

Table 4.6.3-7 PK parameters for diclofenac, IV route

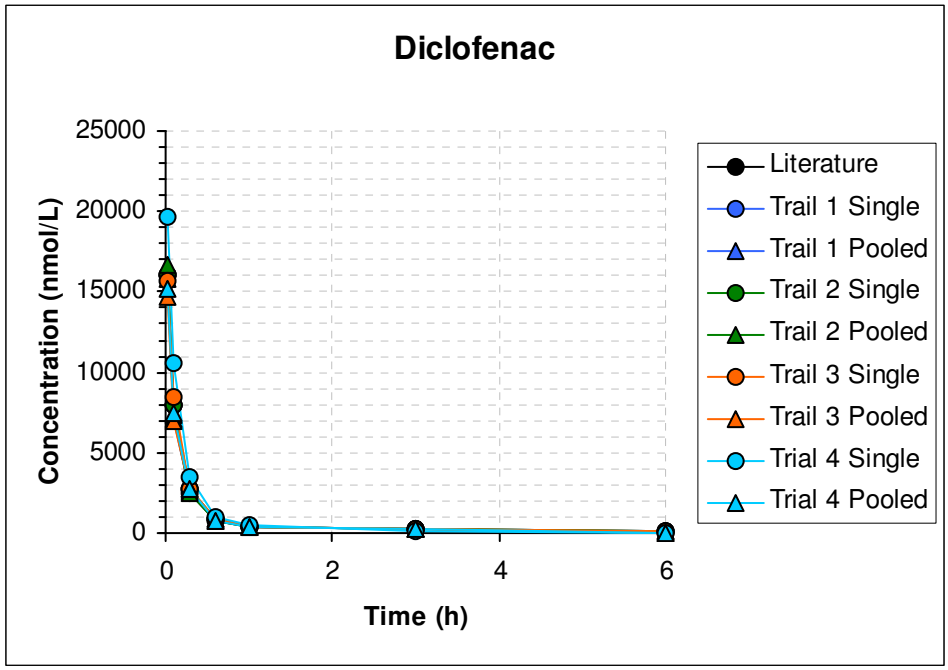


Figure 27 PK curves for diclofenac, IV route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V (L/kg)	CL (L/kg)
Trial 1	19%	2%	0%	7%	5%	26%	6%
Trial 2	18%	4%	0%	7%	6%	25%	6%
Trial 3	26%	6%	0%	4%	5%	21%	5%
Trial 4	2%	22%	0%	20%	20%	28%	25%

Table 4.6.3-8 PK parameters for diclofenac, deviation from single, IV route

As illustrated in the PK curves for diclofenac, the deviation between single and pooled samples are very small. But still, some of the PK parameters exceed the acceptance criterion, but this because the PK curves are so abrupt in the beginning.

Naproxen:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast (hr*nmol/L)	AUCinf_pred (hr*nmol/L)	AUCextr (%)	V (L/kg)	CL (L/kg)
Literature		5.1	35000	0.03	98961	173351	42.9	0.13	0.017
Trial 1 HPLC	Single	5.3	38450	0.03	115540	209510	44.9	0.11	0.014
	Pooled	6.5	40479	0.03	123072	252683	51.3	0.11	0.012
Trial 2 HPLC	Single	5.6	38046	0.03	110813	208195	46.8	0.12	0.014
	Pooled	5.5	43541	0.03	124785	232376	46.3	0.10	0.013
Trial 3 UPLC	Single	5.6	36197	0.03	117826	220704	46.6	0.11	0.014
	Pooled	6.1	33502	0.03	113549	222703	49.0	0.12	0.013
Trial 4 UPLC	Single	5.6	36130	0.1	107075	200182	46.5	0.12	0.015
	Pool	4.9	33880	0.03	108672	186856	41.8	0.11	0.016

Table 4.6.3-9 PK parameters for naproxen, IV route

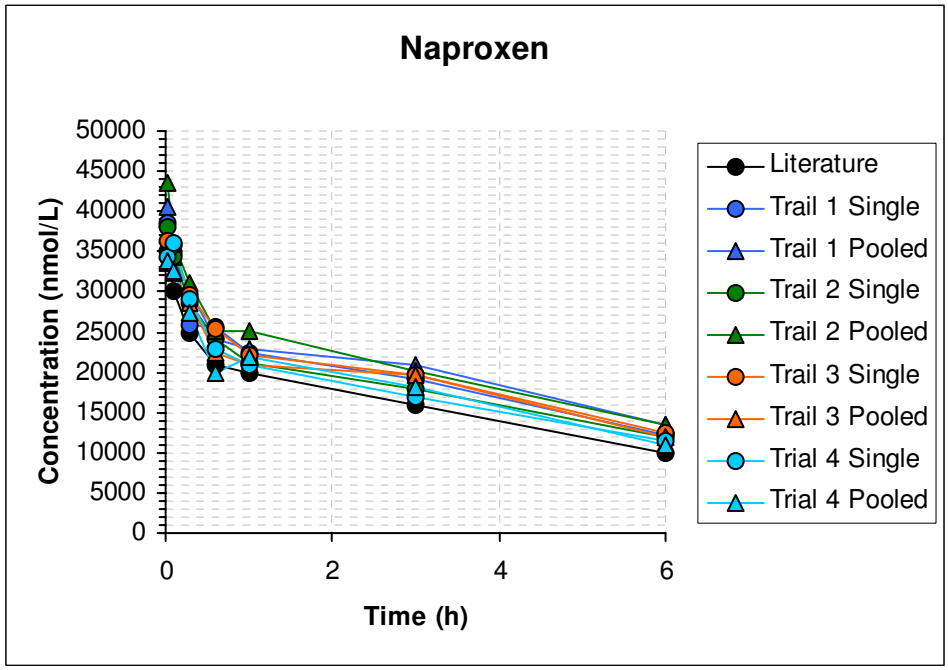


Figure 28 PK curves for naproxen, IV route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V (L/kg)	CL (L/kg)
Trial 1	22%	5%	0%	7%	21%	2%	14%
Trial 2	2%	14%	0%	13%	12%	12%	7%
Trial 3	9%	7%	0%	4%	1%	7%	7%
Trial 4	13%	6%	70%	1%	7%	7%	7%

Table 4.6.3-10 PK parameters for naproxen, deviation from single, IV route

The PK curves for naproxen show some more variation between the single and pooled samples. But, if we look at the PK parameters, they are all over very satisfying, and in analogy to imipramine, the Tmax in the PK curve is completely in the beginning of the curve.

Rofecoxib:

		$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast (hr*nmol/L)	AUCinf_pred (hr*nmol/L)	AUCextr (%)	V (L/kg)	CL (L/kg)
Literature		1.2	5500	0.03	2870	2920	1.7	1.8	1.0
Trial 1 HPLC	Single	0.8	5416	0.03	2836	2852	0.6	1.2	1.1
	Pooled	0.8	4751	0.03	2960	2977	0.6	1.2	1.0
Trial 2 HPLC	Single	0.9	5693	0.03	3110	3142	1.0	1.2	1.0
	Pooled	1.5	4789	0.03	2639	2735	3.5	2.3	1.1
Trial 3 UPLC	Single	0.9	5951	0.03	3213	3242	0.9	1.2	0.9
	Pooled	0.9	5178	0.03	2761	2786	0.9	1.3	1.1
Trial 4 UPLC	Single	1.1	5194	0.03	2518	2555	1.4	1.9	1.2
	Pool	1.2	4545	0.03	2432	2484	2.1	2.2	1.2

Table 4.6.3-11 PK parameters for rofecoxib, IV route

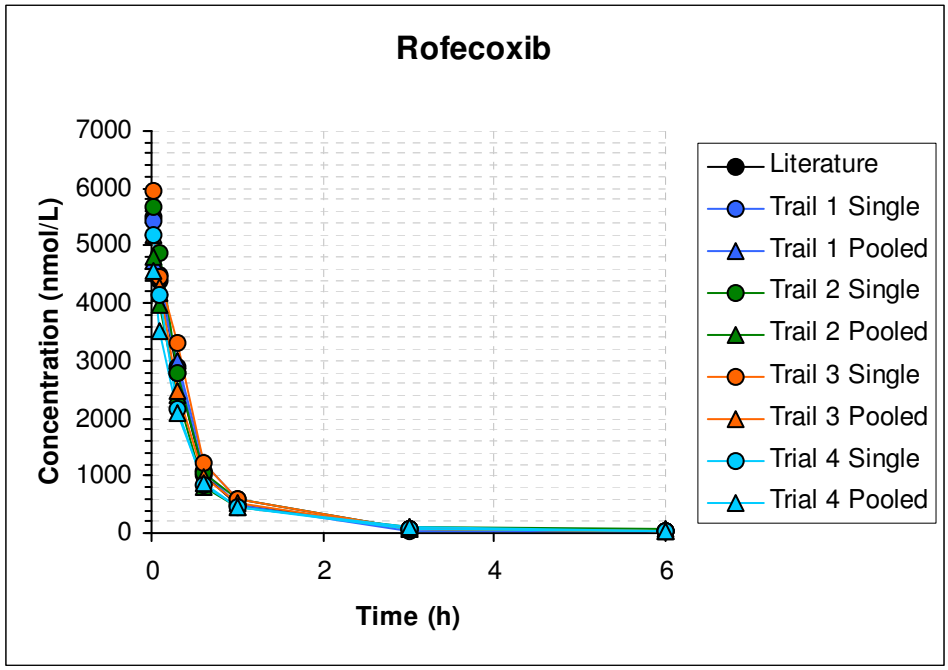


Figure 29 PK curves for rofecoxib, IV route

Deviation from Single

	$T_{1/2}$ (h)	Cmax (nM)	Tmax (h)	AUClast	AUCinf_pred	V (L/kg)	CL (L/kg)
Trial 1	0%	12%	0%	4%	4%	4%	4%
Trial 2	68%	16%	0%	15%	13%	91%	16%
Trial 3	0%	13%	0%	14%	14%	17%	16%
Trial 4	8%	12%	0%	3%	3%	11%	3%

Table 4.6.3-12 PK parameters for rofecoxib, deviation from single, IV route

The PK curves for rofecoxib show very little variation between single and pooled samples, but because of the heavy slope in the beginning of the curve, some variations can be found for the PK parameters.

As shown in the PK curves for the reference compounds, the differences between the trials are small, but for imipramine and naproxen, they show some more variation with larger scattering between the trials. But, even though there are some variations for these compounds, some of the trials still are quite similar both compared with the literature values and the difference between single and pooled samples.

4.7 PK curves for reference compounds with formulations

This was an experiment to investigate the impact of various formulations on the spiked PK (PO) curves of the reference compounds.

The formulations used in this experiment were:

- Cyclodextrine
- Gluconic acid
- Meglumine
- Dimethyl acetamide (DMA)
- Polyethylene glycol 400 (PEG 400)
- Hydroxypropyl methyl cellulose (HPMC) + polyoxyethylene sorbitanmonostearate (Tween80®)
- Microcrystalline cellulose (MCC)/ Sodium Carboxymethylcellulose (NaCMC) + Lipoid S100®*

*Lipoid S100® is a phospholipid formulation used for drugs with poor water solubility for parenteral application.

In the first experiment, both single and pooled spiked PK curves were made. Plasma samples were spiked with the reference compounds corresponding to the concentrations of literature PK values as done earlier. But, in contrast to the earlier experiments where blank plasma was used, now plasma containing formulation was used. Blank plasma was spiked with a 5% formulation mixture of cyclodextrine, gluconic acid and meglumine.

Together with the single PK curves, also pooled PK curves of the three basic and the three acidic compounds respectively, were prepared. Also, standard curves without formulation were prepared to be able to quantify the results.

The samples were analysed on a UPLC-MS system. The same samples were analysed twice on order to detect variations in the system.
There were small variations between the runs, but the results were satisfying for all reference compounds, indicating that the formulations (cyclodextrine, gluconic acid and meglumine) do not give significant suppression or enhancement. The results for naproxen and rofecoxib from the second run are listed in table 4.7-1. The figure shows the measured concentration values [nM] for both single and pooled samples, together with the literature values. The deviation from the literature values and the deviation between single and pooled concentration values are also listed in percentage deviation.

Naproxen Conc. [nM]				Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.25	35000	43045	37298	19%	6%	-15%
0.5	43000	39450	50614	-9%	15%	22%
0.75	43000	42607	48632	-1%	12%	12%
1	43000	43067	50633	0%	15%	15%
1.5	45000	41925	49737	-7%	10%	16%
2.5	45000	39640	50685	-14%	11%	22%
6	40000	39866	44220	0%	10%	10%
24	3000	3130	3545	4%	15%	12%

Rofecoxib Conc. [nM]				Deviation from literature		Deviation from single
Time (h)	Literature	Single	Pool	Single	Pool	Pool
0.25	70	66	61	-6%	-14%	-7%
0.5	150	141	165	-6%	9%	14%
0.75	270	252	268	-7%	-1%	6%
1	300	272	310	-10%	3%	12%
1.5	450	431	435	-5%	-3%	1%
2.5	600	662	560	9%	-7%	-18%
6	450	449	429	0%	-5%	-5%
24	70	65	69	-8%	-1%	6%

Table 4.7-1 Concentrations for naproxen and rofecoxib, including the percentage deviation from literature values, and the deviation between single and pooled values

In the second experiment, spiked, and then pooled PK curves for both basic and acidic compounds were prepared. Totally eight basic pooled PK curves and eight acidic PK curves were prepared, and to each curve, a different formulation was added. There were totally seven different formulations, and also a curve with blank plasma were

analysed as a control. The amount of formulation in the samples was approximately 5% that were added to blank plasma.
This experiment was performed on three different LC systems;

- UPLC, 1-column system, acetonitrile gradient
- HPLC, Quattro Ultima, 2-column system, MeOH gradient
- HPLC, Quattro Ultima, 1-column system, acetonitrile gradient

In the UPLC system, the results showed some variations, the results for the basic compounds for the 1.5 hour time point are listed in table 4.7-2. The figure shows the percentage deviation in measured concentrations between the different formulations from the blank plasma curve. The results from the other time points can be found in Appendix B.

1.5 h	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG400	HPMC/Tween	MCC/NaCMC+ Lipoid S100
Diazepam	3%	6%	14%	5%	-13%	-1%	21%
Imipramine	24%	0%	25%	16%	-53%	-14%	21%
Propranolol	35%	-9%	29%	29%	-118%	-20%	41%

Table 4.7-2 Deviation between spiked PK curves with different formulations from the spiked PK curve with blank plasma (no formulation), results from UPLC

It is difficult to draw a conclusion from this experiment alone; the deviation depends both on formulation and reference compound. However, we can se that PEG 400 gives a significant suppression, whereas the other formulations do not involve the same great impact.

The HPLC systems showed less variation. All over, the acidic reference compounds did not seem to be significally affected by any of the formulations even though there exist some values that varies more than others. In table 4.7-3, the percentage deviation between the different formulations and the curve without formulation are listed for the acidic reference compounds. The table shows only the one-hour time point for each compound, the other measured time points can be found in Appendix B. These samples were analysed on the 2-column HPLC system with a MeOH gradient.

1.0 h	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG400	HPMC/Tween	MCC/NaCMC+ Lipoid S100
Diclofenac	12%	5%	6%	9%	11%	10%	-14%
Naproxen	-1%	-11%	21%	16%	0%	22%	8%
Rofecoxib	-21%	-2%	7%	3%	16%	5%	-9%

Table 4.7-3 Percentage deviation between the formulations and the control (blank), results from HPLC

For the basic reference compounds (diazepam, imipramine and propranolol), there were also some variations, but over all, only PEG 400 seemed to give significant suppression. This could be observed for both HPLC analysis systems (1 and 2 columns).

4.8 Validation experiments

The last experiment in this study was a validation experiment, which was necessary to be able to evaluate the other results as mentioned in the theory part. The primary objective in this experiment was to evaluate the variability in the analytical method.

Standard curves of the six reference compounds were prepared in spiked rat plasma, range 10 – 20000 nM.
Six quality controls (QC) in four concentration levels followed by three blanks were prepared, also in spiked rat plasma.
The concentrations of the controls were:

- LLQC [25 nM]
- LQC [125 nM]
- MQC [1250 nM]
- HQC [12500 nM]

The reference compounds were divided into two cassette groups of basic (diazepam, imipramine and propranolol) and acidic (diclofenac, naproxen and rofecoxib) compounds. Standard curves were prepared in both discrete/single and cassette/pooled mode. The controls were prepared in cassette mode, and also in discrete mode for the compounds diazepam and diclofenac.

This resulted in four analysis plates with controls:

- Basic compounds, cassette mode
- Acidic compounds, cassette mode
- Diazepam, discrete mode
- Diclofenac, discrete mode

The analyze system for this experiment was a UPLC-MS/MS, Waters Acquity Quattro Premier XE system, and the experiment were performed at Waters corporation in Sollentuna, Sweden in collaboration with AstraZeneca.
To evaluate the variation over a longer time period, the analysis plates were analysed six times subsequent for each plate (Run1 – Run6), and then again one more time (Run7) after 24 –72 hours. The pooled, basic compounds were only analysed five times subsequently (Run1 – Run5), and then again after 72 hours (Run7).
The time between the runs for the cassette mode plates were approximately 4 hours and 2,5 hours for the discrete mode.
The acceptance criterion was set to ± 25% from the first run, and not more than 3 out of 20 samples could exceed this limit of 25% deviation.
The deviations from Run1 for all the analytes are listed in Appendix C.

The deviation for the basic compounds (diazepam, imipramine and propranolol) were quite satisfying even though some control values falls out of the limit of 25% deviation from Run1. This is most apparent for propranolol, but comparing Run1 for propranolol, the concentration values seems lower than for all the other runs, and can therefore be an explanation to this. This is illustrated in table 4.8-1, showing the measured concentration values for the controls of propranolol. The mean values for each control (n =6) are shown below each run.

Propranolol:

	Literature	Run1	Run2	Run3	Run4	Run5	Run7
LLQC	25	16	22	20	17	21	18
	25	10	10	10	17	10	10
	25	9	10	8	13	13	10
	25	10	12	14	11	11	10
	25	10	15	13	12	16	10
	25	12	13	11	11	15	10
	Mean	**11**	**14**	**13**	**13**	**14**	**11**
LQC	125	68	90	94	89	90	78
	125	64	104	98	98	104	86
	125	73	104	108	104	98	91
	125	73	108	99	97	111	90
	125	74	103	102	107	95	78
	125	84	112	115	123	111	99
	Mean	**73**	**104**	**103**	**103**	**102**	**87**
MQC	1250	662	945	925	909	919	754
	1250	650	900	876	910	885	730
	1250	681	978	953	940	942	739
	1250	770	1120	1047	1047	1075	908
	1250	892	1286	1236	1321	1246	1125
	1250	951	1300	1239	1280	1269	1108
	Mean	**768**	**1088**	**1046**	**1068**	**1056**	**894**
HQC	12500	7657	9209	9264	9247	9031	8141
	12500	7156	11058	9150	9131	9194	8217
	12500	7656	9914	9506	9504	9771	8384
	12500	7702	9726	9844	9669	9678	8942
	12500	8571	10590	10541	10387	10361	9932
	12500	10398	12511	12677	12792	12474	12231
	Mean	**8190**	**10501**	**10164**	**10122**	**10085**	**9308**

Table 4.8-1 Literature and measured concentration values for propranolol, cassette mode

For the acidic compounds (diclofenac, naproxen and rofecoxib), the results are not as good as for the basic compounds. Distinctly are the lower concentration values remarkably poor. An elucidating reason for this is that the sensitivity in the analysis system is poorer for acidic compounds than for basic compounds. That is also the reason for why the lowest concentrations in the standard curve are missing (non value, NV). This can be illustrated by comparing the chromatograms for both the basic and the acidic compounds as shown in figure 30 and 31.

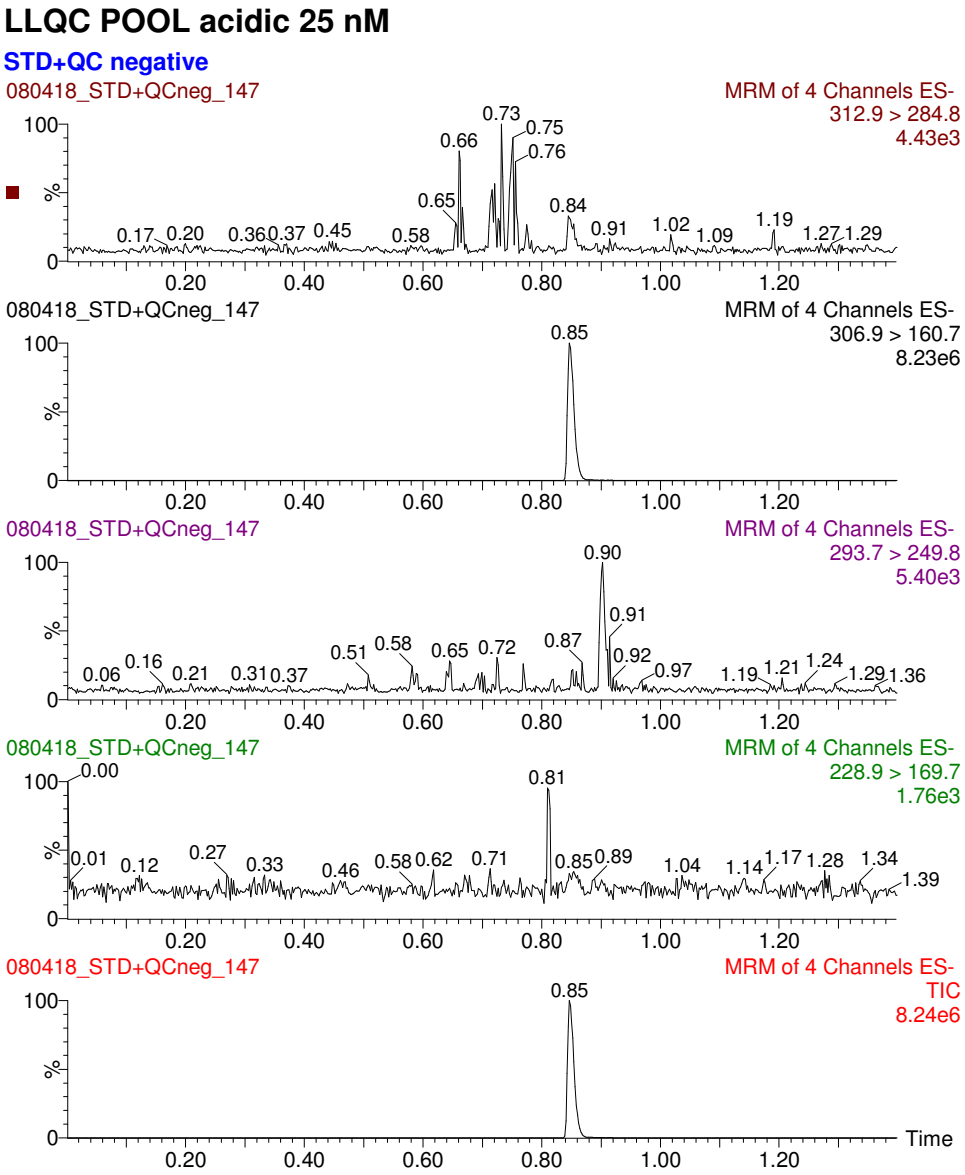


Figure 30 Chromatograms for the acidic compounds, cassette mode

The chromatograms are showing the acidic compounds rofecoxib (top), warfarin (IS), diclofenac, naproxen and the TIC (bottom). The response for rofecoxib, diclofenac and naproxen are very poor, between 1.73e3 (naproxen) and 5.40e3 (diclofenac), and they are below LOQ.

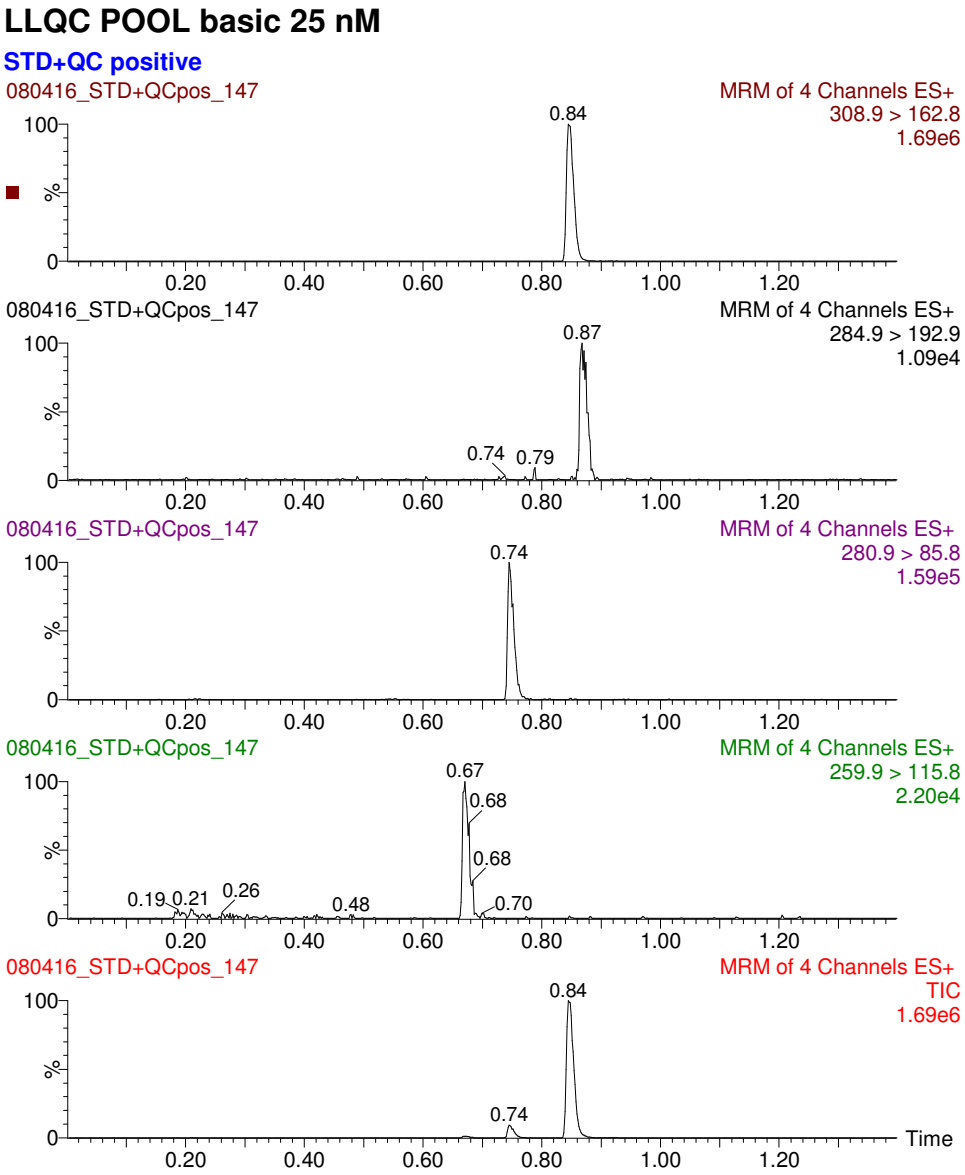


Figure 31 Chromatograms for the basic compounds, cassette mode

The chromatograms are showing the basic compounds, warfarin (IS) on the top, followed by diazepam, imipramine, propranolol and the TIC chromatogram at the bottom.

Compared to the acidic compounds of the same concentration [25 nM], the response is almost up to 100 times better for the basic compounds. The response diversifies from 1.09e4 (diazepam) to 1.59e5 (imipramine). The response for warfarin is high in both positive and negative mode as expected, because it is the internal standard. It is even higher in the negative mode, which also emphasizes that the response for the acidic reference compounds is poor.

To summarize the results from the validation experiment, the mean concentration values [nM] for the controls in each run are listed in table 4.8-2 and 4.8-3.

Diazepam (single):

	Run1	Run2	Run3	Run4	Run5	Run6	Run7
LLQC	27	27	28	26	27	24	25
LQC	140	139	146	144	141	139	135
MQC	1543	1517	1574	1573	1556	1521	1440
HQC	14769	14463	14638	14430	14531	14482	14684

Diazepam (pool):

	Run1	Run2	Run3	Run4	Run5	Run7
LLQC	23	25	26	23	26	23
LQC	111	115	122	116	120	122
MQC	1129	1182	1225	1148	1179	1225
HQC	12300	12678	12581	12484	12545	12969

Imipramine (pool):

	Run1	Run2	Run3	Run4	Run5	Run7
LLQC	19	20	20	20	21	25
LQC	94	112	111	111	111	113
MQC	925	1098	1081	1098	1057	1065
HQC	9405	11367	10854	10987	10637	11168

Propranolol (pool):

	Run1	Run2	Run3	Run4	Run5	Run7
LLQC	11	14	13	13	14	11
LQC	73	104	103	103	102	87
MQC	768	1088	1046	1068	1056	894
HQC	8190	10501	10164	10122	10085	9308

Table 4.8-2 Mean concentration values [nM] for the controls from the basic reference compounds

Diclofenac (single):

	Run1	Run2	Run3	Run4	Run5	Run6	Run7
LLQC	28	17	23	27	16	27	29
LQC	149	150	167	153	149	161	160
MQC	1310	1360	1447	1364	1288	1389	1327
HQC	13332	14216	14073	14735	14252	14251	14006

Diclofenac (pool):

	Run1	Run2	Run3	Run4	Run5	Run6	Run7
LLQC	14	16	15	14	23	12	28
LQC	115	131	123	115	124	123	105
MQC	1283	1158	1146	1204	1176	1159	1027
HQC	14070	12116	14629	14408	13241	13743	14124

Naproxen (pool):

	Run1	Run2	Run3	Run4	Run5	Run6	Run7
LLQC	28	69	21	58	81	52	99
LQC	107	150	147	149	167	140	175
MQC	1399	1403	1457	1436	1487	1359	1316
HQC	15776	13552	15698	16270	17251	16029	15424

Rofecoxib (pool):

	Run1	Run2	Run3	Run4	Run5	Run6	Run7
LLQC	13	29	NV	16	20	19	18
LQC	151	151	128	148	148	130	147
MQC	1591	1571	1663	1575	1396	1452	1537
HQC	12259	10178	11329	11140	10357	11486	11551

Table 4.8-3 Mean concentration values [nM] for the controls from the acidic reference compounds

The percentage difference between the highest and the lowest mean concentration values from the quality controls are listed in table 4.8-4 and 4.8-5.

	Diazepam(single)	Diazepam(pool)	Imipramine(pool)	Propranolol(pool)
LLQC	13%	10%	25%	21%
LQC	8%	9%	17%	30%
MQC	9%	8%	16%	29%
HQC	2%	5%	17%	22%

Table 4.8-4 Percentage difference between highest and lowest mean concentration value of the quality controls, basic reference compounds

	Diclofenac(single)	Diclofenac(pool)	Naproxen(pool)	Rofecoxib(pool)
LLQC	NV	NV	NV	NV
LQC	11%	20%	39%	15%
MQC	11%	20%	11%	16%
HQC	10%	17%	21%	17%

Table 4.8-5 Percentage difference between highest and lowest mean concentration value of the quality controls, acidic reference compounds

From table 4.8-4 and 4.8-5, we can see that the differences from the basic reference compounds are quite small, even though they differ from 2% - 30%. The difference tends to be smaller with higher concentrations. This is as expected because of higher precision with higher concentrations. This is also the case for the acidic reference compounds, and as mentioned earlier, the lowest quality controls (LLQC) are below the LOQ for the acidic compounds, and should therefore be excluded.

If we exclude the LLQC for the acidic reference compounds, the results are quite good. Out of all the results (Appendix C), only 109 of a total of 1255 results exceed the limit of the acceptance criterion ± 25%. The criterion was also that not more than 3 out of 20 samples should exceed the acceptance criterion. 3 out of 20 correspond to 15% aberration, whilst 109 of 1255 correspond to 8.7% aberration.

In table 4.8-6 to 4.8-13, the results from this experiment are summarized to show the interday accuracy and precision for the reference compounds.

Diazepam(single):

	LLQC 25nM	LQC 125nM	MQC 1250nM	HQC 12500nM
mean (n=7)	26.3	140.6	1532	14571
SD	1.4	3.6	46.5	127.5
RSD (%)	5.3	2.6	3.0	0.9
Accuracy(%)	105.2	112.5	122.6	116.6

Table 4.8-6 Accuracy and precision for diazepam (discrete)

Diazepam(pool):

	LLQC 25nM	LQC 125nM	MQC 1250nM	HQC 12500nM
mean (n=6)	24.3	117.7	1181.3	12592.8
SD	1.5	4.4	39.2	223.1
RSD (%)	6.2	3.7	3.3	1.8
Accuracy(%)	97.2	94.2	94.5	100.7

Table 4.8-7 Accuracy and precision for diazepam (cassette)

Imipramine(pool):

	LLQC 25nM	LQC 125nM	MQC 1250nM	HQC 12500nM
mean (n=6)	20.8	108.7	1054	10736.3
SD	2.1	7.2	65.4	699
RSD (%)	10.1	6.6	6.2	6.5
Accuracy(%)	83.2	87	84.3	85.9

Table 4.8-8 Accuracy and precision for imipramine (cassette)

Propranolol(pool):

	LLQC 25nM	LQC 125nM	MQC 1250nM	HQC 12500nM
mean (n=6)	12.7	95.3	986.7	9728.3
SD	1.4	12.7	127.8	850
RSD (%)	11	13.3	13	8.7
Accuracy(%)	50.8	76.2	78.9	77.8

Table 4.8-9 Accuracy and precision for propranolol (cassette)

Diclofenac(single):

	LLQC 25nM	LQC 125nM	MQC 1250nM	HQC 12500nM
mean (n=7)	NV	155.6	1355	14123.6
SD	NV	7.1	53.3	419.9
RSD (%)	NV	4.6	3.9	3.0
Accuracy(%)	NV	124.5	108.4	113

Table 4.8-10 Accuracy and precision for diclofenac (discrete)

Diclofenac(pool):

	LLQC 25nM	LQC 125nM	MQC 1250nM	HQC 12500nM
mean (n=7)	NV	119.4	1164.7	13761.6
SD	NV	8.4	76.4	854
RSD (%)	NV	7.0	6.6	6.2
Accuracy(%)	NV	95.5	93.2	110.1

Table 4.8-11 Accuracy and precision for diclofenac (cassette)

Naproxen(pool):

	LLQC 25nM	LQC 125nM	MQC 1250nM	HQC 12500nM
mean (n=7)	NV	147.9	1408.1	15714.3
SD	NV	21.8	58.3	1120.5
RSD (%)	NV	14.7	4.1	7.1
Accuracy(%)	NV	118.3	112.6	125.7

Table 4.8-12 Accuracy and precision for naproxen (cassette)

Rofecoxib(pool):

	LLQC 25nM	LQC 125nM	MQC 1250nM	HQC 12500nM
mean (n=7)	NV	143.3	1540.7	11185.7
SD	NV	9.9	89.8	718.8
RSD (%)	NV	6.9	5.8	6.4
Accuracy(%)	NV	114.6	123.3	89.5

Table 4.8-13 Accuracy and precision for rofecoxib (cassette)

As expected, the precision (RSD) tends to be better with higher concentrations because of smaller relative variations for all compounds. As we have seen earlier in this experiment, there are small variations between runs, but some compounds have a greater variation from the theoretical values (Accuracy). Although, the accuracy falls within the ±25% acceptance criterion for most of the controls.

This experiment indicates that there are some variations in the analytical method even though the variations are quite even. The dispersion between runs of the same samples shows some variation, but overall, they falls within a limit of ± 25%. This implies that even though not all the results in this whole study fulfilled the ± 25% acceptance criterion, this difference is not related to the LC-technique, but rather to the variability in the analytical method.

5. Conclusions

The results from this study indicate that cassette analysis can be used. Examining differences between six reference compounds analysed in discrete/single and cassette/pooled mode ratifies this. Results from the *in vivo* studies with in house AstraZeneca compounds, and the results from the validation experiment substantiate the assumptions. A general ± 25% acceptance criterion was set for deviations between single and pooled samples. When spiking PK (IV) profiles with the reference compounds to be analysed on HPLC, only 4 out of 42 samples exceeded the acceptance criterion. A comparison of single and pooled standard curves for the AZ compounds showed small variations, the deviations varied from –6% to 13%.

This assessment indicates that there are no large discrepancies between samples analysed in discrete or cassette mode. There are no large discrepancies between the analytical results from UPLC and HPLC either. By the use of cassette analysis instead of discrete analysis, the throughput increases considerably. The throughput increases additionally with use of cassette analysis in combination with UPLC-MS/MS instead of HPLC-MS/MS, and can then increase the throughput at least five times. Because of the increased throughput, there will also be massive savings in time for analysis and costs connected to this.

However, there are some variations in the analysis that not fulfilled the ± 25% acceptance criterion. This difference is on the other hand not related to the LC-technique, but rather to variability in the analytical method , scattering in the analysis system, and variations in the sample preparation. The basis for this assumption can be found in the validation experiment, which also have the same variations. Thereby, there can always be some variations in the analysis, regardless of the samples are single or pooled.

Because of additional dilution in the sample preparation step for the pooled samples, there is a small loss of sensitivity compared with the single samples. However, the additional dilution is only 1.6 times higher for the pooled samples, and is therefore only of concern when working with very small concentrations.

Some formulations can give ion suppression or ion enhancement, for instance PEG 400. When using formulations where the impact on the analytes is unknown, an ion suppression check should be performed. Also, before pooling of unknown compounds, a check of how the compounds affects each other compared to single analysis should be performed. Even though there are more issues to consider when pooling samples, the earnings are much greater.

6. References

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Appendix A

4.3.1 Reducing the amount of acetonitrile used for protein precipitation

Measured area and concentration for the reference compounds with and without formulation (F). Adding different amounts of ACN in the sample preparation, 4.25 minute method:

Diazepam, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
356	483	5	5
707	752	10	9
2451	2125	33	29
8318	7889	113	114
28923	27065	393	397
90270	89858	1249	1338
221414	225405	3199	3447
593258	586286	10171	9727

Diazepam, 300 µL ACN

Area	F_Area	Conc.	F_Conc.
569	522	5	5
1183	1013	11	10
3376	2815	34	30
11371	10411	116	113
36000	34328	373	377
112063	120583	1192	1353
275623	284207	3109	3336
718650	712598	10565	9828

Diazepam, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
591	596	5	5
1237	1092	10	10
4041	3487	34	31
13259	13158	113	116
45514	44735	392	397
134525	139757	1192	1269
324390	350967	3084	3363
798207	852928	10809	9874

Imipramine, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
542	827	5	15
793	571	9	10
2409	1728	33	31
7824	5616	113	100
27268	22247	401	393
87079	74061	1298	1265
210520	237545	3197	3710
619837	752911	10069	9718

Imipramine, 300 µL ACN

Area	F_Area	Conc.	F_Conc.
1131	680	5	5
1798	987	11	9
4109	2549	30	30
14801	8788	121	114
47716	28260	401	375
138823	99914	1195	1346
351309	260885	3163	3587
963581	662591	10288	9606

Imipramine, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
496	560	5	5
1025	1175	10	10
3287	3050	33	27
11054	12515	113	111
36631	46029	376	407
120469	142508	1258	1263
299087	413732	3259	3682
789903	1068544	10094	9604

Propranolol, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
763	1004	5	6
1161	1210	9	8
3342	3141	31	29
11792	11091	114	114
41770	38101	410	406
129456	127041	1297	1376
316659	329457	3300	3636
829417	821774	9923	9491

Propranolol, 300 µL ACN

Area	F_Area	Conc.	F_Conc.
1044	907	5	5
1803	1527	11	10
4722	4243	32	30
16062	15252	118	114
52610	49386	395	375
149523	173350	1151	1353
391393	416485	3193	3425
1013540	988082	10392	9678

Propranolol, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
807	958	5	5
1470	1739	9	10
4911	5048	31	30
17776	18805	114	115
62857	65636	408	406
190066	194951	1268	1237
449662	499545	3209	3384
1068286	1162215	10143	9867

Diclofenac, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
25	NV	5	NV
61	NV	11	NV
156	154	26	34
820	240	137	46
1671	2784	272	366
8805	9004	1479	1154
22598	29387	3573	3740
56516	81307	9662	9731

Diclofenac, 300 µL ACN

Area	F_Area	Conc.	F_Conc.
87	101	NV	NV
260	213	10	10
495	431	30	31
1379	1237	115	107
4114	3877	370	382
13803	13128	1285	1330
42128	35121	3548	3446
101248	94407	9765	9832

Diclofenac, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
168	89	5	5
296	187	12	10
666	634	31	35
1734	2148	93	118
7136	5975	458	351
19557	20715	1183	1256
49638	47725	3255	3188
121550	121313	10142	10224

Naproxen, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
NV	NV	NV	NV
NV	NV	NV	NV
48	25	34	34
172	38	121	42
413	622	284	362
2003	2083	1418	1156
5319	7019	3511	3754
14111	20471	9779	9742

Naproxen, 300 µL ACN

Area	F_Area	Conc.	F_Conc.
NV	48	NV	10
53	126	10	34
150	129	40	34
325	367	104	107
1080	1176	364	376
3587	4062	1230	1307
10558	10847	3220	3295
30563	33505	10128	9992

Naproxen, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
49	118	NV	20
146	60	10	10
290	203	33	38
657	610	97	119
2178	1635	406	342
7271	5645	1275	1202
19940	14338	3608	3235
51861	41958	9678	10166

Rofecoxib, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
NV	NV	NV	NV
NV	NV	NV	NV
45	18	34	34
188	43	119	49
486	652	291	368
2261	2127	1391	1152
6121	6865	3618	3749
14160	18052	9646	9702

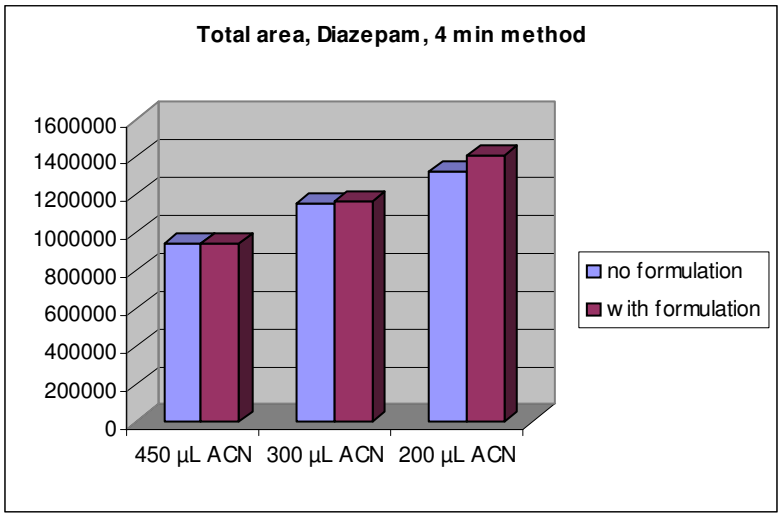
Rofecoxib, 300 µL ACN

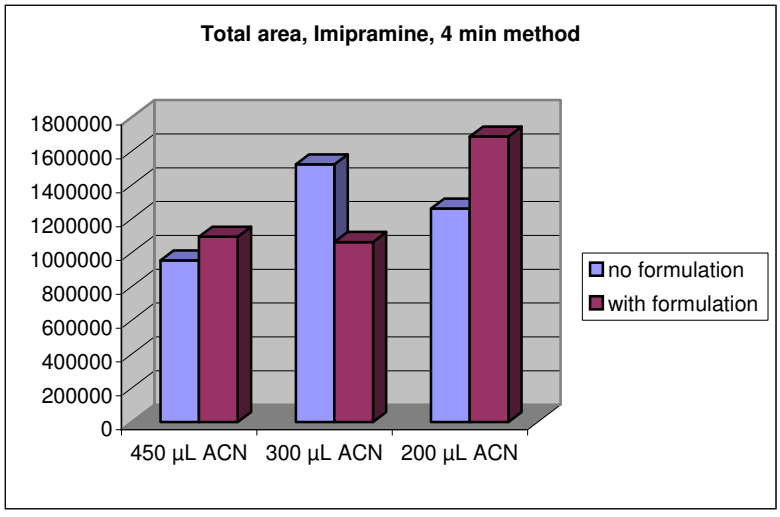
Area	F_Area	Conc.	F_Conc.
NV	22	NV	NV
NV	86	NV	10
118	141	35	30
299	355	94	105
1278	1112	398	399
4199	3449	1329	1310
11595	9248	3372	3522
27645	22053	9858	9723

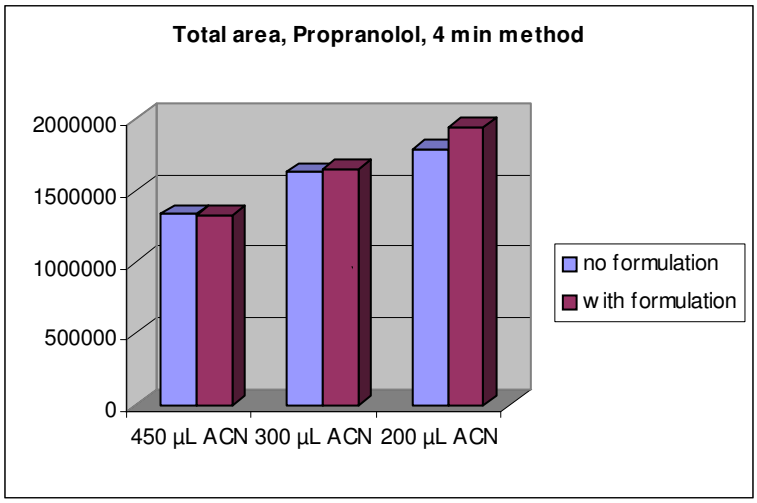
Rofecoxib, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
26	64	5	15
43	45	9	10
173	113	39	31
453	474	111	142
1363	1191	384	387
4180	3187	1102	1084
11506	7092	3269	2721
29360	19019	10345	12723

Total area for all compounds, 4.25 minute method:



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1000000
500000
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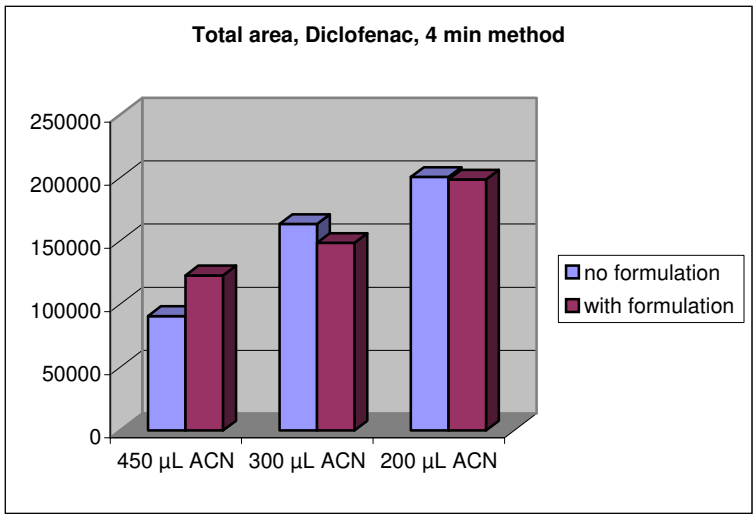
450 µL ACN
300 µL ACN
200 µL ACN
no formulation
with formulation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100000
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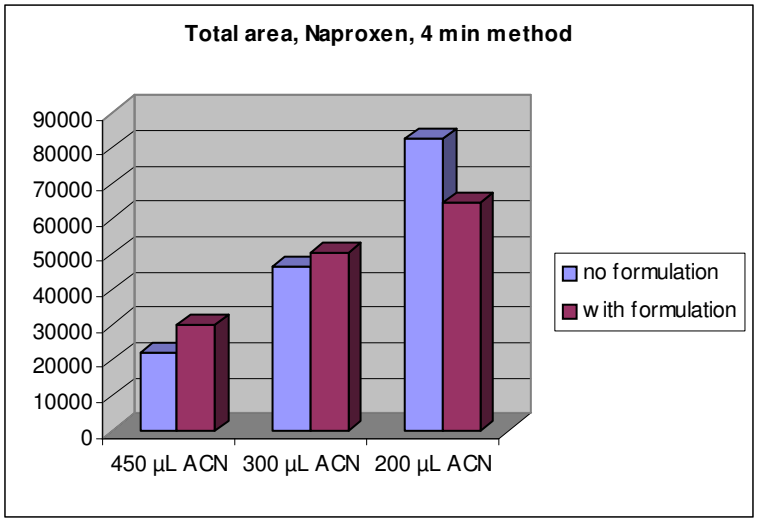
450 µL ACN
300 µL ACN
200 µL ACN
no formulation
with formulation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90000
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60000
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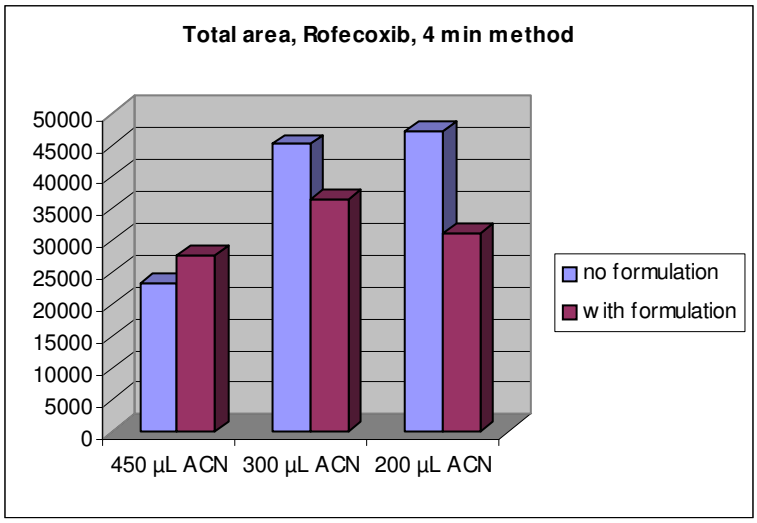
40000
30000
20000
10000
0
450 µL ACN
300 µL ACN
200 µL ACN
no formulation
with formulation



Measured area and concentration for the reference compounds with and without formulation (F). Adding different amounts of ACN in the sample preparation, 7.5 minute method:

Diazepam, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
687	556	5	5
1040	864	9	9
3522	2727	34	31
11206	9727	112	112
38798	34547	396	403
122915	112727	1279	1332
297437	278752	3221	3376
798803	748783	10082	9815

Diazepam, 300 µL ACN

Area	F_Area	Conc.	F_Conc.
589	738	5	5
1169	1423	10	10
3372	4178	31	31
12149	15179	113	113
40754	49415	383	372
135498	178675	1287	1380
342223	399986	3330	3227
942078	1032575	9949	9950

Diazepam, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
1193	1123	5	5
1714	1942	9	9
5351	6294	34	32
16859	22617	115	118
57501	71381	403	378
169316	234397	1215	1277
419358	563060	3148	3261
1144281	1363855	10285	10060

Imipramine, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
361	526	5	5
620	1002	9	11
1920	2476	29	28
6740	8226	100	95
27312	36288	402	421
100108	124457	1418	1418
277517	314705	3638	3458
887176	986637	9669	9751

Imipramine, 300 µL ACN

Area	F_Area	Conc.	F_Conc.
130	696	5	5
320	1354	10	10
1033	4508	29	31
4207	15308	112	106
14982	53535	393	368
51090	209830	1318	1434
138813	508302	3463	3440
431622	1484713	9827	9744

Imipramine, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
405	1172	6	5
508	2040	8	9
1492	6493	33	31
4907	23665	117	113
15641	78207	382	373
52596	291046	1277	1390
152039	714669	3565	3416
457734	2043731	9778	9781

Propranolol, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
927	762	5	5
1430	1245	9	10
4274	3377	33	31
13791	10720	111	105
48849	37225	400	371
153568	139704	1286	1404
376608	347018	3289	3519
983713	929943	9971	9656

Propranolol, 300 µL ACN

Area	F_Area	Conc.	F_Conc.
761	1023	5	5
1280	1777	9	10
4079	5170	31	31
14726	18600	112	114
53260	61087	409	380
167540	213732	1304	1364
415828	487803	3340	3265
1105752	1227900	9876	9901

Propranolol, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
1385	1461	5	5
2328	2358	10	9
6941	7878	32	33
23608	27227	112	116
83923	87935	406	382
253683	288021	1262	1290
612665	685577	3237	3290
1501892	1585411	10089	9966

Diclofenac, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
24	0	0	0
95	340	10	32
249	356	35	34
657	418	101	42
1843	2835	295	361
9593	8491	1496	1154
24459	28042	3695	3784
70560	76273	9599	9709

Diclofenac, 300 µL ACN

Area	F_Area	Conc.	F_Conc.
167	194	5	4
271	291	12	13
677	652	37	39
1593	1353	97	95
5107	4550	324	353
20007	16132	1358	1230
46188	40592	3423	3165
112128	109962	9823	10292

Diclofenac, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
251	250	5	4
382	566	11	15
841	1031	33	33
2078	3466	93	120
9437	8268	452	295
22589	31096	1132	1200
60905	70669	3384	2963
150216	183973	10017	11295

Naproxen, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
12	0	0	0
33	46	10	38
62	40	38	34
144	65	114	53
285	512	249	381
1583	1505	1403	1139
4541	5049	3791	3599
13608	16243	9646	9874

Naproxen, 300 µL ACN

Area	F_Area	Conc.	F_Conc.
81	31	16	5
62	39	10	9
121	103	31	37
345	243	116	103
998	836	354	383
3422	2956	1266	1285
9831	7822	3642	3253
28397	25390	9761	10038

Naproxen, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
53	37	0	4
142	88	11	15
173	168	19	34
509	575	98	120
2122	1337	471	284
5922	5312	1352	1180
16576	14528	3788	3272
45792	45063	9537	10198

Rofecoxib, 450 µL ACN

Area	F_Area	Conc.	F_Conc.
26	0	11	0
26	27	10	11
56	47	35	25
158	71	121	42
307	608	249	410
1781	1735	1447	1227
4634	5452	3662	3802
13577	14706	9678	9611

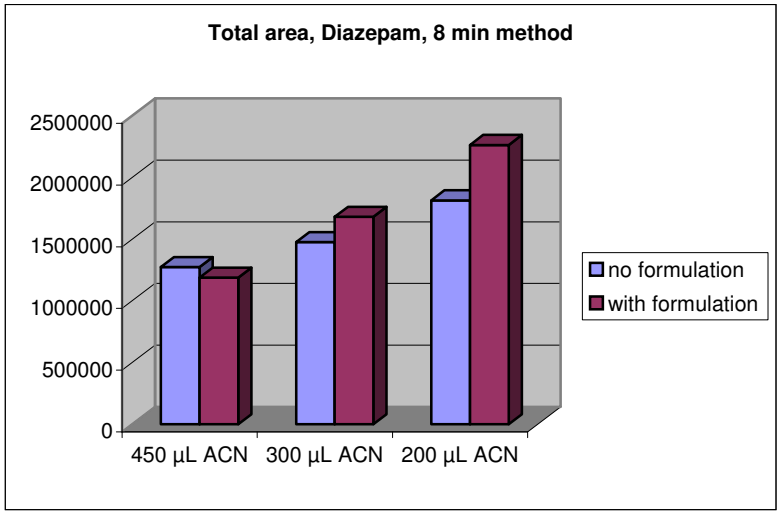
Rofecoxib, 300 µL ACN

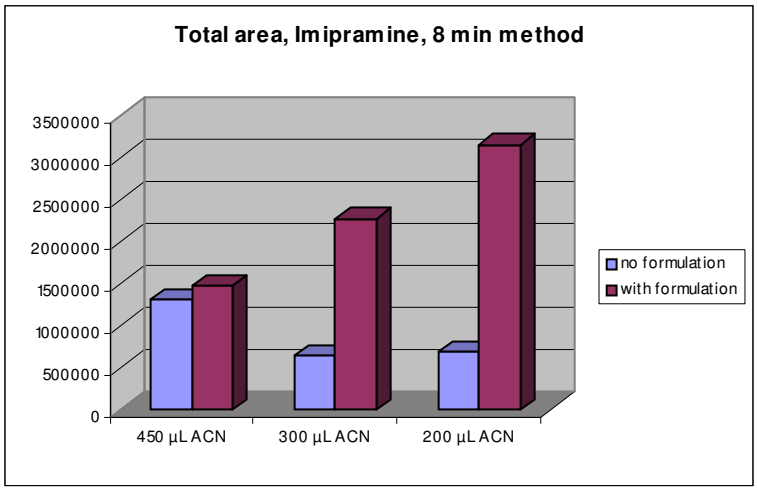
Area	F_Area	Conc.	F_Conc.
38	39	5	5
51	45	11	9
88	95	23	30
339	282	115	115
1137	911	400	406
3761	3066	1392	1324
9287	8011	3621	3442
23334	21219	9562	9785

Rofecoxib, 200 µL ACN

Area	F_Area	Conc.	F_Conc.
51	31	5	5
64	65	9	12
165	168	32	36
524	551	118	118
1752	1730	411	378
4846	4711	1191	1100
12688	10909	3440	2759
31050	30586	9888	12293

Total area for all compounds, 7.5 minute method:




Total area, Imipramine, 8 min method
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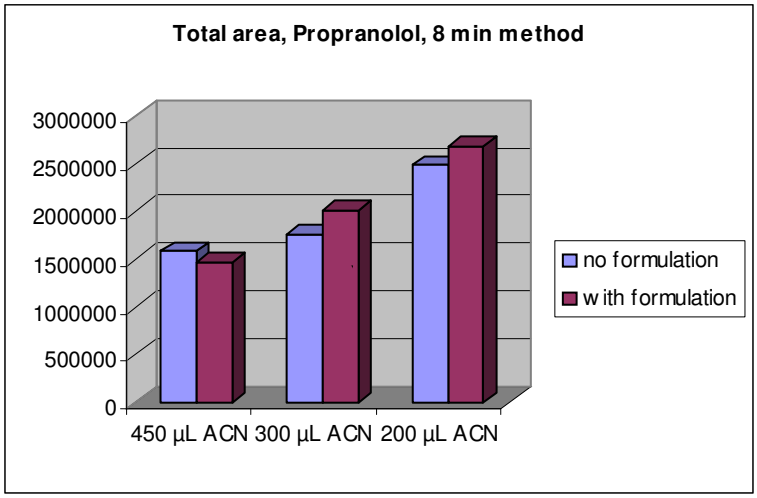
3000000
2500000
2000000
1500000
1000000
500000
0
450 µL ACN
300 µL ACN
200 µL ACN
no formulation
with formulation


Total area, Propranolol, 8 min method
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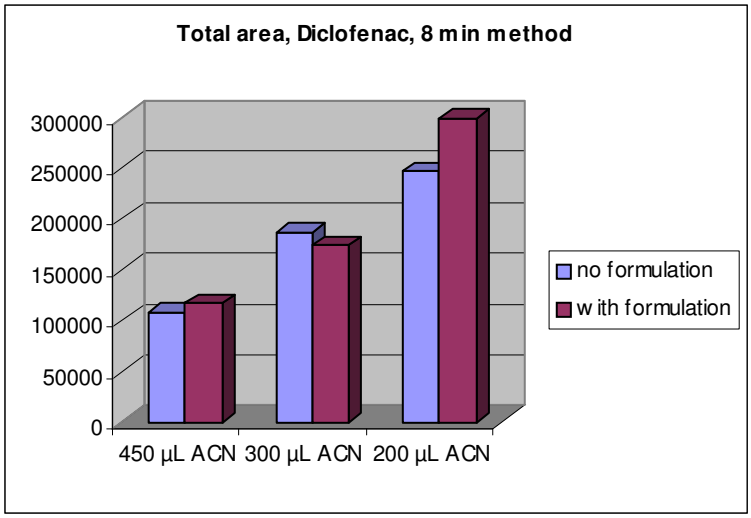
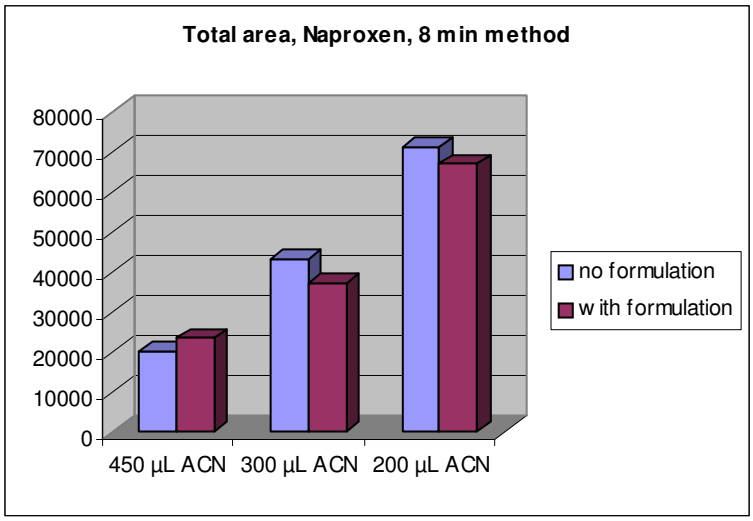
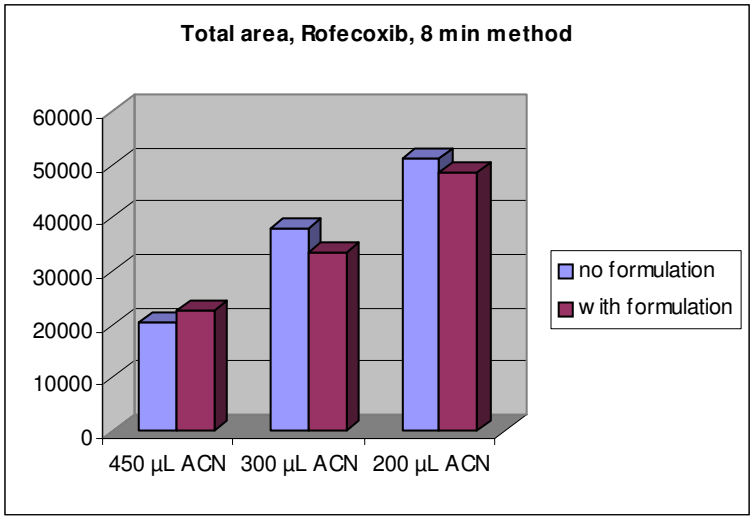
2000000
1500000
1000000
500000
0
450 µL ACN
300 µL ACN
200 µL ACN
no formulation
with formulation


Total area, Diclofenac, 8 min method
300000
250000
200000
150000
100000
50000
0
450 µL ACN
300 µL ACN
200 µL ACN
no formulation
with formulation


Total area, Naproxen, 8 min method
80000
70000
60000
50000
40000
30000
20000
10000
0
450 µL ACN
300 µL ACN
200 µL ACN
no formulation
with formulation


Total area, Rofecoxib, 8 min method
60000
50000
40000
30000
20000
10000
0
450 µL ACN
300 µL ACN
200 µL ACN
no formulation
with formulation

Appendix B

4.7 PK curves for reference substances with formulations

Deviations between spiked PK curves with different formulations from the spiked PK curve with blank plasma (no formulation), results from UPLC:

Diazepam							
Time(h)	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG 400	HPMC/Tween	MCC/NaCMC+Lipoid S100
0.25	-12%	1%	10%	2%	4%	7%	11%
0.5	-37%	0%	17%	-3%	-18%	0%	8%
0.75	-12%	-6%	9%	-2%	-10%	0%	13%
1	-10%	3%	12%	2%	-17%	-1%	12%
1.5	3%	6%	14%	5%	-13%	-1%	21%
2.5	-13%	-5%	4%	-3%	-21%	-12%	8%
6	-35%	-35%	26%	18%	-35%	-10%	-5%
24	-50%	25%	40%	25%	-50%	0%	40%

Imipramine							
Time(h)	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG 400	HPMC/Tween	MCC/NaCMC+Lipoid S100
0.25	11%	23%	29%	25%	16%	-1%	25%
0.5	32%	29%	39%	29%	-44%	5%	35%
0.75	31%	19%	28%	18%	-38%	-8%	28%
1	28%	10%	30%	21%	-51%	-13%	26%
1.5	24%	0%	25%	16%	-53%	-14%	21%
2.5	-28%	-25%	16%	9%	-30%	-22%	16%
6	12%	-2%	26%	14%	-41%	-15%	23%
24	16%	-3%	16%	15%	-97%	-20%	14%

Propranolol							
Time(h)	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG 400	HPMC/Tween	MCC/NaCMC+Lipoid S100
0.25	26%	23%	37%	37%	18%	8%	34%
0.5	22%	12%	38%	30%	-65%	-1%	35%
0.75	35%	16%	42%	40%	-43%	-2%	36%
1	16%	-5%	33%	22%	-91%	-17%	24%
1.5	35%	-9%	29%	29%	-118%	-20%	41%
2.5	29%	-33%	37%	43%	-71%	-9%	0%
6	57%	-50%	0%	25%	0%	25%	50%
24	0%	0%	0%	0%	0%	0%	0%

Concentrations for diclofenac, naproxen and rofecoxib, including the percentage deviation between the formulations and the control (blank), results from HPLC:

Diclofenac Conc. [nM]

Time (h)	Blank	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG 400	HPMC/Tween	MCC/NaCMC+ Lipiod S100
0.5	1638	1261	1565	1506	1437	1636	1526	1549
0.75	1169	1106	1095	1209	1211	1297	1228	952
1	774	875	816	821	847	865	864	678

Deviation from blank plasma

Time (h)	Blank	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG 400	HPMC/Tween	MCC/NaCMC+ Lipiod S100
0.5		-30%	-5%	-9%	-14%	0%	-7%	-6%
0.75		-6%	-7%	3%	3%	10%	5%	-23%
1		12%	5%	6%	9%	11%	10%	-14%

Naproxen Conc. [nM]

Time (h)	Blank	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG 400	HPMC/Tween	MCC/NaCMC+ Lipiod S100
0.5	4354	3914	3383	4635	3859	4799	4062	5005
0.75	4560	3955	3656	5363	4346	4668	5224	4742
1	4172	4126	3745	5293	4956	4180	5320	4524

Deviation from blank plasma

Time (h)	Blank	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG 400	HPMC/Tween	MCC/NaCMC+ Lipiod S100
0.5		-11%	-29%	6%	-13%	9%	-7%	13%
0.75		-15%	-25%	15%	-5%	2%	13%	4%
1		-1%	-11%	21%	16%	0%	22%	8%

Rofecoxib Conc. [nM]

Time (h)	Blank	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG 400	HPMC/Tween	MCC/NaCMC+ Lipiod S100
0.5	210	129	167	156	161	211	197	151
0.75	189	157	160	195	219	259	255	193
1	246	203	241	264	254	294	258	225

Deviation from blank plasma

Time (h)	Blank	Cyclodextrine	Gluconic acid	Meglumine	DMA	PEG 400	HPMC/Tween	MCC/NaCMC+Lipiod S100
0.5		-63%	-26%	-35%	-31%	0%	-7%	-39%
0.75		-21%	-18%	3%	14%	27%	26%	2%
1		-21%	-2%	7%	3%	16%	5%	-9%

Appendix C

4.8 Validation experiments

Percentage deviation from Run1 for diazepam, both single and pooled:

DIAZEPAM, SINGLE

	Run2	Run3	Run4	Run5	Run6	Run7
STD	-1%	2%	-1%	1%	-2%	-2%
	2%	-8%	4%	-3%	5%	7%
	0%	-2%	-4%	-2%	1%	-1%
	0%	3%	-1%	1%	-3%	0%
	-1%	2%	1%	1%	0%	-2%
	-1%	1%	1%	2%	-1%	-3%
	0%	1%	0%	0%	-1%	-1%
	0%	-1%	0%	0%	1%	2%
LLQC	-3%	2%	-10%	1%	-12%	-12%
	0%	1%	-3%	-16%	-8%	-10%
	6%	4%	-7%	-1%	-7%	-13%
	-12%	0%	-9%	-2%	-25%	-18%
	6%	0%	2%	5%	-7%	-3%
	-2%	6%	-4%	-6%	-17%	-1%
LQC	1%	3%	3%	-2%	-2%	-5%
	1%	5%	4%	3%	0%	-2%
	-1%	10%	7%	4%	6%	-2%
	-3%	3%	1%	2%	-3%	-5%
	0%	5%	2%	-2%	-1%	1%
	0%	1%	0%	-1%	-4%	-6%
MQC	-4%	2%	9%	0%	-2%	-5%
	0%	3%	-1%	-1%	-3%	-6%
	-3%	1%	0%	0%	-3%	-12%
	-2%	1%	-1%	0%	-1%	-4%
	-1%	3%	3%	4%	-1%	-7%
	-1%	2%	1%	3%	2%	-10%
HQC	-3%	-4%	-6%	-4%	-4%	-3%
	-1%	-1%	-2%	-2%	-3%	-3%
	-2%	-2%	-3%	-3%	-2%	-6%
	-3%	-4%	-2%	0%	-3%	5%
	-2%	3%	-2%	1%	1%	11%
	-2%	2%	1%	-1%	-1%	-6%

DIAZEPAM, POOLED

Run2	Run3	Run4	Run5	Run7
-1%	6%	1%	0%	3%
3%	-22%	-5%	1%	-9%
-1%	1%	2%	-3%	-4%
0%	6%	1%	-1%	4%
-3%	0%	-2%	2%	2%
2%	6%	1%	0%	2%
-1%	4%	3%	3%	4%
0%	-4%	-2%	-2%	-3%
-9%	0%	15%	-5%	-23%
13%	1%	-6%	22%	0%
16%	16%	4%	-2%	-6%
-4%	7%	3%	2%	4%
12%	14%	-7%	16%	18%
-1%	18%	-26%	22%	10%
6%	12%	3%	9%	12%
9%	13%	12%	13%	18%
-1%	3%	3%	2%	1%
6%	13%	-1%	10%	15%
5%	6%	7%	3%	4%
-2%	7%	2%	10%	1%
5%	11%	5%	4%	11%
3%	8%	2%	3%	6%
2%	6%	-1%	3%	5%
4%	9%	0%	5%	8%
8%	10%	1%	5%	9%
5%	5%	3%	5%	8%
-2%	3%	2%	-1%	5%
12%	3%	2%	3%	8%
5%	3%	3%	4%	2%
1%	3%	1%	3%	7%
-1%	1%	-1%	2%	3%
1%	0%	1%	1%	5%

Percentage deviation from Run1 for imipramine and propranolol, pooled:

	IMIPRAMINE, POOLED					PROPRANOLOL, POOLED				
	Run2	**Run3**	**Run4**	**Run5**	**Run7**	**Run2**	**Run3**	**Run4**	**Run5**	**Run7**
STD	2%	3%	2%	0%	1%	7%	5%	5%	6%	3%
	-7%	-10%	-8%	1%	-5%	-22%	-13%	-15%	-20%	-8%
	-2%	-3%	-4%	-5%	-5%	-8%	-15%	-10%	-9%	-3%
	3%	3%	2%	0%	4%	1%	1%	-1%	-1%	2%
	0%	3%	1%	1%	1%	10%	12%	10%	12%	6%
	6%	5%	7%	3%	2%	14%	12%	13%	12%	2%
	5%	6%	8%	5%	6%	10%	11%	12%	12%	6%
LLQC	0%	6%	-1%	10%	12%	28%	17%	6%	21%	8%
	8%	5%	7%	5%	20%	2%	-3%	40%	-1%	-1%
	10%	3%	10%	16%	24%	6%	-13%	26%	30%	3%
	6%	5%	8%	12%	32%	11%	28%	3%	6%	-9%
	10%	10%	15%	13%	30%	30%	22%	14%	35%	2%
	14%	12%	10%	17%	30%	9%	-10%	-10%	19%	-22%
LQC	11%	11%	11%	12%	16%	24%	27%	23%	24%	12%
	15%	16%	15%	17%	19%	38%	34%	34%	38%	25%
	14%	13%	13%	11%	14%	30%	33%	30%	26%	20%
	18%	14%	14%	13%	17%	32%	25%	25%	34%	19%
	18%	16%	19%	18%	16%	28%	27%	31%	23%	5%
	20%	21%	22%	20%	23%	25%	27%	32%	24%	15%
MQC	16%	16%	16%	12%	12%	30%	28%	27%	28%	12%
	15%	14%	16%	11%	12%	28%	26%	29%	26%	11%
	16%	16%	15%	13%	10%	30%	29%	28%	28%	8%
	14%	13%	12%	12%	10%	31%	26%	26%	28%	15%
	18%	15%	21%	13%	18%	31%	28%	32%	28%	21%
	15%	13%	14%	13%	16%	27%	23%	26%	25%	14%
HQC	11%	14%	12%	10%	12%	17%	17%	17%	15%	6%
	35%	15%	19%	14%	20%	35%	22%	22%	22%	13%
	17%	15%	15%	15%	13%	23%	19%	19%	22%	9%
	10%	13%	13%	9%	15%	21%	22%	20%	20%	14%
	13%	11%	15%	10%	19%	19%	19%	17%	17%	14%
	11%	12%	14%	12%	15%	17%	18%	19%	17%	15%

Percentage deviation from Run1 for diclofenac, both single and pooled:

DICLOFENAC, SINGLE

	Run2	Run3	Run4	Run5	Run6	Run7
STD	-1%	7%	-3%	-1%	-2%	-3%
	6%	-37%	12%	7%	10%	12%
	-6%	5%	2%	-10%	-2%	-5%
	-4%	1%	-13%	-3%	-7%	-5%
	-2%	4%	1%	-3%	0%	-5%
	NV	NV	NV	NV	NV	NV
	1%	3%	1%	3%	2%	4%
	-2%	-4%	0%	-3%	-1%	-3%
LLQC	-82%	1%	-3%	-39%	-45%	-6%
	-10%	-5%	-13%	-13%	-22%	12%
	-39%	-43%	19%	-230%	21%	11%
	-20%	-11%	5%	-156%	36%	18%
	-60%	-46%	7%	-76%	0%	-27%
	-421%	-38%	-46%	-104%	-33%	1%
LQC	14%	27%	21%	21%	26%	20%
	10%	2%	-9%	0%	4%	-5%
	1%	11%	5%	-4%	3%	12%
	1%	11%	0%	-24%	-2%	-1%
	-2%	7%	-13%	4%	5%	14%
	-33%	2%	2%	-3%	6%	-2%
MQC	-1%	4%	2%	-7%	0%	2%
	5%	10%	2%	-3%	6%	3%
	-2%	5%	3%	-7%	3%	-2%
	8%	17%	9%	0%	8%	0%
	7%	13%	5%	6%	10%	4%
	4%	7%	3%	-2%	6%	2%
HQC	3%	2%	0%	4%	3%	-3%
	7%	7%	4%	7%	7%	5%
	8%	6%	6%	6%	11%	10%
	6%	6%	7%	8%	3%	11%
	7%	6%	3%	7%	7%	2%
	5%	4%	28%	7%	8%	3%

DICLOFENAC, POOLED

Run2	Run3	Run4	Run5	Run6	Run7
NV	NV	NV	NV	NV	NV
-5%	-1%	3%	2%	4%	7%
12%	4%	-15%	-8%	-24%	-48%
17%	-2%	-3%	1%	2%	8%
-9%	-1%	5%	3%	7%	16%
-10%	4%	8%	1%	4%	-3%
-17%	-5%	-4%	1%	0%	-2%
9%	1%	-1%	-3%	-3%	-6%
-250%	37%	3%	28%	-48%	37%
64%	-267%	47%	85%	63%	64%
-10%	70%	62%	71%	64%	81%
-79%	-56%	-135%	-151%	-495%	15%
28%	-17%	6%	13%	-110%	58%
49%	-37%	-65%	43%	0%	41%
0%	-21%	-56%	-13%	-2%	-40%
13%	13%	19%	-7%	23%	-7%
13%	10%	-5%	15%	-1%	12%
21%	-12%	10%	-1%	5%	1%
0%	23%	3%	11%	-11%	-20%
26%	14%	6%	29%	18%	-18%
-10%	-17%	-12%	-20%	-24%	-34%
-11%	-7%	-7%	1%	-11%	-30%
-10%	-14%	-8%	-10%	-10%	-36%
-10%	-12%	-2%	-10%	-8%	-27%
-12%	-14%	-6%	-9%	-7%	-10%
-12%	-9%	-5%	-9%	-7%	-16%
-18%	-6%	-1%	-21%	-7%	-20%
-8%	7%	0%	-6%	-8%	-17%
-18%	10%	2%	-12%	0%	21%
-16%	-2%	4%	4%	4%	-12%
-21%	5%	2%	1%	-3%	5%
-15%	4%	7%	-8%	-3%	1%

Percentage deviation from Run1 for naproxen and rofecoxib, pooled:

NAPROXEN, POOLED

	Run2	Run3	Run4	Run5	Run6	Run7
STD	NV	NV	NV	NV	NV	NV
	NV	NV	NV	NV	NV	NV
	-8%	-31%	-4%	0%	-5%	-4%
	27%	-1%	16%	0%	19%	14%
	-7%	3%	-7%	4%	-6%	1%
	-10%	12%	-4%	-4%	-8%	-10%
	-12%	3%	-2%	-2%	-3%	-3%
	6%	-3%	1%	1%	2%	2%
LLQC	61%	-71%	47%	58%	49%	71%
	54%	-224%	63%	69%	37%	74%
	78%	-89%	63%	78%	65%	80%
	36%	-7%	15%	35%	26%	49%
	35%	-183%	28%	56%	22%	64%
	84%	74%	84%	89%	79%	89%
LQC	28%	17%	-5%	25%	15%	29%
	34%	50%	48%	33%	44%	62%
	30%	38%	41%	28%	3%	43%
	52%	48%	53%	47%	25%	52%
	30%	52%	48%	49%	63%	43%
	2%	-108%	-50%	35%	-44%	5%
MQC	-2%	-4%	-5%	-5%	-8%	-8%
	-3%	-2%	-18%	5%	10%	-19%
	-2%	17%	11%	9%	9%	-14%
	5%	4%	15%	12%	0%	1%
	-2%	-4%	10%	6%	-10%	3%
	6%	9%	-3%	8%	-24%	-4%
HQC	-10%	-3%	6%	-7%	3%	-3%
	-20%	1%	3%	-1%	-4%	-1%
	-10%	-1%	-2%	11%	6%	0%
	-18%	-9%	-2%	15%	-1%	-4%
	-27%	6%	7%	24%	3%	0%
	-16%	1%	6%	3%	1%	-6%

ROFECOXIB, POOLED

	Run2	Run3	Run4	Run5	Run6	Run7
	NV	NV	NV	NV	NV	NV
	-3%	NV	4%	-5%	-8%	7%
	4%	-19%	-19%	11%	19%	-28%
	9%	10%	18%	0%	-8%	0%
	-1%	-4%	-3%	-1%	-10%	20%
	-6%	10%	1%	-11%	-4%	-5%
	-13%	0%	-8%	-8%	-4%	-3%
	5%	-2%	3%	10%	5%	0%
	65%	NV	50%	77%	70%	47%
	52%	NV	-98%	3%	-32%	-4%
	66%	NV	-14%	33%	68%	24%
	68%	NV	33%	53%	15%	72%
	51%	NV	55%	1%	28%	-202%
	25%	NV	-3%	17%	-8%	-2%
	24%	9%	20%	5%	36%	5%
	-3%	-5%	-1%	-30%	-22%	2%
	10%	-18%	-1%	9%	-41%	-3%
	11%	-44%	14%	4%	2%	8%
	-40%	-57%	-30%	-3%	-73%	-33%
	-7%	-4%	-18%	-4%	-46%	4%
	-3%	3%	1%	-19%	-19%	5%
	-2%	11%	9%	1%	-1%	-4%
	-9%	-11%	-20%	-27%	-20%	-9%
	5%	6%	1%	-16%	-5%	-4%
	0%	6%	0%	-13%	-8%	-8%
	0%	9%	3%	-12%	-6%	0%
	-18%	-9%	-9%	-19%	0%	3%
	-24%	-7%	-13%	-28%	-11%	2%
	-17%	-5%	-10%	-17%	-8%	-17%
	-14%	-6%	-1%	-9%	1%	3%
	-24%	-12%	-14%	-15%	-13%	-22%
	-26%	-11%	-14%	-20%	-13%	-17%