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Mass spectrometry and n-in-one analytics in early drug discovery: Combinatorial chemistry libraries, lipophilicity and absorption screening

Abstract 3
List of original publications 4
1. Introduction 7
2. Literature review 8
2.1 Drug discovery of compounds and early screening 8
2.1.1 Drug discovery of compounds and early tests 8
2.1.2 *In silico* tests for lipophilicity and absorption 9
2.1.3 *In vitro* tests for lipophilicity and absorption 10
2.1.4 *In silico* with *in vitro* 13
2.2 N-in-one measurements 14
2.2.1 The principle of n-in-one 14
2.2.2 Cocktail dosing, mixtures (biological n-in-one) 15
2.2.3 Pooled cocktail analysis (analytical n-in-one) 18
2.2.4 Pooled analysis of samples (analytical n-in-one) 18
2.2.5 Parallel and other analytical solutions 19
2.3 The separation of liquid chromatography mass spectrometry 20
2.3.1 Definitions for specificity, sensitivity and selectivity 20
2.3.2 Mass spectrometric separation 21
2.3.3 Liquid chromatography methods in early studies 24
2.3.4 Mass spectrometry compared to other detectors 25
3. Aims of the studies 27
4. Experimental 28
4.1 Chemicals and reagents 28
4.2 Instruments and methods 32
4.2.1 Nitrophenoxy benzoate study 32
4.2.2 IAM study 33
4.2.3 Caco-2 study 35
5. Results and discussion 37
5.1 Nitrophenoxy benzoate study 37
5.1.1 Fragmentation 37
5.1.2 Combinatorial library screening 42
5.2 IAM study 43
5.2.1 Retention factors of IAM data 43
5.2.2 IAM columns 43
5.2.3 ESI suitable buffers 44
5.2.4 UV versus MS 44
5.2.5 Single versus n-in-one 45
5.3 Caco-2 study 47
5.3.1 LC-MS/MS method 47
6. Conclusions 50
7. References 52

Abstract

Compound libraries of pharmaceutical companies include many novel compounds. In the optimization of biological activity and other drug properties, efficient approaches are needed. The n-in-one strategy in drug discovery was introduced 20 years ago. In this strategy, multiple samples are mixed or pooled prior to measurement or analysis. This allows the most promising compounds to be rapidly identified and then analysed. Although it is not a new approach, several new reviews of n-in-one pharmacokinetics have recently been published. Knowledge of cocktail dosing, sample pooling and other analytical methods has increased, and it is relevant to understand the role of n-in-one in drug development. Study plans and proper analytical methods are important for n-in-one, as with any new drug discovery technologies that are introduced. According to recent publications, n-in-one without proper method planning and validation has several risks, compound-compound interactions being the most serious.

This thesis describes current and past n-in-one methods and presents three early experimental studies using mass spectrometry and the triple quadrupole instrument, on the application of n-in-one in drug discovery. In the first study, the fragmentation patterns of ten nitrophenoxy benzoate compounds were characterised and the presence of the compounds was determined in a combinatorial library. The influence of one or two nitro substituents and the alkyl chain length of methyl to pentyl on collision-induced fragmentation was studied, and interesting structure-fragmentation relationships were detected. Two nitro group compounds increased fragmentation compared to one nitro group, whereas less fragmentation was noted in molecules with a longer alkyl chain. The most abundant product ions were nitrophenoxy ions, which were also tested in the precursor ion screening of the combinatorial library. In the second study, the immobilized artificial membrane chromatographic method was transferred from ultraviolet detection to mass spectrometric analysis and a new method was developed. Mass spectra were scanned and the chromatographic retention of compounds was analysed using extract ion chromatograms. Comparisons were made between two different buffers as the mobile phase, between single compound analysis and n-in-one of nine compounds, and fast lipophilicity screening with gradient elution was also used. In the final study, a new method was developed for the quantitation of ten compounds in Caco-2 samples using liquid chromatography tandem mass spectrometry. Compounds were separated by liquid chromatography and quantified by selected reaction monitoring using mass spectrometry. This method was used in evaluation of n-in-one in Caco-2 samples.

Better understanding of n-in-one is needed in order to apply the strategy appropriately. These studies provided good examples of how methods for mixture analysis and mass spectrometry can be developed and tested for compound identification, method transfer and quantitation. In these new methods, several scanning modes of the triple quadrupole instrument are exemplified. The high sensitivity and selectivity that can be achieved using liquid chromatography mass spectrometry are especially important for new analytical methods using n-in-one.

List of original publications

This licentiate thesis is based on the following three original publications, which will be referred to in the text by A, B and C:

A. H. Kangas, R. Franzen, J. Tois, J. Taskinen and R. Kostiainen, Effect of nitro groups and alkyl chain length on the negative ion tandem mass spectra of alkyl 3-hydroxy-5-(4'-nitrophenoxy) and alkyl 3-hydroxy-5-(2',4'-dinitrophenoxy) benzoates, Rapid Commun. Mass Spectrom. 1999, 13, 1680-1684

B. H. Kangas, T. Kotiaho, T. Salminen and R. Kostiainen, N-in-one determination of retention factors for drugs by immobilized artificial membrane chromatography coupled to atmospheric pressure chemical ionization mass spectrometry, Rapid Commun. Mass Spectrom. 2001, 15, 1501-1505

C. L. Laitinen, H. Kangas, A.M. Kaukonen, K. Hakala, T. Kotiaho, R. Kostiainen, J. Hirvonen, N-in-One Permeability studies of heterogeneous sets of compounds across Caco-2 cell monolayers, Pharmaceutical Research 2003, 20 (2), 187-197

The author has been the responsible scientist in the analytical studies of presented publications. In publications A and B she was also the main author. The compounds in publication A were synthesised by Robert Franzen and Jan Tois, who also performed NMR measurements on the compounds and measured the yields of the synthesis. The *Ab initio* calculations for publication A were offered by Jyrki Taskinen.

In publication C, the author had a smaller role. She developed the final analytical method and analysed the first part of the samples. In addition, the author trained researcher Kati Hakala during the analysis and the final part of the study was then analysed by Kati Hakala. Furthermore, publication C was mainly written by Leena Laitinen, who was the responsible scientist in the permeability studies, the Caco-2 cell tests and the interpretation of the cell results using n-in-one. Therefore, the Caco-2 tests are only discussed in literature survey.

ABBREVIATIONS

λ_{max}	absorption maximum e.g. in ultraviolet spectra
ADME	pharmacokinetic parameters of a drug: absorption, distribution, metabolism and excretion
AUC	area under the curve of a concentration versus time plot
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photo-ionisation
CAD	charged aerosol detection
CID	collision-induced dissociation
CYP	cytochrome P450 enzymes
CLND	chemiluminescent nitrogen detection
ESI	electrospray ionization
ELSD	evaporative light scattering detection
FIA	flow injection analysis
Caco-2	human colon adrenocarcinoma cell line
HBSS	Hank's balanced salt solution (an isotonic solution of inorganic salts present in approximately the correct physiological concentrations)
HSA	human serum albumin
%HIA	human intestinal absorption values
IAM	immobilised artificial membrane
LC	liquid chromatography
LLOQ	lower limit of quantitation
Log D	logarithm of the partition coefficient between an organic solvent and an aqueous phase (representative of both ionized and neutral forms in a given pH)
Log P	logarithm of the partition coefficient between an organic solvent and an aqueous phase (lipophilicity of the neutral form of a molecule)
cLog P	computerized estimation of Log P
$[M+H]^+$	protonated molecule
$[M-H]^-$	deprotonated molecule
MDCK	Mardin-Darby canine kidney cells
MDR1	multidrug resistance drug transporter, p-glycoprotein

MS	mass spectrometry / mass spectrometer
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
MUX	multiplexed electrospray interface
Mw	molecular weight
NMR	nuclear magnetic resonance
PAMPA	parallel artificial membrane permeability assay
pKa	the negative logarithm of a dissociation constant for acids, Ka (used for acid-base properties)
SRM	selected reaction monitoring
TOF	time of flight
RSD	relative standard deviation
UGT	UDP-glucuronosyl transferase enzymes
UPLC	ultra pressure liquid chromatography
UV	ultraviolet
QSAR	quantitative structure activity relationship
QqQ	triple quadrupole instrument

1. Introduction

Drug discovery aims to identify compounds having the greatest potential as early as possible. N-in-one is a methodology whereby this process is accelerated by using mixtures. The definition of n-in-one includes studies that mix or pool compounds before measurement or analysis. The planning of such studies requires more effort than single compound studies, and the analysis of n-in-one samples also requires better methods than usual. This thesis presents new analytical methods for early drug discovery and n-in-one. The main focus was to develop mass spectrometric methods for combinatorial libraries, lipophilicity and absorption screening. These methods should differentiate and quantify the analytes in the presence of other components in the sample. The lack of specificity in previous analytical procedures was compensated by mass spectrometric analysis. The studies were conducted using a triple quadrupole mass spectrometric instrument. One qualitative study was carried out on a component of a combinatorial chemistry series. In addition, a lipophilicity screening method and quantitative method for absorption screening were tested using a set of molecules consisting of commercial small organic drugs. This thesis includes three separate studies, all presenting new mass spectrometric methods. The suitability of these analytical methods for n-in-one applications was tested. New applications such as these can improve the efficacy and speed of early drug research.

2. Literature review

2.1 Drug discovery of compounds and early screening

2.1.1 Drug discovery of compounds and early tests

New compounds for drug discovery are sought from natural sources or chemical synthesis (Edwards, P.J. et al., 2006, Newman, D.J. et al., 2003). Natural sources include, for example, microbiological fermentations, plant extractions or animal toxins. In chemical synthesis, various methods, such as combinatorial chemistry and microwaves, are used. Microwaves provide a catalyst for the reactions (Kappe, O.C. and Dallinger, D., 2003). Combinatorial chemistry is the methodology where compounds are prepared using a primary core structure (linker molecule) and selections of additional building blocks. It uses different reactions and several building blocks to increase the size of compounds. The number of compounds, N, can be calculated using $N = bcd$, where b, c and d are building blocks in each reaction vessel (Gallop, M.A. et al., 1994).

Drug developers know that money can be saved if unpromising candidates are removed from compound selection at an early stage (Posner, B.A., 2005, Venkatesh, S. and Lipper, R. A., 2000, DiMasi J.A., 2002). It is estimated that the major reasons for clinical failures in 2000 were the lack of efficacy (30% of failures), clinical safety (30%), animal safety (30%) and pharmacokinetics and bioavailability reasons (10%) (Chackalamannil, S. and Desai, M., 2007). Pharmacokinetic and bioavailability problems have decline over the years, because scientists have encouraged the testing of solubility, dissolution, absorption (lipophilicity), transporters or metabolism to improve pharmacokinetics (Yu, H. and Adedoyin, A., 2003). Better understanding and multiple absorption, distribution, metabolism and elimination (ADME) tests in the drug development process explain the improved success rates.

The permeability of compounds across cell membranes is important for drug absorption. Most drug candidates have passive transcellular transport as the main absorption mechanism, although not all. A relatively recent review by Shah (Shah, P., 2006) listed the drug absorption routes and mechanisms as follows: 1) passive transcellular route, 2) passive paracellular route, 3) carrier mediated transport, 4) carrier mediated efflux and 5) vesicular transport. Lipophilic compounds are mainly transported via the transcellular pathway. Hydrophilic drugs (Mw < 200) and peptides partition poorly into cell membranes and are therefore transported via the paracellular pathway. Apart from passive diffusion, transporter proteins and carriers are involved in drug absorption. Some compounds enter the intestinal lumen using carriers (apical efflux) to limit intestinal absorption, and some macromolecular drugs are absorbed via vesicular transport.

Nowadays, as confirmed by several researchers, absorption, distribution, metabolism and elimination (ADME) properties are characterised earlier and in parallel with pharmacological efficacy (Bedersford, A.P. et al., 2002, Chu, I. and Nomeir, A.A., 2006, Feng, W.Y., 2004, Stahl, M. et al., 2006, Yu, H. and Adedoyin, A., 2003). Di and Kerns presented the optimization of drug properties as illustrated in Figure 1 (Di, L. and Kerns, E. H., 2003). In the figure, some common *in vitro* tests are listed, such as permeability, physiochemical and metabolism tests. The main physiochemical tests are to determine acid-base properties and lipid/water solubility. The lipid solubility, as the

logarithm of the partition coefficient between an organic solvent and an aqueous phase (Log P), is important for drug permeability. The acid-base properties of compounds, which influence their solubility, are usually expressed as the negative logarithm of a dissociation constant (pKa). For example, basic compounds become ionic in acidic solutions; furthermore, ions are usually more water soluble. Therefore, permeability varies for acidic or basic compounds at different pH values.

In vivo pharmacokinetic tests, usually animal tests in early phases, are also mentioned in the same figure. During *in vivo* experiments, more of the investigated substance is needed, especially for larger laboratory animals. Usually, at the beginning of drug discovery, only a small quantity of the compound to be tested is available and rapid *in vitro* methods are preferred. The ADME tests are numerous and are not thoroughly reviewed here. Nevertheless, the following methods for lipophilicity and absorption screening are briefly presented: the partition coefficient (Log P), immobilised artificial membrane (IAM) chromatography and the parallel artificial membrane permeability assay (PAMPA), as well as human colon adrenocarcinoma (Caco-2) and Mardin-Darby canine kidney (MDCK) cell assays. However, not all varieties of reverse phase chromatography and capillary electrophoresis methods (Henchoz, Y. et al, 2009) are discussed.

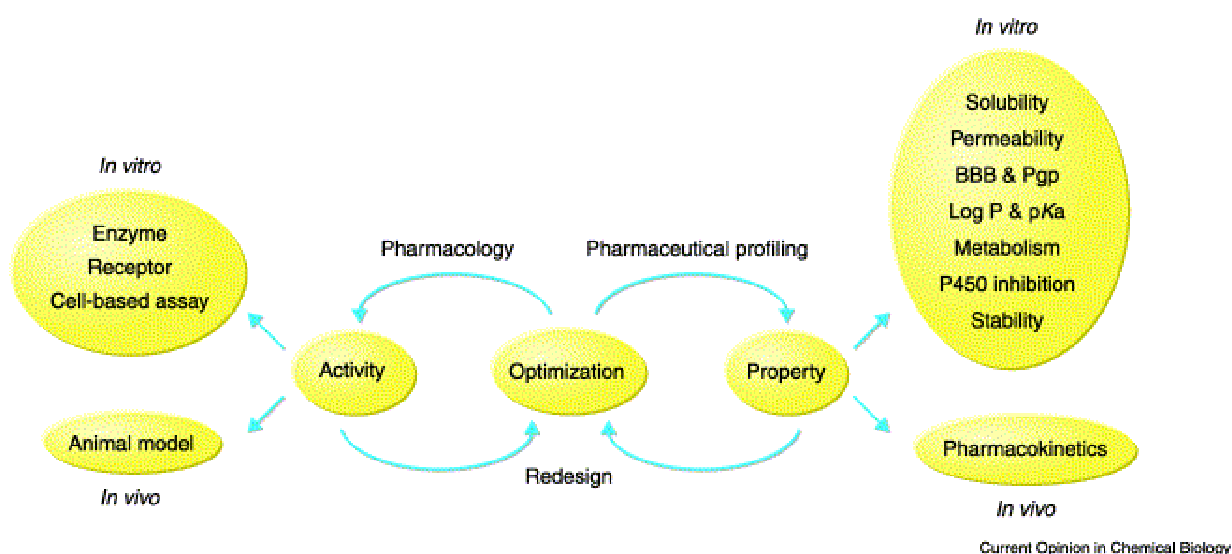


Figure 1. Optimization in drug development using a parallel approach presented by Di, L. and Kerns, E. H., 2003: the absorption, distribution, metabolism and elimination (ADME) of a compound are measured in parallel with activity screening.

2.1.2 *In silico* tests for lipophilicity and absorption

Physiochemical values have been presented for optimizing the lipophilicity and absorption properties of drug candidates. Some of the physiochemical properties of a compound can directly be estimated from the compound structure or calculated by using computers, usually presented as *in silico* methods. Poor absorption is apparent with candidates having more than 5 hydrogen bond donors, 10 hydrogen bond acceptors, a molecular weight in excess of 500 daltons, and log P above +5, known as Lipinski's "Rule of 5" (Lipinski, C.A., et al., 1997). A slightly wider range for lipophilicity is given by Ghose, where the probability of succeeding is expressed to be one order of magnitude higher if log P is between -0.4 and 5.6 (Ghose, A.K. et al., 1999). The molecular surface

area values have also been used to predict oral drug absorption (Bergström, C. et al., 2003). Passive diffusion is sometimes expressed as a combination of lipophilicity, polar surface area, molecular volume, flexibility and hydrogen bonding (Balimane, P.V. et al., 2006). Some of the *in silico* studies as mentioned in review of Dickins and Waterbeemd (Dickins, M. and van de Waterbeemd, H., 2004) work closely with *in vitro* methods. These cannot all be all presented here, but a combinationing of some of the data sets is presented a little further.

2.1.3 *In vitro* tests for lipophilicity and absorption

The partition coefficient (Log P), immobilised artificial membrane (IAM) chromatography and parallel artificial membrane permeability assay (PAMPA) are commonly used *in vitro* tests for passive transcellular transport. Human colon adrenocarcinoma (Caco-2) and Mardin-Darby canine kidney (MDCK) cell assays are usually used if more information on permeability is needed, such as passive paracellular routes or active carrier mediated transport mechanisms. Table 1 presents a list of common *in vitro* lipophilicity and permeability tests. The list of lipophilic phases starts from the shake flask method to determine the octanol/water partition coefficient, which still remains the reference method for lipophilicity. The following tests are simplifications of the lipid bilayer of a cell membrane, and finally at the end are listed methods that use the monolayers of cell cultures. As the complexity of the lipid material increases, more information on permeability and drug absorption can also be expected.

Table 1. Lipophilic phases used in the partition coefficient (Log P), immobilised artificial membrane (IAM) chromatography and parallel artificial membrane permeability assay (PAMPA), human colon adrenocarcinoma (Caco-2) and Mardin-Darby canine kidney (MDCK) cell assays for lipophilicity and absorption screening.

***In vitro* test**	**Lipophilic phase**	**Ref.**
Log P	octanol	
IAM	silica as solid support + phosphatidyl choline (PC) + with of without glycerol linkers	(Barbato, F. et al., 2004)
PAMPA	hydrophobic filter support + mixture of (egg-) lecithin + an inert organic solvent (1,7-octadiene or n-octanol)	(Praveen V. et al., 2006)
bio-mimetic PAMPA	hydrophobic filter / porous chemical filter support + lipid composition (PS, PI, PC, PE and CHO, springomyelin) + an inert organic solvent (1,7, octadiene / n-octanol) + additional pumps giving sink conditions	(Kiyohiko S. et al., 2003, Corti, G. et al., 2006)
Caco-2	cell monolayer + P-Glycoprotein transporters involved	(Praveen, V. et al., 2006)
MDCK	cell monolayer + leakier paracellular pathways + might have transfected efflux transporters	(Shah, P. et al., 2006)

L-α-phosphatidylserine (PS), L-α-phosphatidylinositol (PI), L-α-phosphatidylcholine (PC), L-α-phosphatidylethalamine (PE), cholesterol (CHO)

The typical reference parameter for lipophilicity is expressed as a logarithm of the partition coefficient between an organic solvent and an aqueous phase (Log P). The traditional organic solvent is n-octanol. Log P represents the lipophilicity of the neutral form of a molecule, while Log D is representative of both ionized and neutral forms at a given pH. Log P and Log D values are experimentally determined using an *in vitro* shake flask method. Since measurements of concentrations are laborious, theoretical Log P methods (cLogP) are often used for lipophilicity values.

Immobilized artificial membrane (IAM and PAMPA) technologies use an immobilized lipid phase instead of octanol and physiological buffers instead of water. In an immobilised artificial membrane (IAM) chromatography column, the immobilized phospholipids mimic the cell membranes and the retention time correlates with the lipophilicity. Figure 2 presents the basis of the IAM model. Several columns with different phases have been developed and tested. Even though there is some variability in the retention factors of different phases, IAM chromatography columns might give as good as or more representative estimations of drug behaviour than the Log P value.

In a computational study for predicting the volume of distribution, two chromatographic sets were combined (Hollosy, F. et al., 2006). The retention of immobilized artificial membrane (IAM) chromatography, as a marker for membrane binding in tissues, and the retention of human serum albumin (HSA) chromatography, as a marker for plasma protein binding, were tested against *in vivo* pharmacokinetic results. The calculations based on IAM results gave better estimations of the human volume of distribution than using computational values of log P (cLog P). Yet another test for predicting drug bioavailability was carried out by combining IAM results with *in vitro* hepatic metabolic clearance (Shin, B. et al., 2009). Users have also reported that the IAM method is simpler and faster compared to traditional Log P measurements (Pehourcq, F. et al., 2003). In addition, the IAM method might also be easily automated and possible impurities of the compounds will not affect the results (Hollosy, F. et al., 2006).

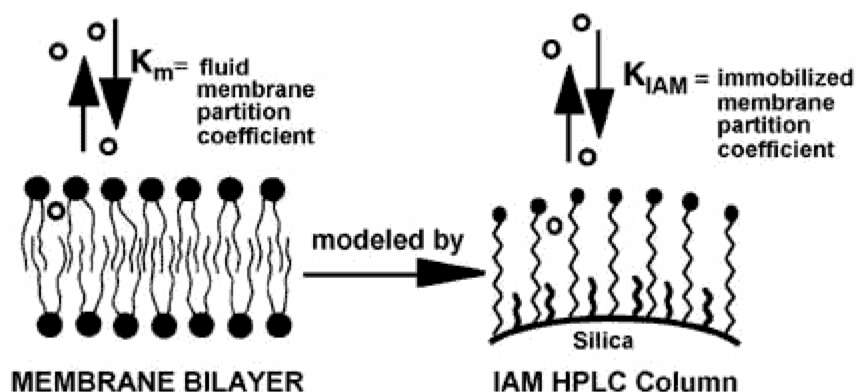


Figure 2. Production of immobilized artificial membrane (IAM) columns, as presented by Regis Technologies: phosphatidyl choline chains are bound to the solid silica structure of the chromatographic column, mimicking the membrane structure (http://www.registech.com/InfoPages/IamInfo/IAMAbstracts.html).

Immobilised artificial membrane (IAM) data are used as an approximation of oral absorption, usually in combination with *in silico* parameters. Without *in silico* data the IAM results give only a moderate correlation compared with Caco-2 results (Stewardt, B.H. and Chan, H.O., 1998) or with human intestinal absorption values (Yen, T.E. et al., 2005). In penetration studies on the blood-brain barrier the IAM method has shown a slightly better correlation. For example, a good correlation for steroids and biogenic amines was achieved between IAM data and *in vivo* blood-brain data (Reichel, A. and Begley, D., 1998). Another test of IAM and blood-brain ratios gave a modest correlation, because cationic compounds were outliers (Salminen T. et al., 1997).

The parallel artificial membrane permeability assay (PAMPA) measures the passive diffusion of compounds using filter support, the phospholipids of egg lecitin and an inert organic solvent such as 1,7-octadiene or n-octanol (Praveen, V. et al., 2006). The PAMPA screening method has increased in popularity because of the possibility to simultaneously analyse multiple samples using high throughput well plates. The rather simple model has a variation known as bio-mimetic PAMPA, which also uses phospholipids materials such as IAM in the lipophilic phase (Sugano, K. et al., 2003). Currently, other filter materials and lipid compositions are also being evaluated. In addition, an unstirred water layer on the membrane has been shown to influence the resistance to drug permeation (Loftsson, T. et al., 2006). Stirring of the test mixture gives a dynamic construction when sink conditions are needed (Corti, G. et al., 2006).

Human colon adrenocarcinoma (Caco-2) permeability tests use living cells that are isolated and introduced on a filter membrane, and after a growing period the cells compose a monolayer structure (Figure 3). First the compound is introduced in a buffer to the apical side of the filter. After incubation, the amount of compound that has passed across the cell monolayer is usually determined from the buffer solutions on the basolateral side. This absorption method is especially informative for passive absorption pathways, but also for transcellular transport mechanisms (Shah, P. et al., 2006, Artursson, P. et al., 2001, Hämäläinen, M.D. et al., 2004). Artursson's research group noted in their 2001 review that the Caco-2 monolayer is ideal in identifying the potential absorption problems of small organic drugs (Artursson, P., et al., 2001). To reduce some quantitative differences in paracellular drug permeation between caco-2 and intestinal absorption *in vivo*, other cell culture models with leakier paracellular pathways have also been employed, such as Mardin-Darby canine kidney (MDCK) cells (Shah, P. et al., 2006). The review article of Shah also pointed out that the cell cultures of Caco-2 cells need a longer culture time: the passage time is 21 days for Caco-2 but only 3-5 days for MDCK cells. Nevertheless, using an extended experimental time, the results of Caco-2 can be qualitatively used for paracellular permeation.

In cell monolayer tests (e.g. Caco-2), different transporters or metabolising enzymes might be involved (Shah, P. et al., 2006, Artursson, P. et al., 2001). The Caco-2 model, for example, sometimes has the capability to show the effects of P-glycoprotein transporters (multidrug resistance drug transporter, MDR1) and some metabolising enzymes such as UGT (UDP-glucuronosyl transferase enzymes). In addition, cells in the monolayer tests can be transfected with P-glycoprotein and other specific efflux transporters to allow a focus on active transport mechanisms. However, each metabolising enzyme (Thompson, T., 2001, Masimirembwa, C., et al., 2003) and transporter (Zhang, L., et al, 2006) has its own specialised tests, in which activity or binding are thoroughly assessed.

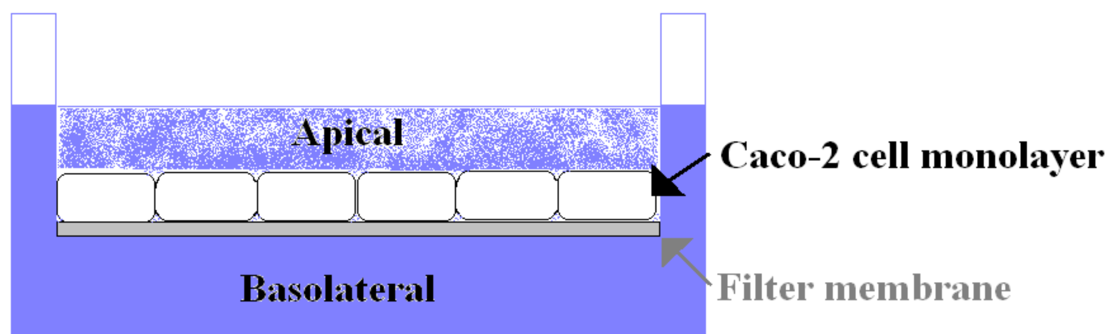


Figure 3. Caco-2 monolayer structure. Compounds are usually added to apical side of the cell monolayer and samples are collected from the basolateral side for permeability analysis.

2.1.4 *In silico* with *in vitro*

Several researchers have claimed that the combination of *in vitro* and *in silico* data provides a valuable drug discovery tool (Nasal, A. et al.., 2002, Matysiak, J., 2007, Yen, T.E. et al., 2005). For example, theoretical Log P (cLog P) values can be achieved more rapidly compared to *in vitro* methods. For improved decision making by medicinal chemists, the theoretical values need some updating with experimental data. However, few studies have presented the combination of *in vitro* and *in silico* data. In one study, the retention factor of an immobilized α_1-acidglycoprotein (AGP) column for antihistamine drugs was measured in analysing the drug binding site of a protein (Kaliszan, R. et al., 1996), and in another, solute descriptors enhanced by immobilised artificial membrane (IAM) data have been calculated using the quantitative structure activity relationship (QSAR) (Sprunger, L et al., 2007). The limitation of QSAR analysis may be the size and quality of *in vitro* data available.

The combination of *in silico* with *in vitro* immobilised artificial membrane (IAM) data is beneficial. Stewards and Chan investigated HIV drug candidates using both IAM and Caco-2 cell permeability tests (Stewardt, B.H. and Chan, H.O., 1998). IAM correlation with Caco-2 permeability improved if calculated molecular weight and hydrogen bonding values were added (r^2 increased from 0.39 to 0.91). Yen and co-workers have also tested human intestinal absorption values and IAM (Yen, T.E. et al., 2005). When the IAM data were combined with molecular descriptors, the correlation improved (R improved from 0.64 to 0.83). This may show that not only lipophilicity explains oral absorption.

Immobilised artificial membrane (IAM) measurements have also been linked with *in vitro* activity tests using quantitative structure activity relationship (QSAR) analysis. In these studies, activity and IAM lipophilicity correlations were calculated for active anti-inflammatory drugs and antitumor drug candidates. Comparison of a small set of anti-inflammatory drugs and cyclooxygenase-2 enzyme inhibition gave a relevant correlation with IAM data (Escuder-Gilabert, L. et al., 2000), revealing that active compounds had the same kind of lipophilicity. A larger set of compounds was investigated using IAM and other molecule descriptors against three antitumor *in vitro* cell tests. A clear difference between three cell-test models of antiproliferation activity was detected. The IAM, as parameter for lipophilicity, only revealed to be a descriptive for activity with the T47D cell model and not in models using SW707 or A549 cells (Matysiak, J. et al., 2007). Hopefully, these two examples show how lipophilicity tests might help in understanding activity results and even guide early drug discovery.

2.2 N-in-one measurements

2.2.1 The principle of n-in-one

Historically, the identification of new pharmacologically active hit compounds in drug discovery has used mixtures and cocktails. Combinatorial chemical synthesis has screened unspecified libraries (mixtures) for pharmacologically active leads, and has identified and purified only active compounds. Activity screening methods have also been developed for mixture analysis, such as immobilised receptors. Unfortunately these experiments still have problems with false positive and false negative results (Shin, Y.G. and van Breemen, R., 2001). Currently, compounds are more often purified before actual activity screening, because purification reduces false positive results (Isbell, J., 2008). Lately, research has tended to focus on parallel systems rather than on combinatorial chemistry mixtures. Nevertheless, Triolo and Kennedy point out in their papers that combinatorial chemistry has had rather significant effects on synthetic planning and analytical solutions (Triolo, A. et al., 2001, Kennedy, J.P. et al., 2008). It is important to understand what other impacts n-in-one has had.

In drug discovery the focus is on the selection of appropriate and potentially valuable compounds for next developmental stage. Single (parallel) and n-in-one approaches are described in the table below (Table 2). In the n-in-one approach, potential compounds are selected as early as possible, but experimental planning is more complex, the result might have some average meaning and validation of the method for mixtures is an important part of the process.

Table 2. Limitations and benefits in the n-in-one approach and traditional single compound analysis in the drug discovery selection process: planning, analysing, interpreting and reporting the results.

Single	**N-in-one**
One simple	Complex
Exact results	Sometimes an average result, but inter-test variability is reduced
Useful for understanding details	A spirit of going forward
Creates a lot of (extra) work	Rapidly provides a bigger picture

Cocktail dosing in pharmacokinetic experiments was started in 1990 by researchers at Glaxo Wellcome (Berman, J. et al., 1997). Recently, two excellent reviews have been published on pharmacokinetic studies using cocktail dosing (Smith, N.F. et al., 2007, He, K. et al., 2008). In the past 20 years, knowledge of the benefits and limitations of n-in-one measurements has increased. Manitpisitkul and White assessed the feasibility of the n-in-one approach in drug discovery (Manitpisitkul, P. and White R.E., 2004). However, their claim that approximately half of drug development companies favour the approach and half are against it appears a little “black & white”. Although the research scope was rather small, it remains clear that use of n-in-one should not be underestimated.

Many methods have been presented as n-in-one (Figure 4), and the definitions of the analysis are as follows:

a) In cocktail dosing, before biological assay, compounds are combined. Mixtures are also referred to as cassettes or cocktails. Compounds are assayed and analysed as a cocktail, which means that biological results should also be interpreted as a mixture.

b) In pooled cocktail analysis, biological assays are conducted separately for each compound. After biological assays, the samples of different compounds are pooled and analysed in as cocktails. Results of biological assay can be interpreted individually, but the analytical method needs to be valid for a mixture analysis.

c) Samples including the same compounds can also be pooled. Compounds are assayed separately and combined before analysis. This reduces chemical analysis and helps if the sample amount is limited. Even though the information for an individual sample is missed, different time points can be summarised as classifying parameters. Different time points are also analysed as mixtures for qualitative analysis.

d) Parallel methods are sometimes also referred to as n-in-one analysis, but in reality they have a singe compound in each vessel. In these studies, parallel or generic analytical methods are usually developed.

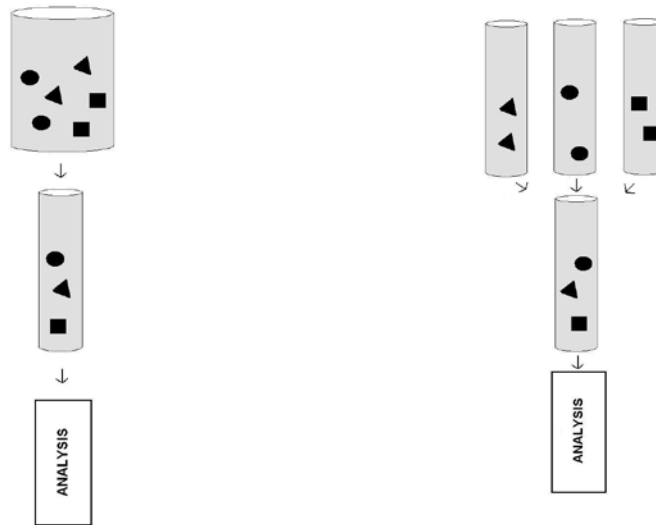
2.2.2 Cocktail dosing, mixtures (biological n-in-one)

In vivo pharmacokinetics is the study of a compound's behaviour in the body. The aim of early studies is to collect results that are exact enough for compound selection, for example using cocktail dosing. To have evidence for this, the pharmacokinetics of nine compounds were tested using single and cocktail dosing by Allen and co-workers (Allen, M.C. et al., 1998). The results for the AUC (area under the curve of a concentration versus time plot) and the Cmax (maximum concentration in plasma) were compared following dosing with single compounds or mixtures of five compounds. The rank order of exposure was similar, even though AUC was 45% higher in cocktails. Cmax was only 9% higher in cocktails than in single dosing, tested using a control compound. Another research group also reported that the AUC and half life were greater using cocktail dosing versus single dosing for some compounds (Smith, N.F. et al., 2007).

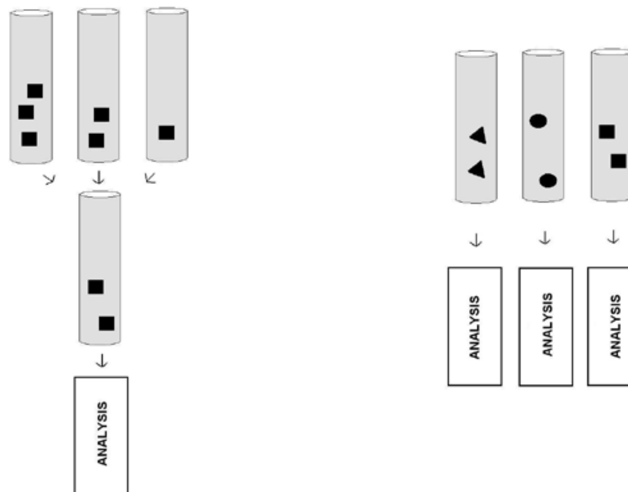
Researchers from the Bristol-Myers-Squibb drug company also investigated the *in vivo* pharmacokinetics of several lead compounds using mixtures (He, K. et al., 2008). The best correlation was achieved for the volume of distribution and renal excretion ($r = 0.91$ and 0.83, respectively), with a lower correlation for systemic clearance ($r = 0.69$) and the lowest for oral bioavailability ($r = 0.53$). In their studies the pharmacokinetics in different animals (rats, dogs and chimpanzees) were significantly correlated between single and cocktail dosing.

Smith et al. reported both positive and negative experiences with cocktail dosing (Smith, N.F. et al., 2007). In some cases, the discovery process was accelerated when n-in-one was used, but in some compound classes, compound-compound interactions appeared and the rank order of the compounds also changed. The group additionally studied *in vitro* metabolism using hepatocytes. They found lower levels of metabolism in microsome incubations with n-in-one than in single

compound experiments. This was interpreted as one or more compounds inhibiting the metabolism of the others. Although the inhibition of metabolism sometimes exists, metabolic clearance studies have been successfully carried out cocktail dosing (Hakala, K. et al., 2005). In addition to small doses, the cocktails have been limited to compounds that do not have metabolic interactions with the main cytocrome P450 enzymes (CYP) (White, R.E. and Manitpisitkul, P., 2001). Smith and coworkers also mentioned that cocktail studies should be regularly validated with different compound classes (Smith, N.F. et al., 2007).



a) Cocktail dosing b) Pooled cocktail analysis



c) Pooled analysis of samples d) Parallel analysis

Figure 4. Nomenclature of single and n-in-one methods: Biological tests are performed n-in-one in cocktail dosing (a). Analytical tests are performed n-in-one when; b) pooled analysis is carried out using cocktails, or c) pooled analysis of samples is conducted. In addition, d) parallel "single" analysis can be used.

Plasma protein binding (unbound/bound concentration ratio of a compound in blood) has an important effect on pharmacokinetics, because pharmacokinetic parameters are usually a function of the unbound fraction of a drug. Human serum albumin (HSA) is the most abundant protein in blood, and protein binding parameters can be calculated from retention factors using an immobilized HSA column. The latest method using an HSA column is comparable with older methods such as ultrafiltration and dialysis (Cheng, Y. et al., 2004). An n-in-one study demonstrated that 10 compounds can be measured using a HSA gradient run (Liu, H. et al., 2002). However, little information was provided on whether the single and n-in-one experiments correlated. It remains unclear whether possible compound-compound interactions exist in protein binding. Moreover, when the validation is not reported, a more important question is whether the results of n-in-one are method-dependent.

Smaller concentrations are favoured in cocktail dosing, because certain undesired compound-compound interactions are minimised. In the example of Caco-2 absorption studies, with low sample concentrations, three different approaches have been presented: single compound, sample pooling and cocktail dosing. In these studies the same results have been obtained with single and n-in-one methods (Bu, H.-Z. et al., 2000). A general expectation nowadays is that coexistence of compounds that are substrates or inhibitors of P-glycoprotein transporters might cause compound-compound interactions. Recently, a mixture of ten compounds, five of which were P-glycoprotein substrates, showed that one had different responses in single compared to cocktail dosing (Smalley, J. et al., 2006). Even though Smalley et al. claimed that the substrate concentration was as low as 3μM, it might be argued that this was too high.

In an article describing how early pharmacokinetic data from mice were investigated using cassette dosing, some of analyses could not be performed. Because of the limited amount of blood, the smaller dose in the cocktail and too low concentrations in blood, analysis was problematic (Watanabe, T., et al, 2006). A serious problem in comparing the results of individual and n-in-one analysis might be the lack of analytical sensitivity. With low concentrations, greater sensitivity is usually needed in analytical methods.

Ethically, cocktail dosing is at its best when it minimizes the number of test animals. As previous examples have shown, cocktail dosing is still controversial because of potential compound-compound interactions, such as the competitive inhibition of drug-metabolizing enzymes, transporter proteins or perhaps even plasma protein binding, which may lead to false negative as well as false positive results (Smith, N.F. et al., 2007). No report has clearly stated that the risks are smaller using *in vitro* tests, but at least fewer parameters then need to be validated. Moreover, *in vitro* testing using cell cultures of human origin might be beneficial compared to some animal models.

Because of the risk of compound-compound interactions, especially in *in vivo* cocktails, attempts have been made to eliminate interaction via the study design. Cocktail dosing is usually limited to small concentrations, only few compounds, and to compounds that are known to have no interactions (White, R.E. and Manitpisitkul, P., 2001). Moreover, as previously mentioned, because of the poor correlation of oral bioavailability between single and cocktail dosing (He, K. et al., 2008), the use of *intra venous* administration and control compounds in the mixtures is encouraged (Huang, R. et al., 2004). Another precaution has been recommended for the administration of cocktails. The formulation of several compounds in a single dosing solution might be challenging if they include poorly soluble substances. Several researchers have noted that solutions should be checked for compound precipitation (He, K. et al., 2008, Smith, N.F. et al., 2007).

In addition to early drug discovery, a similar approach to n-in-one has been employed in later phases. Mixtures of several compounds for different cytochrome P450 (CYP) isoforms were evaluated and presented in a research paper by Turpeinen and coauthors (Turpeinen, M. et al., 2005). Drug-drug interactions are tested using biological n-in-one. Such interactions are usually thoroughly studied before clinical trials. To achieve better success rates in drug development, some tests might also be included earlier, such as inhibition of CYP3A4 enzymes (He, K. et al., 2008).

In addition to the validation of different compound series suggested earlier, n-in-one has also been used for the method validation of Caco-2 experiments. An article by Koljonen and coworkers (Koljonen, M. et al., 2006) reported that it is beneficial to standardise the permeability experiment, because of variability between Caco-2 cell batches and potential P-glycoprotein candidates. The control compounds could be, for instance, some of the 20 compounds suggested for passive permeability classification in FDA guidelines (Waiver of *in vivo* bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system, 2000). These also include verapamile, a known marker of P-glycoprotein transport. One selection of these compounds has also been published with the quantitative liquid chromatography tandem mass spectrometry (LC/MS/MS) methods (Hakala, K. et al., 2003).

2.2.3 Pooled cocktail analysis (analytical n-in-one)

A further attempt to eliminate interaction problems is pooled cocktail analysis, in which different compounds are pooled just before analysis. In Pfizer research, three different pharmacokinetic samples of plasma or brain tissue were pooled and analysed using cocktails (Atherton, J. et al., 1999). Chromatographic separation with fluorometric detection was performed and validation gave accurate and repeatable results for all n-in-one analysis. The literature also shows that serum or plasma samples of three rats have been pooled and then analysed using liquid chromatography and ultra violet detection (LC-UV). Data for AUC curves were obtained in a time required by conventional single compound analysis (Kuo, B.-S. et al., 1998, Singh, R.P. et al., 2005).

Hsieh and co-workers pooled plasma samples two times to increase throughput (Hsieh, Y. et al., 2002). Six samples of equal time points, but different compounds, were pooled. These samples were pooled with samples from another study, making a total of 12 compounds in a single cocktail. However, small amounts of plasma might be difficult to pipette. In addition, each time the concentrations of compounds in plasma will decrease. For repeatability and sensitivity reasons, at some point, extra pooling is too much. Smaller animals, such as mice, have a limited blood volume. The use of a smaller sample volume is appreciated when the rest of the sample is needed for other studies.

2.2.4 Pooled analysis of samples (analytical n-in-one)

Metabolic stability has been estimated for compounds using *in vitro* microsome incubations and pooled analysis (Zhao, S. et al., 2005). The same compound but different time points were pooled before analysis. To accelerate the analysis, the curve values (t1/2) were calculated using fewer data points. This allowed the researchers to select sample volumes convenient for their experimental approach. In addition, a certain amount of the samples was saved for other studies, such as qualitative screening of metabolites. The n-in-one method was also compared with the single approach and correlated well.

In addition to *in vitro* metabolic research, *in vivo* pharmacokinetic studies have used plasma pooling of different time points. When plasma samples of all time points have been pooled, the concentration achieved has been equivalent to the area under curve (AUC) (Hop, C.E. et al., 1998). Furthermore, analysis has been reduced from seven data points to three, and both AUC and Cmax values could be derived with less sample analysis (Han, H.-K. et al., 2006).

2.2.5 Parallel and other analytical solutions

If experiments are performed in single vials, parallel or other analytical techniques can be used to increase the throughput, such as automated sample preparation procedures using 96-well plate formats. For example, Jenkins and co-workers reported automatic liquid handling in a well plate format and liquid chromatography - mass spectrometry (LC-MS) analysis for metabolic assays (Jenkins, K.M. et al., 2004).

The multiplexed electrospray interface (MUX) was described as analytical n-in-one method by Fang and co-workers (Fang, L. et al., 2002). In this method, eight chromatographic columns were connected to one pump and one mass spectrometer. Eight compounds or eight time points in total could be analysed at once and each run was recorded in an individual file. MUX technology with four sprayers has also been used for cytochrome P450 enzyme (CYP) inhibition studies at Schering-Plough Research (Chu, I. and Nomeir, A.A., 2006).

The principle of n-in-one can be interpreted in different ways. Sadagopan and Ohkawa with their colleagues have reported on generic method development for plasma analysis. Generic methods are ideal for the early discovery phase, because the compounds in one compound family are usually similar (Sadagopan, N. et al., 2005, Ohkawa, T. et al., 2003). Both teams have described their method as being valid for multiple analytics. Some generic methods have already been developed for mixtures in cassette dosing (Mensch, J. et al., 2007). Metabolic screening tests are also interpreted as n-in-one if several unknown metabolites are screened (Li, A.C. et al. 2007). Furthermore, biomarker screening might be seen as n-in-one analytics in drug discovery (Geoghegan, K. and Kelly, M.A., 2005) when several markers are followed in a single run.

2.3 The separation of liquid chromatography mass spectrometry

2.3.1 Definitions for specificity, sensitivity and selectivity

The terms specificity, sensitivity and selectivity are commonly used in diagnostic tests and in the validation of drug analysis. Here, three definitions presented in the guidelines of the Food and Drug Administration (FDA) are quoted; the first is also included in the guidelines of the International Conference on Harmonisation (ICH) on technical requirements for the registration of pharmaceuticals for human use.

The following definition is presented in the FDA documents Guidance for Industry, Validation of Analytical Procedures: Definition and Terminology, Guidance for Industry, Text on Validation of Analytical Procedures, ICH-Q2A, in addition to the ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2 (R1):

"***Specificity*** is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.
Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).
This definition has the following implications:
Identification: to ensure the identity of an analyte.
Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.
Assay (content or potency): to provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample."

Guidance for Industry and FDA Staff, Statistical Guidance on Reporting Results from Studies Evaluating Diagnostic Tests states:

"In studies of diagnostic accuracy, the ***sensitivity*** of the new test is estimated as the proportion of subjects with the target condition in whom the test is positive. Similarly, the ***specificity*** of the test is estimated as the proportion of subjects without the target condition in whom the test is negative (see the Appendix for an example of this calculation)."

Guidance for Industry, Bioanalytical Method Validation presents:

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

These are also the definitions used in the following, even though the analytical applications are different. The reasons why liquid chromatography mass spectrometry (LC-MS) methods can be seen as sensitive and selective for early drug discovery analysis are exemplified in next chapters. Mass spectrometry was the selected instrument for the analysis in this thesis, because it is more specific than many other detectors.

2.3.2 Mass spectrometric separation

Liquid chromatography – tandem mass spectrometry (LC-MS/MS) using triple quadrupole (QqQ) instruments provides high sensitivity and selectivity for n-in-one samples. The mass spectrometric separation involves mass-to-charge ratio (m/z) analysis, fragmentation and aspect of ionisation. The following examples are for triple quadrupole instruments, which were used in the experimental studies of this thesis.

The flow from liquid chromatography (LC) is introduced to a mass spectrometer using interfaces. The two most common interfaces used with LC are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). Although a universal detector is what is wanted, ionisation of mass spectrometry causes limitations. The negative ion mode is usually better utilised for acidic compounds and the positive ion mode for basic compounds. ESI, where ion formation is also in the liquid phase, is more compatible for ionic molecules and polar compounds (containing heteroatom, acid and basic organic compounds). APCI, where ions are formed mostly in the gaseous phase, is more often used with small, moderately polar or nonpolar compounds. Thermally labile compounds cannot be detected by APCI. Atmospheric pressure photo ionisation (APPI) is a new technique that is suitable for also for some neutral non-polar compounds. Gas phase ionisation involves the transfer or abstraction of protons, adduct attachments, charge exchange and electronic capture reactions. In ESI ionisation, which is softer ionisation than APCI, adducts and cluster ions are also more often formed. Ionisation energy and proton affinity provide better understanding of ionisation efficiency in the LC-MS methods. Usually, these differences are well understood by the method developer. Ionisation with ESI, APCI and APPI interfaces are discussed in detail in a new review by Kostiainen and Kauppila (Kostiainen, R. and Kauppila, T., 2009).

The ionisation efficiency depends on how well ions are formed. Non-volatile buffers and matrix compounds (such as salts, proteins and other analytes) might enhance or suppress the ionisation and even disturb the analysis (Taylor, P.J., 2005). Buffers commonly used in liquid chromatography may strongly suppress ionisation in ESI, leading to significantly decreased sensitivity. APCI tolerates much higher buffer concentrations than ESI ionisation (Schuhmacher, J. et al., 2003). The three-dimensional structure of the ionisation probe and the electronics also influence the matrix effects in mass spectrometry (Mei, H. et al., 2003). If the matrix suppression is intense, sample pre-treatment before MS analysis should considered. Sample cleaning and separation by liquid chromatography is introduced in next chapter, while other sample pre-treatments, which depends on the sample matrix, are not discussed here.

A mass spectrometer distinguishes compounds according to their mass per charge ratios (m/z). Only isobaric compounds have the same exact mass, which is mainly problematic with some peptides. A single quadrupole has one quadrupole mass selector in which the characteristic ions of a molecule are detected. Triple quadrupoles (QqQ) use two quadrupole mass selectors, and between them there is a quadrupole chamber for collision-induced dissociation (CID). Isobaric compounds with different fragmentation patterns can also be separated in QqQ.

Triple quadrupole (QqQ) instruments can be operated in different modes (Figure 5):

a) Positive or negative ions of introduced compounds can be detected as a mass spectrum. The molecule ion of a compound, $[M-H]^-$ or $[M-H]^+$, is important for compound recognition. By directly introducing a complex mixture and analysing ions characteristic of a compound, it is possible without any other separation method to determine the presence of the compound.

This is documented in the combinatorial library screening, where the use of the molecular weight with isotopic pattern information gave tools to manage huge amount of identification, purity and synthetic yield information gathered. Yates and co-workers have used estimated and analyzed peak intensities as a prioritising tool and the best synthesised libraries were possible to screen first (Yates, N. et al., 2001).

However, it is important to understand that exact mass measurements and the identification of elemental compositions are mainly performed using magnetic sector instruments, fourier transform ion cyclotron resonance mass spectrometers and time of flight (TOF) instruments, and not with quadrupole instruments. Triple quadrupole mass spectrometers are commonly integrated with liquid chromatography and employed as detectors. The extracted ion chromatograms of mass spectra are able to show profiles of different ions, even for co-eluting peaks.

b) Mass fragmentation can be studied using a product ion scan, also known as a daughter ion scan. The selected molecular ion gives fragments during collision and all product ions are detected. This is usually used for qualitative analysis, in which it provides structural information.
The usual molecular ions in the ionisations of electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) have an even electron structure, and they are more stable than odd electron ions. Therefore, before fragmentation, molecular ions often form the biggest peaks in the spectra, which is advantageous. Characteristic isotope patterns may also be used in interpreting the spectrum of an unknown compound. Typical fragmentation reactions include single-bond cleavages, fragmentations with hydrogen rearrangements and skeletal reorganization with the extrusion of stable neutrals, although hydrogen rearrangement reactions usually need specific geometries to appear (Williams, D.H.; and Fleming, I., 1989).

Structural information from the product ion scan depends not only on the chemical structure, but also on the collision energy and multivariate physicochemical properties, for example charge localization and stabilisation of the compound. The collision-induced dissociation (CID) of gas phase ions is an energy-selected system; statistically, the weakest bond splits first. *Ab initio* calculations have been used to estimate the energy differences, as reported by Armentrout and Baer, as well as Lifshitz (Armentrout, P.B. and Baer, T., 1996, Lifshitz, C., 2001). In these calculations, the energies, structures and vibrational frequencies of stable and transient species are used in virtual estimation together with fragmentation data.

c) A precursor ion scan, also known as a parent ion scan, analyses all the ions that produce the wanted fragment ion (m/z) in collision.

d) A neutral loss scan selects all ions whose fragmentation gives the wanted neutral molecule loss in collision, i.e. the neutral fragment. Precursor and neutral loss modes are both used to detect compound classes. These modes are used for screening phase 1 metabolites (Li, A.C. et al. 2007 and Xu, X. et al. 2004) and conjugative phase 2 metabolites (e.g. glucuronides) (Liu, D.Q. et al. 2002). Glutathione conjugate screening is important in finding possible reactive metabolites (Shuguang, M. and Subramanian, R., 2006). These modes also give a signal for some compound classes, such as acylcarnitines, in complex combinatorial chemistry mixtures (McClellan, J.E. et al., 2002).

e) In monitoring selective reactions, the wanted molecular ion fragments during collision and the selected ion are detected. Several reactions are quite regularly monitored simultaneously after each other. Selective reaction monitoring (SRM) is mainly used in quantitative research. Quantitation with triple quadrupole instruments has usually increased sensitivity compared to single quadrupole instruments, because the signal/noise ratio is improved.

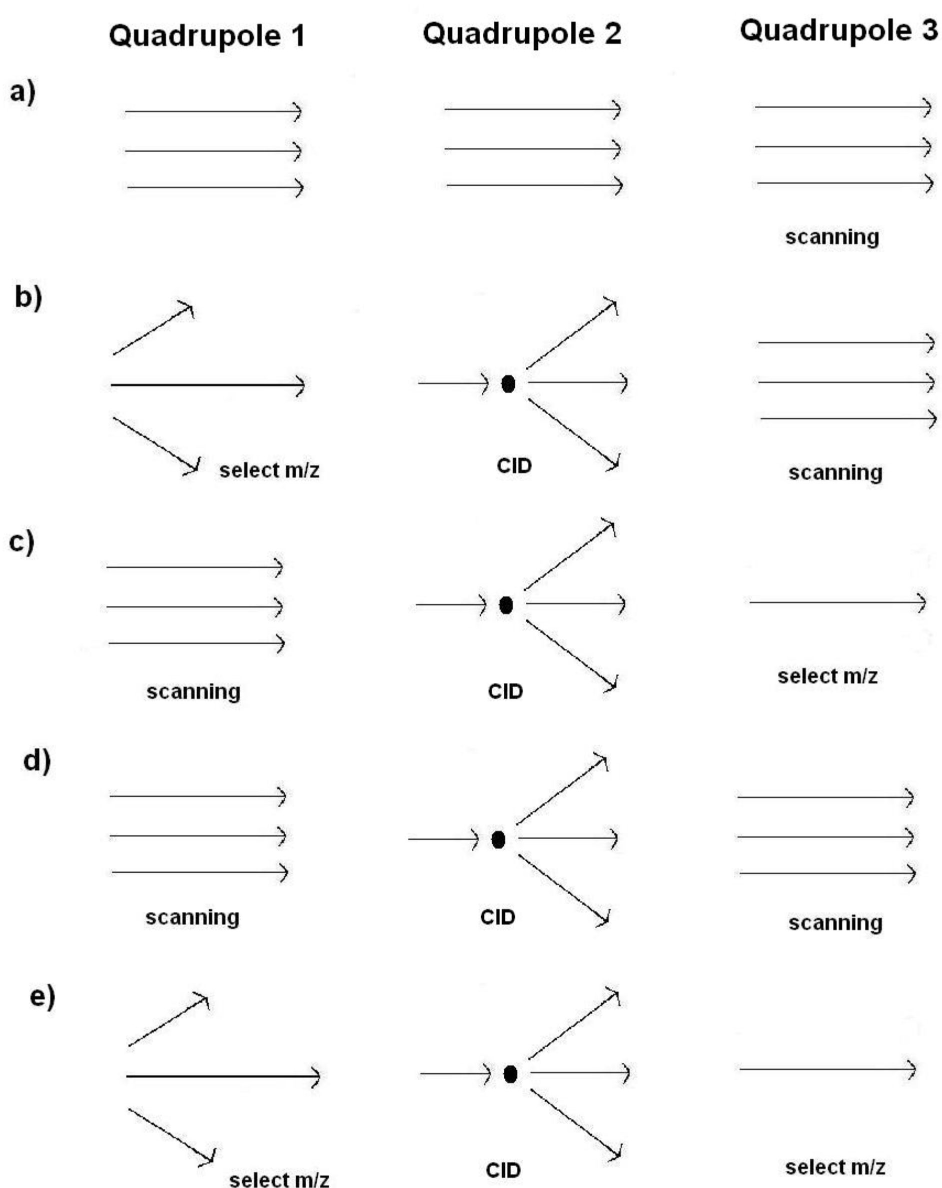


Figure 5. Different scanning modes using a triple quadrupole mass spectrometer: a) mass spectrum, b) product ion scan, c) precursor ion scan, d) neutral loss scan and e) selected reaction monitoring; (CID = collision-induced dissociation)

2.3.3 Liquid chromatography methods in early studies

Analytes in a complex mixture can be isolated before detection using chromatographic separation. The isolation of compound increases both the sensitivity and selectivity. This is especially important for mass spectrometric analysis because chromatographic separation can, among other effects, limit signal suppression caused by the matrix effect. Chromatography is based on retention and resolution (k and α). Even though basis of the separation lies in chromatographic columns and different stationary phases, this chapter presents practical aspects of method development other than solid phase chemistry. Unger et al. explain some common chromatographic parameters in their review (Unger, K. et al., 2008), and these are presented in Table 3. These equations are a simple and straightforward for improving analytical methods. Programs for method development use parameters in their simulation of a chromatographic run.

During 30 years (from 1970 to 2000) the particle sizes of columns have dramatically diminished from 30 µm to less than 2 µm. A smaller particle size usually gives better resolving power (Rs) by affecting the column plate number (N). Raising the flow-rate (u) gives more rapid analysis. Unfortunately, with smaller particle columns (d_p), the consequently higher back pressure (Δp) might be a problem. For high pressures the ultra pressure liquid chromatography (UPLC) has been developed. All new methods developed with UPLC instruments have been published, including those in the ADME area (Mensch, J. et al., 2007, Pedgraglio, S. et al., 2007, Plumb, R.S. et al., 2008).

Table 3. Basic chromatographic parameters and their equations, as presented here, can help in method development.

Retention factor (k): $k = (t_r - t_0)/t_0$, tr = retention time, t0 = void volume time
Resolution (Rs): $Rs = (1/4)\ (\alpha-1)\ (N^{1/2} k) / (1+k)$; k = retention factor, N = column plate number, α = separation factor (k_2/k_1)
Kinetic properties and peak dispersion (H and N): H = theoretical plate height, N = number of theoretical plates
Hydrodynamic properties such as pressure change (Δp): $\Delta p = \Phi \eta L u / d_p^2$; Φ = column resistance factor, η = viscosity of the eluent, L = column length, u = linear velocity of eluent, d_p = average particle diameter of the packing material

A shorter column (L) gives a smaller pressure. Therefore, small cartridge columns have been used for Caco-2 permeability samples to provide rapid sample pre-treatment before MS detection (Bu, H.Z. et al., 2000). This method cleaned the sample matrix, but the compounds were only separated by mass spectrometry and not using chromatography. In addition to short columns, lower viscosities (η) of the mobile phase have also been used to lower the pressure. Furthermore, higher column

temperatures, when effective, are used to change the column pressure. Even if columns with different dimensions and materials can be selected, Zhao and co-workers admitted in their article that solvent composition and column temperature modifications are usually decided on method development for practical reasons (Zhao, J. and Can, P.W., 1999). This depends on how much time is available for method development.

The main streams in the development of chromatographic columns during the last 50 years have involved two types of sorbent materials: packed particle beds and monolith structures. In addition, nonporous particle columns with porous surface have been developed and used as Halo columns (Marchetti, N . et al., 2007). Monolithic columns are porous rods (Unger, K.K. et al., 2008). A similar hydrodynamic equation to that for particle columns is invalid for monolith columns, but faster separation can be achieved at low pressures. Monolith columns are important for high flow rate analysis, as mentioned in P450 inhibition screening assays (Peng, S.X. et al., 2003).

Small particle columns with mass spectrometry gives better sensitivity compared to traditional detectors when only a small sample amount is available (Plumb, R.S. et al., 2008). The basic chromatographic equations, which are valid for particle columns, can be used especially for up- or downscaling of methods, and this is an advantage for rapid method development.

2.3.4 Mass spectrometry compared to other detectors

In characterising synthetic compounds, as combinatorial libraries, mass spectrometry (MS) is used with other detectors. Comparable results are achieved by evaporative light scattering (ELSD), chemiluminescent nitrogen (CLND), corona charged aerosol (CAD, charged aerosol detection) or nuclear magnetic resonance detectors (NMR) (Yurek, D.A. et al., 2002, Zhang B. et al., 2008). If no pure analytical standards are present, the capability of CLND to quantify the molar amount of compounds containing nitrogen is beneficial. NMR detectors, which give elevated qualitative information, have the disadvantage of high expense. ELSD can also be used, although the nebulization has to be optimised and the temperature should be below the melting point of the detected compounds. CAD is newer, more sensitive compared to ELSD and a competitor for traditional ultraviolet (UV) detectors. In a combination of ultraviolet (UV) and MS, product identification is achieved by MS and the relative purity of products is determined by UV (Fang, L. et al., 2002). Sometime, UV detection gives better linearity in the high concentration range, but products and side-products often also possess equal absorption. Letot and co-workers criticised that results for the total amount of a compound might be inexact if the compound elutes in the void volume of the column and is excluded from UV or MS data (Letot, E. et al., 2005). However, this problem is quite common for all methods using chromatographic columns.

In quantitative analysis, ultra violet (UV) or fluorescence detection methods give good analytical parameters such as linearity, accuracy, precision and repeatability. A problem with UV is its poor sensitivity, and sometimes also the poor selectivity. Cocktail analysis easily increases the limit of detection when the total sample volume is fixed. Moreover, small substrate concentrations are common in the cocktails, because of the risk of compound-compound interactions. Rajanikanth and co-workers noted that signals might overlap and metabolites of compounds might interfere in both fluorescence and UV methods, especially *in vivo* cocktail dosing studies (Rajanikanth, M. et al., 2001). Some methods use both mass spectrometric and UV/fluorescence detection. In a Caco-2 study, the fluorescence and mass spectrometry (MS) methods had 5-10 times better sensitivity compared to UV (Bu, H-Z. et al., 2000). Different detector sensitivities were employed in study by Palmgren and coworkers, where UV and fluorescence were combined. In the analysis of Caco-2

samples, fluorescence detection was used for low concentrations and UV detection for higher concentrations (Palmgren, J.J et al., 2004). However, the lack of fluorescence is a limitation and restricts its use. In mixture pharmacokinetics, with cocktail dosing or plasma pooling, the possibility of interference from drug metabolites increases. More selective method, as mass spectrometry, is an advantage, even though ion suppression of the matrix and unknown metabolites has to be kept in mind (Sadagopan, N. et al., 2005, Ohkawa, T. et al., 2003).

The use of mass spectrometry (MS) compared to ultra violet (UV) detection has increased in the past few years. As described earlier, the parallel artificial membrane permeability assay (PAMPA) has grown in popularity over Caco-2 absorption samples because of its easier implementation in high throughput analysis, as also reported by Praveen et al. (Praveen, V. et al., 2006). Even though Hämäläinen and coworkers noted that UV readers are fast, they still may have limited sensitivity, and components that are unstable may give unspecific results (Hämäläinen, M.D. et al., 2004). When PAMPA permeability studies were analysed with UV, Mench and coworkers also had difficulty in determining the tested compounds, mainly because of poor solubility or the low UV response. When they changed to an automated LC-MS instrument, the capability to estimate PAMPA coefficients rose by over 50% (Mensch, J. et al., 2007). Several other mixture analyses using mass spectrometry have been developed for metabolism studies, as for the UDP-glucuronosyl transferase (UGT) isoenzyme with cocktails (Hakala, K. al., 2005).

3. Aims of the studies

New drug discovery technologies, such as analysis using n-in-one, require proper planning and appropriate analytical methods. The aims of the studies reported here were to investigate ways of using n-in-one measurement, mixing and pooling of samples to give better sample throughput and to describe analytical solutions in n-in-one. The compounds in the studies were test compounds: a component from a synthesised combinatorial library or a mixture of commercial drug molecules. Selected compounds for lipophilicity and absorption screening tests included acids and bases, and they were small organic drugs. Three separate mass spectrometric studies were designed to give good examples of new methods in the field of n-in-one and drug discovery analytics: combinatorial chemistry libraries, lipophilicity and absorption screening.

The more specific aims of the research (A-C) were

A. To characterise mass spectrometric fragmentation patterns for ten nitrophenoxy benzoate compounds made by combinatorial chemistry synthesis, to study the influence of nitro substituents and alkyl chain length on collision-induced fragmentation and to demonstrate the precursor ion scan spectra of compounds. Compounds in the study were individually synthesised but pooled before the last library screening analysis.

B. To compare immobilized artificial membrane (IAM) chromatography retention factor k (log k_{IAM} values) using phosphate buffer and ammonium acetate buffer with ultraviolet (UV) detection and single compound analysis. Furthermore, to develop IAM chromatography with mass spectrometric (MS) detection for nine compounds, and to compare IAM chromatography retention factor k (log k_{IAM} -values) of single compound analysis and UV with n-in-one analysis and MS using atmospheric pressure chemical ionization (APCI). In addition, to demonstrate fast lipophilicity screening using MS, n-in-one and a gradient run of nine small organic drugs, including both acidic and basic compounds.

C. To develop a rapid and sensitive liquid chromatographic method with mass spectrometric (SRM) detection for ten small organic drugs, including both acidic and basic compounds, using electrospray ionization (ESI) for Caco-2 analysis.

4. Experimental

4.1 Chemicals and reagents

Nitrophenoxy bentzoates were synthesized by the synthetic group of the Division of Pharmaceutical Chemistry. The general procedure for the synthesis of the compounds was the following: The alkyl 3,5-dihydroxybenzoates needed for the subsequent etherification procedure were prepared by acid-catalysed etherification of commercially available 3,5-dihydroxy benzoic acid. Reaction with p-dinitrobenzene or o,p-dinitrobenzenechloride in the presence of potassium carbonate in acetone produced the corresponding alkyl 3-hydroxy-5-(4'-nitrophenoxy) benzoates or alkyl 3-hydroxy-5-(2',4'-dinitrophenoxy) benzoates, respectively (Figure 6) (Bird, C.W. and Latif, M., 1980). A full interpretation of the ^{1}H and ^{13}C NMR shifts and coupling constants of spectra recorded on a Varian Fourier transform NMR spectrometer at 500 and 125 MHz revealed that the target compounds had been successfully synthesised in total yields of 27 - 54%.

Compounds used in immobilized artificial membrane (IAM) and Caco-2 studies were purchased. The structures of studied compounds are presented in Figure 7 and the chemicals and reagents are listed in Tables 4 and 5.

Compound	R_1	R_2
A1	CH_3	H
A2	C_2H_5	H
A3	C_3H_7	H
A4	C_4H_9	H
A5	C_5H_{11}	H
B1	CH_3	NO_2
B2	C_2H_5	NO_2
B3	C_3H_7	NO_2
B4	C_4H_9	NO_2
B5	C_5H_{11}	NO_2

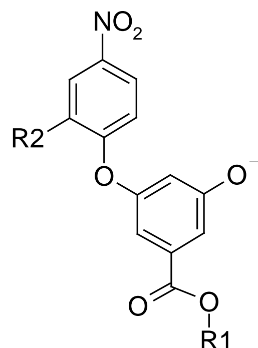


Figure 6. Structures of nitrophenoxy benzoate compounds studied in publication A.

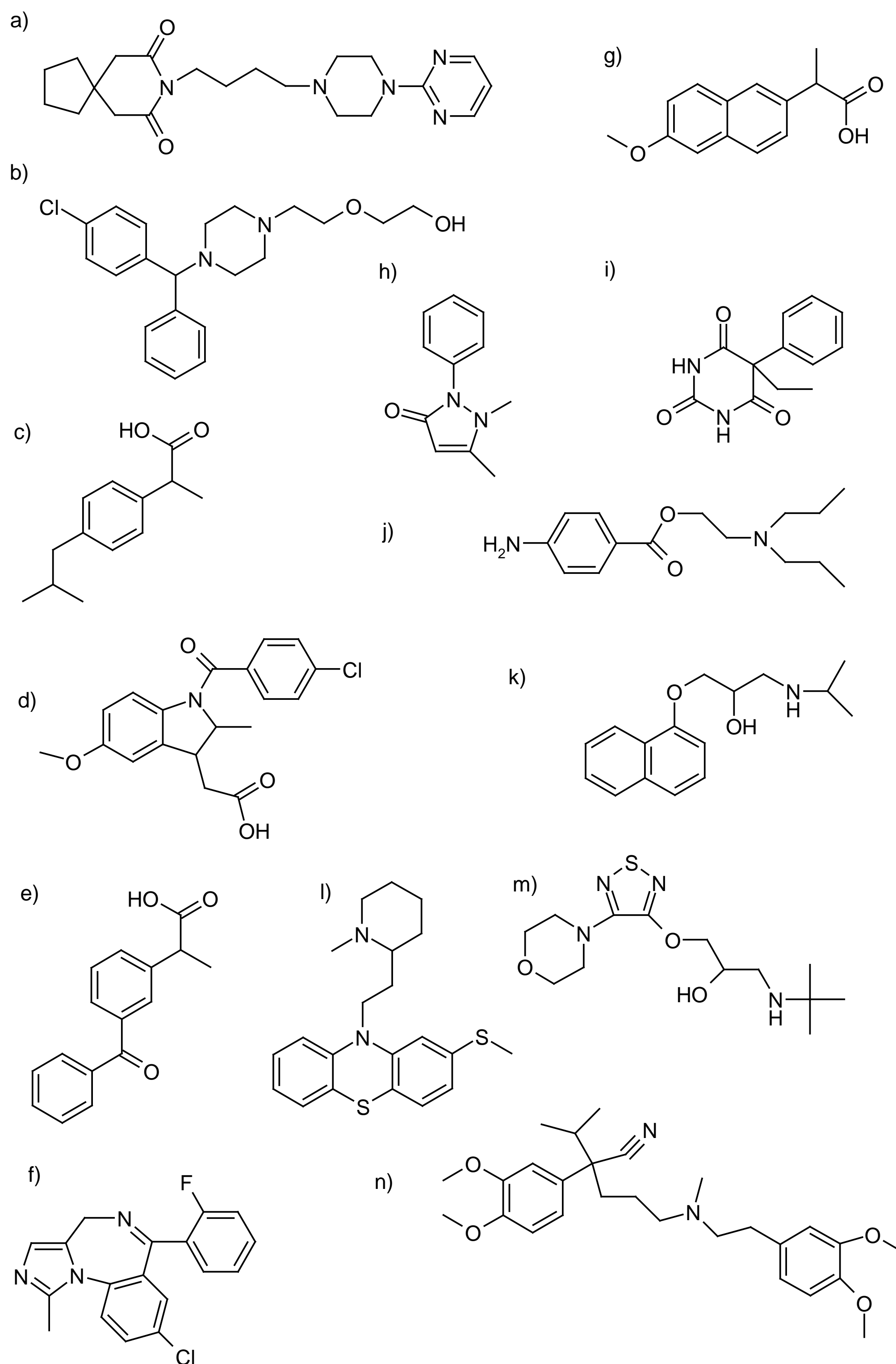


Figure 7. Compounds and their structures studied in publications B and C were the following:
a) buspirone, b) hydroxyzine, c) ibuprofen, d) indomethacin, e) ketoprofen, f) midazolam, g) naproxen, h) phenazon, i) phenobarbital, j) procaine, k) propranolol, l) thioridazine, m) timolol and n) verapamil

Table 4. Compounds studied in immobilized artificial membrane (IAM) chromatography and in the Caco-2 study, publications B and C. The negative logarithm of the dissociation constant Ka (pKa), HA for acids and HB+ for bases, is also presented (Lemke, T. et al. 2008).

Compound	pKa	Manufacturer/Purchased	Publication
Buspirone	–	Sigma Chemical Co, St Louis, MO, USA	C
Hydroxyzine	2.0 (HB+)	Sigma Chemical Co, St Louis, MO, USA	C
Ibuprofen	5.2 (HA)	University Pharmacy, Helsinki, Finland	B
Indomethacin	4.5 (HA)	Orion Oyj, Espoo, Finland	B
Ketoprofen	4.8 (HA)	Medifon, Helsinki, Finland	B
Ketoprofen	(same)	ICN Biomedical Inc., Aurora, OH, USA	C
Midazolam	6.2 (HB+)	Hoffman-la Roche, Basle, Switzerland	B, C
Naproxen	4.2 (HA)	ICN Biomedical Inc., Aurora, OH, USA	C
Phenazon (Antipyrine)	1.5 (HB+)	Aldrich Chemical Company Inc, Milwaukee, WI, USA	C
Phenobarbital	7.4 (HA)	University Pharmacy, Helsinki, Finland	B
Procaine hydrochloride	6.8 (HB+)	University Pharmacy, Helsinki, Finland	B, C
Propranolol hydrochloride	10.4 (HB+)	Orion Oyj, Espoo, Finland	B
Propranolol hydrochloride	(same)	ICN Biomedical Inc., Aurora, OH, USA	C
Thioridazine hydrochloride	9.5 (HB+)	Orion Oyj, Espoo, Finland	B
Timolol maleate	8.8 (HB+)	University Pharmacy, Helsinki, Finland	B
Timolol maleate	(same)	Orion Oyj, Espoo, Finland	C
Verapamil hydrochloride	3.5 (HB+)	ICN Biomedical Inc., Aurora, OH, USA	C

Table 5. The list of reagents used: All organic solvents and other reagents were of analytical or chromatographic grade.

Reagents	**Manufacturer**	**Publication**
Milli-Q water from water purification system	Millipore, Molsheim, France	A, B, C
methanol	Rathburn, Walkerburn, UK	A
acetonitrile	Rathburn, Walkerburn, Scotland	B, C
ammonium hydroxide	Merck, Darmstadt, Germany	A, B
potassium dihydrogen phosphate	Merck, Darmstadt, Germany	B
disodium hydrogen phosphate dihydrate	Merck, Darmstadt, Germany	B
phosphoric acid	Merck, Darmstadt, Germany	B
potassium chloride	Merck, Darmstadt, Germany	B
sodium chloride	Merck, Darmstadt, Germany	B
ammonium acetate	Merck, Darmstadt, Germany	B, C
sodium nitrite	Riedel-de Haën, Seelze, Germany	B
formic acid	Merck, Darmstadt, Germany	C
Dulbecco's phosphate-buffered saline	Gibco Invitrogen Corp., Life Technologies Ltd. Paisley, Scotland	C
Hank's balanced salt solution (HBSS)	Gibco Invitrogen Corp., Life Technologies Ltd. Paisley, Scotland	C
HEPES solution (10mM)	Gibco Invitrogen Corp., Life Technologies Ltd. Paisley, Scotland	C

4.2 Instruments and methods

The methods were flow injection (FIA) and liquid chromatography (LC) analysis using ultra violet (UV) or mass spectrometric (MS) detection. The instruments are listed in Table 6. The methods used are briefly described here with Tables 7-10. More detailed descriptions of the instrumental methods can be found in the original publications (A-C).

Table 6. List of the instruments used for flow injection analysis (FIA), liquid chromatography (LC), ultra violet (UV) and mass spectrometric (MS) detection.

Method	Instrument	Manufacturer	Publication
FIA	Micro syringe pump	Harvard Apparatus, USA	A
	Rheodyne 7725 manual injector	Cotati, Ca, USA	A
LC	Liquid chromatograph, HP/Agilent 1100 series	Hewlett-Packard GmbH, Waldbronn, Germany	B, C
UV	Ultraviolet detector, HP/Agilent	Hewlett-Packard GmbH, Waldbronn, Germany	B
MS	Sciex API300, mass spectrometer	Sciex, Toronto, Canada	A, B
	Sciex API3000, mass spectrometer	Sciex, Toronto, Canada	C
	ESI (pneumatically assisted electrospray) ionisation probe	Sciex, Toronto, Canada	A, C
	APCI (atmospheric pressure chemical ionisation) probe	Sciex, Toronto, Canada	B

4.2.1 Nitrophenoxy benzoate study

FIA-MS

A sample of concentration 48 - 61 nmol/mL was introduced using FIA with a flow rate of 5 µl/min (Table 7). Higher orifice voltages (50-120 V) were used in the fragment ion MS/MS experiments in order to examine fragmentation pathways. The scan range was m/z 170-500 (3.1 sec/scan, dwell time 1.0 ms) for the mass spectrum and m/z 30-500 (3.1 sec/scan, dwell time 0.65 ms) for the product ion scan. The spectra were recorded by accumulating 10 spectra.

Table 7. Experimental parameters for flow injection analysis and mass spectrometry (FIA-MS) in nitrophenoxy benzoate study, article A.

FIA-MS	
Sample introduction:	Sample concentration 48 - 61 nmol/mL
	Flow rate 5µl/min
	Sample loop 10 µl
	Solvent water: methanol (50:50), pH 11.6, adjusted by ammonium hydroxide
Mass spectrometric parameter:	Capillary voltage 5000 V
	Orifice voltage 30 V, (50-120 V in high orifice voltage studies)
	Collision energy 25 V
	Nebulisen gas: Synthetic air (80% N_2, 20% O_2)
	Curtain and Collision gas: Nitrogen

4.2.2 IAM study

UV and MS

The ultraviolet (UV) detection method used the individual wavelength λ_{max} presented in Tables 8 and 9. Mass spectra of the analytes were recorded by directly injecting 100 µl (100 µM) of the individual compound solutions into the mass spectrometer from the liquid chromatography equipment. The mass spectrum was measured in the range m/z 150–550 (0.4 s/scan). Selected ion chromatograms in the experiments determining log k_{IAM} values were performed by monitoring the intensities of protonated or deprotonated molecules of the analytes (Table 8). The final LC-UV or MS methods are summarised in Table 9.

Table 8. Ultraviolet detection wavelength λ_{max} and protonated (positive) or deprotonated (negative) ions in the mass spectrum of the compounds in the immobilised artificial membrane (IAM) chromatography study.

	Compound	UV λ_{max}	Positive or negative ions in mass spectrum
I	Ibuprofen	220 nm	205 m/z (neg)
II	Indomethacin	275 nm	356 m/z (neg)
III	Ketoprofen	260 nm	253 m/z (neg)
IV	Midazolam	215 nm	326 m/z (pos)
V	Phenobarbital	235 nm	231 m/z (neg)
VI	Procaine	230 nm	237 m/z (pos)
VII	Propranolol	288 nm	260 m/z (pos)
VIII	Thioridazine	262 nm	371 m/z (pos)
IX	Timolol	295 nm	317 m/z (pos)

Table 9. Experimental parameters for immobilised artificial membrane (IAM) chromatography using ultraviolet or mass spectrometry detection in publication B.

IAM UV or MS	
Chromatographic conditions:	IAM.PC.DD2, 30 x 4.6 mm, (12 μm), Regis Technologies Inc., Morton Grove, IL, USA
	Column temperature 30 °C
	Flow rate 1 ml/min
Ultraviolet detection:	UV detection wavelength was individual compound (λ_{max})
Mass spectrometric parameter:	Mass spectrum recording
	Dwell time: 0.1 ms
	Charge needle current 3 μA
	Probe temperature: 450 ºC
	Orifice voltage: 15 V
	Collision energy: 25 V
	Nebulizer gas: Synthetic air (80% N_2, 20% O_2)
	Curtain gas: Nitrogen

LC: Two different buffers

Two different buffers were used in IAM using an ultraviolet detector:

a) 10 mM phosphate buffer with 0.2 g/l of potassium chloride and 8 g/l of sodium chloride and pH adjusted to 7.4 with phosphoric acid, and

b) 10 mM ammonium acetate buffer with pH adjusted to 7.4 with ammonium hydroxide.

All mass spectrometric IAM experiments were performed with 10 mM ammonium acetate buffer (pH 7.4).

LC: Isocratic and gradient elution

Isocratic experiments were performed without acetonitrile and with four different amounts of acetonitrile (10, 20, 30 and 35%) in the mobile phase. Acetonitrile was used for the elution of lipophilic compounds (indomethacin, midazolam, propranolol and thioridazine), which require an organic modifier if they are to be eluted within a reasonable time (30 min). Sample solutions for these experiments were prepared in 10% acetonitrile and 90% aqueous buffer with pH 7.4 and contained one analyte each (concentration 0.5 or 1 mM).

A fast gradient was also used in the mass spectrometric study. The gradient program was identical to that described by Valko et al. (Valko, K. et al., 2000), that is: 0–1.5 min, 0% acetonitrile; 1.5–10.5 min, 0–100% acetonitrile; 10.5–11.5 min, 100% acetonitrile; 11.5–12.0 min, 100–0% acetonitrile; 12–20 min, 0% acetonitrile. The mixture of all the analytes (concentration of each analyte 100 μM) to be analysed in the IAM–APCI/MS experiments was prepared in 10% acetonitrile and 90% ammonium acetate buffer (pH 7.4).

Calculation of the retention factor

Retention factors, k, for the five different isocratic mobile phase compositions (0, 10, 20, 30 and 35% acetonitrile) were calculated according to the equation:

$$k = (t_r - t_0)/t_0.$$

The t_0 value (void time of the column) was measured by injection of sodium nitrite. The log k_{IAM} values (log k_w, theoretically corresponds to 0% acetonitrile) of the lipophilic compounds (indomethacin, midazolam, propranolol and thioridazine) were obtained from the linear plots of log k (y) vs. per cent of acetonitrile (x) by extrapolating to 0% acetonitrile.

4.2.3 Caco-2 study

Study samples

The Caco-2 cells (ATCC) were cultured according to Andersberg and co-workers (Andersberg, E.K. et al., 1992) on polycarbonate filters of 0.4 µm pore size on 12-well plates (Corning Costar Corp., Cambridge, MA) at a seeding density of 75 000 cell/cm^2. Cells were used in experiments between passages 29-30. Apical-to-basolateral (AP-BL) and basolateral-to-apical (BL-AP) transport of the studied drugs was followed over 120 minutes (15 min intervals). The pH of the apical transport medium (HBSS) was 5.5 and that of the basolateral medium was 7.4.

LC-MS/MS

A standard stock mixture containing 0.1 mg/ml of each study compound was diluted to an appropriate concentration with Hank’s balanced salt solution (HBSS) at pH 5.5 or 7.4. Caco-2 analysis was performed with a liquid chromatography mass spectrometry system using pneumatically assisted electrospray ionisation (ESI). The liquid chromatographic set-up consisted of a binary pump, a column switching system and an autosampler (Table 10). The gradient program was 0.1 min–1.5 min, 10% acetonitrile; 1.5–6.5 min, 10–100% acetonitrile; 6.5–10 min, 100% acetonitrile; 10.0–11.0 min, 100–10% acetonitrile; 11–20 min, 10% acetonitrile. The eluent was introduced to waste for 1.5 min, after which the flow was directed by column switching to mass spectrometry.

Table 10. Experimental parameters for liquid chromatography and mass spectrometry for Caco-2 samples, article C.

LC-MS/MS in Caco-2	
Chromatographic conditions:	Purospher STAR® C_{18} 5.5 x 2mm (3 µm) -column, Merck, Darmstadt, Germany
	Mobile phase: 15 mM ammonium acetate pH 3.5 : acetonitrile gradient
	Column temperature: +30 °C.
	The injection volume: 50 µl
	Flow rate: 350 µl/min
Column switching:	Flow directed to waste: 0-1.5 min
Sample filtration:	Millex HV (0.45µm) polyvinylidene difluoride (PVDF) membrane filtration, Millipore Corp., Bedford, USA
Sample dilution:	With HBSS buffer (if needed)
Splitter:	Flow was split with the ratio 1:6
Mass spectrometric parameter:	Selected reaction monitoring (SRM)
	Dwell time: 70 ms /each reaction
	Ionspray voltage: 5500 V
	Declustering/orifice potential, collisional energy and collision cell exit potential were individually optimised.
	Nebulizing, curtain and collision gas: nitrogen

5. Results and discussion

5.1 Nitrophenoxy benzoate study

5.1.1 Fragmentation

In routine analytical work, as Rivier and Pavlic with co-workers describe, standardised mass spectra libraries are a tool for the automatic characterisation of compounds (Rivier, L. et al., 2003, Pavlic, M. et al., 2006). With high resolution instruments, where standard compounds are absent, the accurate mass, nitrogen rules and mass detect filter rules assist (Tiller, P.R. et al., 2008). When the given instrument does not offer high mass accuracy, information on fragmentation is needed. Most of all, supporting information is required when the presence of the targeted compounds in combinatorial libraries is determined.

The fragmentation of ten nitrophenoxy bentzoate compounds was investigated here. Several earlier studies have described positive (Benoit, F. and Holmes, J.L, 1970, Schwarz, H., 1974, Martens, J. et al., 1975, Brophy, J.J. et al., 1979, Zitrin, S. and Yinon, J., 1976, Brown, P., 1970) and negative (Brown, C.L. and Weber, W.P., 1970, Bowie, J.H., 1971, Bowie, J.H. and Nussey, B., 1972, Bowie, J.H. and Nussey, B, 1974, Bowie, J.H. and Ho, A.C., 1974, Bowie, J.H. and Stapleton, B.J., 1975, Wilson, J.C. and Bowie, J.H., 1975, Bowie, J.H., 1978, Bowie, J.H., 1974) ion spectra for different types of nitroaryl compounds. In these studies the fragmentation from $M^{-\cdot}$ and $M^{+\cdot}$ ions has been well characterised. However, the fragmentation of odd-electron molecular ions produced by electron impact differs from that of even-electron deprotonated molecules produced by ESI.

The only ion in the negative ion ESI/MS spectra of alkyl 3-hydroxy-5-(4'-nitrophenyl) benzoates (A1-A5) and alkyl 3-hydroxy-5-(2',4'-nitrophenyl) benzoates (B1-B5) is $[M-H]^-$. The most probable deprotonation site is the phenolic hydroxy group. The product ion spectra of the $[M-H]^-$ ions of A1-A5 are alike, as are those of B1-B5. However, the spectra of the A series compounds differ significantly from those of the B series compounds (Tables 11 and 12). The fragmentation is clearly more abundant with compounds B1-B5 than with A1-A5, indicating the increased instability of the $[M-H]^-$ ion in compounds B1-B5 due to the presence of a second electron-withdrawing nitro substituent. The relative abundance of $[M-H]^-$ increases and the amount of fragmentation decreases with an increase in the length of the alkyl chain from methyl to pentyl: the energy transferred in the collision to the internal energy of the precursor ion decreases as the alkyl chain length increases, because the number of degrees of freedom in the molecule increases.

The product ion spectra of $[M-H]^-$ of both A1-A5 and B1-B5 show an abundant nitrophenoxy ion, at m/z 138 and m/z 183, respectively. This is formed by the loss of 3-hydroxybenzoate (Schemes 1 and 2). The extensive charge delocalization and stabilisation explain the high abundance. The relative abundance of the ion is significantly decreased with an increasing length of the alkyl chain, since the internal energy is distributed over a larger number of bonds and less energy is focused on the ether bond.

Table 11. Product ion spectra of $[M-H]^-$ of alkyl 3-hydroxy-5-(4'-nitrophenoxy)benzoates A1-A5

	Compounds				
Ion, m/z (rel. abund. %)	A1	A2	A3	A4	A5
$[M-H]^-$	288 (100)	302 (100)	316 (100)	330 (100)	344 (100)
$[M-H-CO]^-$	244 (37)	258 (20)	272 (20)	286 (13)	300 (9)
$[M-H-alkene]^-$	-	274 (15)	274 (14)	274 (6)	274 (6)
$[M-H-alkene-CO_2]^-$	-	230 (11)	230 (9)	230 (6)	230 (6)
$[M-H-alkene-CO_2-H_2O]^-$	212 (8)	212 (4)	212 (4)	212 (2)	212 (0.4)
$[M-H-alkene-CO_2-H_2O-C_2H_2]^-$	185 (10)	186 (13)	186 (6)	186 (5)	186 (3)
$[[M-H-alkene-CO_2-H_2O-C_4H_2]^-$	162 (22)	162 (20)	162 (11)	162 (7)	162 (6)
nitrophenoxy ion	138 (99)	138 (87)	138 (68)	138 (22)	138 (19)
other ions	246 (8)	260 (8)	-	288 (5)	302 (2)
	228 (5)	228 (4)	-	228 (3)	-
	214 (5)	-	-	-	-
	-	184 (4)	184 (4)	184 (3)	184 (1)
	187.5 (4)	136 (5)	-	-	-

Table 12. Product ion spectra of $[M-H]^-$ of alkyl 3-hydroxy-5-(2',4'-dinitrophenoxy)benzoates B1-B5

	Compounds				
Ion, m/z (rel. abund. %)	B1	B2	B3	B4	B5
$[M-H]^-$	333 (1)	347 (1)	361 (4)	375 (5)	389 (6)
$[M-H-HNO_2]^-$	286 (71)	300 (65)	314 (100)	328 (100)	342 (100)
$[M-H-HNO_2-alkene]^-$	-	272 (13)	272 (12)	272 (12)	272 (2)
$[M-H-HNO_2-alkene-CO_2]^-$	227 (10)	228 (33)	228 (32)	228 (20)	228 (18)
$[M-H-HNO_2-alkene-CO_2-NO]^-$	-	198 (3)	198 (3)	198 (1)	198 (2)
dinitrophenoxy ion	183 (100)	183 (100)	183 (95)	183 (78)	183 (49)
other ions	167 (7)	167 (5)	167 (5)	167 (4)	167 (4)
	-	-	181 (4)	-	-

The ion $[M-H-HNO_2]^-$ is very intense in the product ion spectra of B1-B5, but does not appear in the spectra of A1-A5 (Tables 11 and 12). The hydrogen between the two strongly electron withdrawing nitro groups in B1-B5 is more acidic and is more likely eliminated along with NO_2 than is the hydrogen adjacent to the nitro group in compounds A1-A5. For the CID reactions of gas phase ions as energy selected systems, *ab initio* calculations may be carried out. *Ab initio* calculations performed with compound B2 at the 3-21G level of theory showed that the fragment ion $[M-H-HNO_2]^-$ is 12.2 kcal/mol more stable when the nitro group is eliminated from the ortho than from the para position.

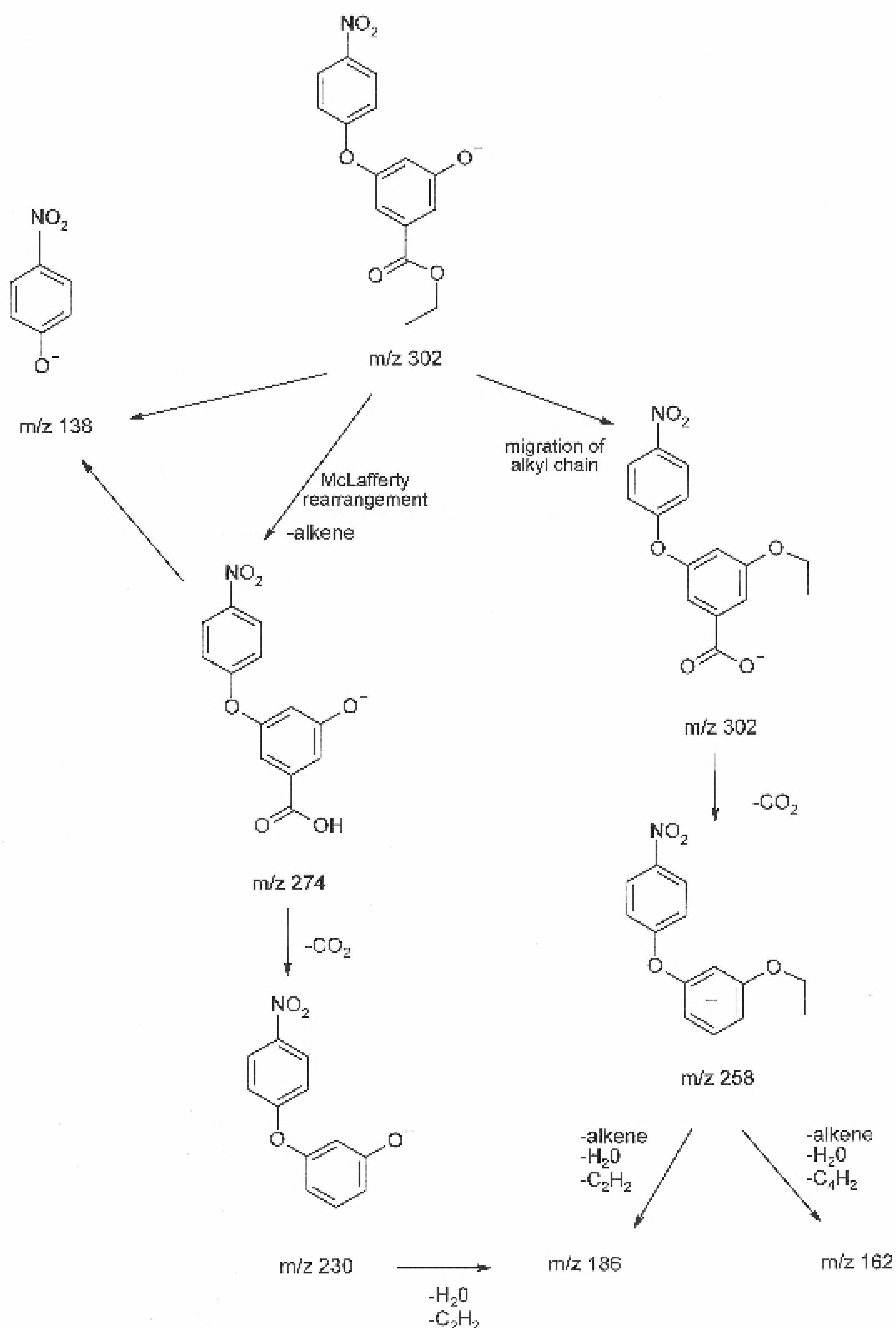
In contrast to the dinitrophenoxy ion (m/z 183), the relative abundance of $[M-H-HNO_2]^-$ in the spectra of B1-B5 increases with the length of the alkyl chain. The energy transferred in the collision activation to the precursor ion decreases as the length of the alkyl chain increases. This decrease in the internal energy has a stronger effect on the formation of nitrophenoxy ions than on the formation of $[M-H-HNO_2]^-$ ions, and allows us to assume that more energy is required for the formation of nitrophenoxy ions than for the formation of $[M-H-HNO_2]^-$ ions. Moreover, this energy

is evidently quite near the level of the internal energy transferred in the collision activation. It follows that when the alkyl chain length is increased, the relative abundance of $[M-H-HNO_2]^-$ is increased at the expense of the relative abundance of the dinitrophenoxy ion.

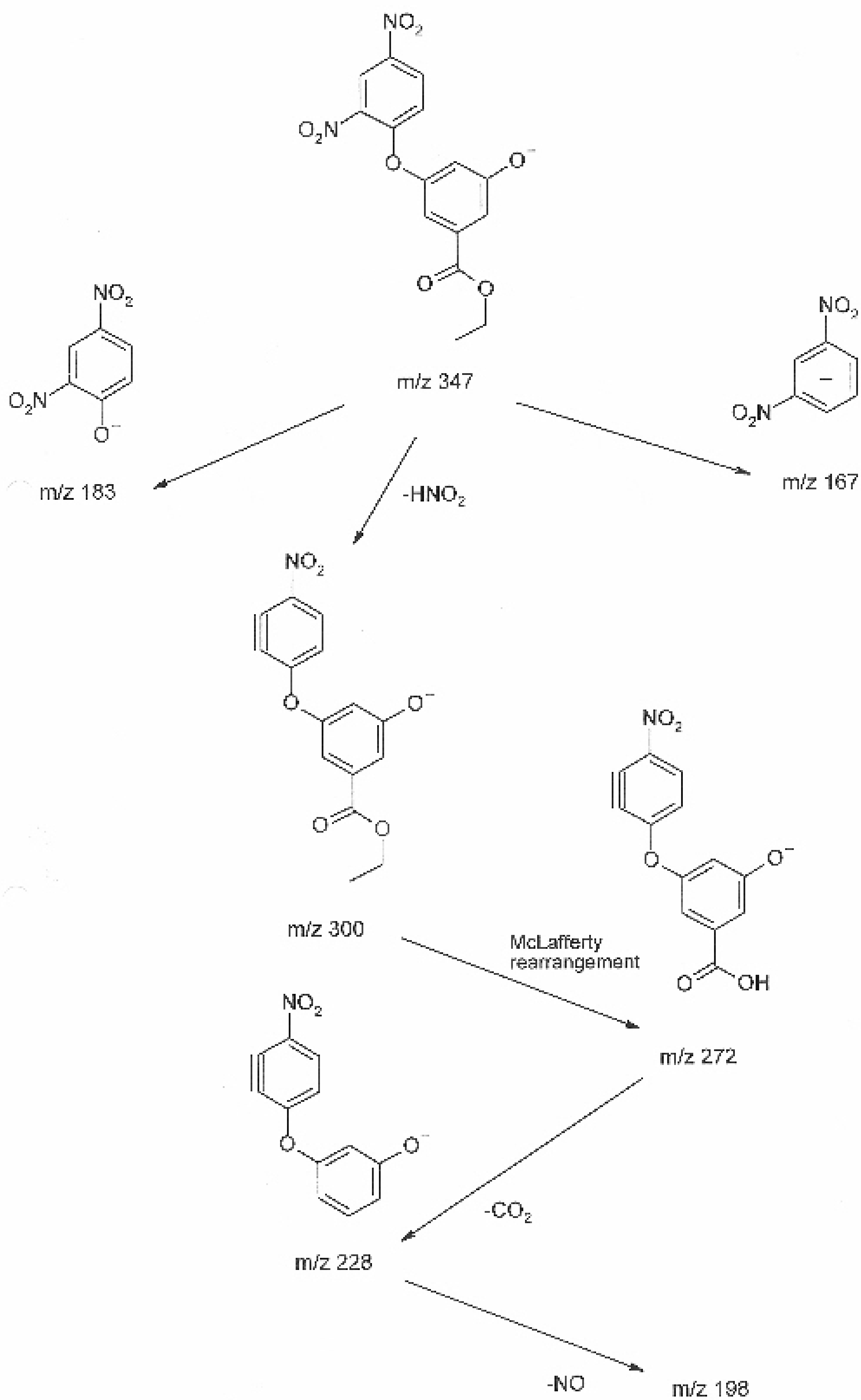
The ion m/z 274 in the spectra of A2-A5 is formed from $[M-H]^-$ by the cleavage of alkene from the ester group after McLafferty rearrangement. The ion m/z 272 in the spectra of B2-B5 is formed in the same way, but after cleavage of HNO_2. Because the hydrogen transfer in McLafferty rearrangement requires a six-member ring transition state, the ions at m/z 274 and m/z 272 do not appear in the spectra of the methyl esters (A1 and B1). The intensities of the ions at m/z 272 and m/z 274 are slightly decreased with an increase in the length of the alkyl chain. The ions at m/z 230 (A1-A5) and m/z 228 (B1-B5) are formed by the loss of CO_2 from the ions at m/z 274 and m/z 272, respectively, and the ions at m/z 230 and m/z 228 are further fragmented by the loss of NO, producing very weak ions at m/z 200 (A1-A5) and m/z 198 (B1-B5), respectively.

The unexpected ion $[M-H-44]^-$ formed by the loss of CO_2 is seen in the spectra of A1-A5, but not in the spectra of B1-B5. Before the loss of CO_2, the alkyl chain migrates from the ester moiety, evidently to the negatively charged phenolate oxygen. The semi-empirical calculations showed that the minimum of the electrostatic potential on the total electron density surface near the phenolate oxygen of the deprotonated molecule is -164.200 kcal/mol for A2 and -158.136 kcal/mol for B2. The lower value for A2 indicates that the nucleophilicity at the phenolate oxygen is significantly higher for A1-A5 than for B1-B5. This is because the electron withdrawing effect of two nitro groups is significantly greater than that of one nitro group. It follows that the higher nucleophilicity of the phenolate oxygen in compounds A1-A5 favours the migration of the alkyl chain to this moiety. The relative abundance of the ion $[M-H-CO_2]^-$ decreases as the length of the alkyl chain increases, since the internal energy transferred in the collision activation also decreases.

The ion m/z 186 in the spectra of A1-A5 is formed via two pathways, i.e. by the loss of 44 amu from m/z 230 and by the loss of 72 amu from m/z 258, shown by fragment ion tandem mass spectrometry. The MS/MS spectrum of m/z 186 reveals the loss of NO (m/z 156) and ion NO_2^- (m/z 46), indicating the presence of a nitro group. However, the structure of the ion m/z 186 cannot be reliably characterised with the available data. The ion m/z 162 is formed from m/z 258 by an unknown mechanism.



Scheme 1. The main CID fragmentation paths for $[M\text{-}H]^-$ of A2.



Scheme 2. The main CID fragmentation paths for $[M-H]^-$ of B2.

5.1.2 Combinatorial library screening

The fragmentations of compounds A1-A5 are alike, as are those of compounds B1-B5. The similarity of fragmentation may often be the case with combinatorial libraries, since the basic structure of the compounds is similar. This feature can be exploited in the rapid assessment of the quality of the synthesis of combinatorial libraries, by employing precursor or neutral loss scans for compound identification. Figure 8 illustrates the precursor ion spectra of the nitrophenoxy ion of A1-A5 and B1-B5. The results rapidly tell whether the targeted compounds are present in the library. Only limited information is provided on the possible synthesis impurities. However, several improvements could be made to the presented method, such as chromatographic separation or the use of other detectors, if more information is needed.

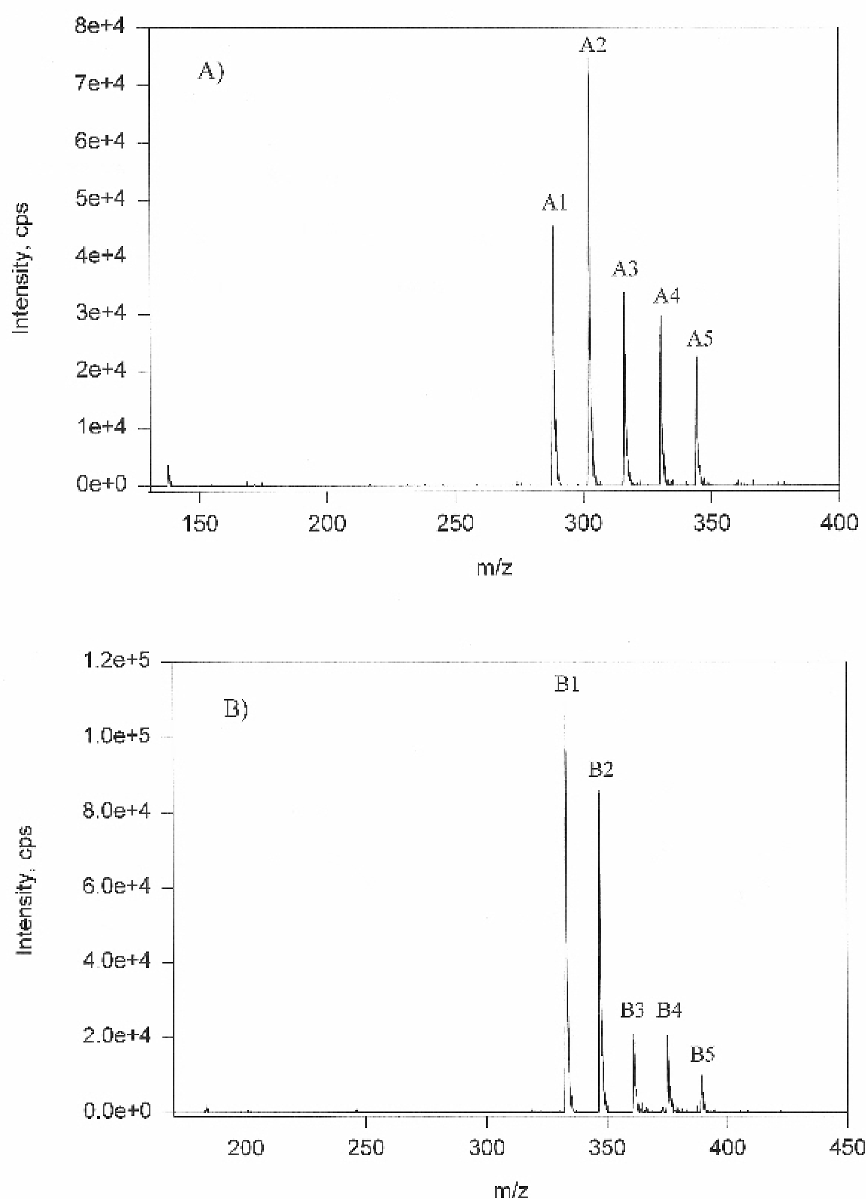


Figure 8. Precursor ion spectra of m/z 138 from nitrophenoxy benzoate compounds, A1-A5 (a), and m/z 183 from dinitrophenoxy benzoate compounds, B1-B5 (b).

5.2 IAM study

5.2.1 Retention factors of IAM data

IAM data approximate the lipophilicity of the compound or the permeability through cell membranes. Permeability studies are used to assess blood-brain penetration (Reichel, A. and Begley, D., 1998) or for the assessment of oral absorption. In oral absorption studies, IAM data improve if combined with *in silico* parameters (Stewardt, B.H. and Chan, H.O., 1998, Yen, T.E. et al., 2005). Lipophilicity values also enable understanding of the behaviour of certain compounds in activity screening (Escuder-Gilabert, L. et al., 2000, Matysiak, J. et al., 2007) or improve the solute descriptors in QSAR analysis (Sprunger, L et al. 2007). The use of IAM results for estimating the volume of distribution was also encouraging (Hollosy, P. et al., 2006). Calculated IAM values using three analytical methods for nine drugs are presented in Table 13.

Table13. Log k_{IAM} values measured with no organic solvent or extrapolated to a zero amount of organic solvent. 10mM phosphate buffer pH 7.4 with a physiological salt concentration was used in the first IAM-UV method (I), and 10mM ammonium acetate buffer pH 7.4 in the second IAM-UV method (II) and in the IAM-APCI-MS method (III).

Log k_{IAM}	IAM-UV (method I)	IAM-UV (method II)	IAM-APCI-MS (method III)
Ibuprofen	1.20	1.07	1.17
Indomethacin	2.16	2.10	1.91
Ketoprofen	0.98	0.82	0.88
Midazolam	2.49	2.44	2.49
Phenobarbital	0.58	0.67	0.74
Procaine	0.84	0.96	1.08
Propranolol	2.16	2.36	2.23
Thioridazine	3.88	3.49	3.43
Timolol	0.95	1.24	1.32

5.2.2 IAM columns

Typical IAM columns contain phosphatidylcholine (PC) analogues chemically bonded to a propylamino-silica core (IAM-PC) (Yang, C.Y. et al., 1996). For example, IAM-PC-DD columns use phosphatidyl choline bound as single-chain, IAM-PC-DD2 as double chain and IAM-PC-MG uses a double chain of phosphatidyl choline with a glycerol linker (Barbato, F. et al., 2004, Ward, R.S. et al., 2003). IAM-PC-DD and DD2 have end-capping of free propylamino residues with C10- and C3-acyl groups and IAM-PC-MG with a glycerol linker is end-capped with methylglycolate (MG). In this study the IAM-PC-DD2 column was used.

Comparison of IAM columns suggests that ionised compounds usually bind more strongly to IAM-PC-MG columns than IAM-PC-DD. The IAM-PC-DD2 column with a double chain structure provides higher retention for neutrals compared to the earlier DD column. Generally, IAM-PC-DD and DD2 columns are more often used in drug membrane permeability studies, while IAM-PC-MG phases are more often used in protein purification (Barbato, F. et al., 2004, Ward, R.S. et al., 2003, Luo,

H.B. et al., 2007). The chromatographic column immobilized with cholesterol has also been tested, but a correlation with physiological membrane penetration was not achieved (Al-Haj, M.A. et al., 1998).

The results of different laboratories should be easy to compare by using retention factors. Nevertheless, reported results may vary because of wide column selection. The repeatability of results over a longer time, however, was not investigated here.

5.2.3 ESI suitable buffers

The phosphate buffers normally used in IAM chromatography are inappropriate in atmospheric pressure chemical ionization (APCI) and electrospray ionisation (ESI), since they rapidly contaminate the ion source of the mass spectrometer. Ammonium acetate is a volatile buffer, widely used in liquid chromatography mass spectrometry, but rarely in IAM chromatography. Recently, Valko et al. (Valko, K. et al., 2000) used 50 mM ammonium acetate in IAM chromatography, but they did not directly compare their results with those obtained in phosphate buffered saline. Buffer concentrations higher than 10 mM may strongly suppress ionisation in ESI, leading to significantly decreased sensitivity. Although APCI tolerates much higher buffer concentrations than ESI, it is advantageous to use low buffer concentrations in APCI as well, to minimize chemical noise in the analysis. Accordingly, we tested the suitability of ammonium acetate as a buffer for IAM chromatography at a concentration of 10 mM.

The first step in the method development was to show, with use of UV detection, that the log k_{IAM} values measured with phosphate (method I) and ammonium acetate buffer (method II) are similar (Table 13). The measurements of the lipophilic compounds (indomethacin, midazolam, propranolol and thioridazine), which required an organic modifier for elution within a reasonable time (30 min), were made with 10, 20, 30 and 35% acetonitrile in the mobile phase. Extrapolation of the retention factor to a zero amount of organic modifier for lipophilic compounds was possible because, as was shown in earlier studies (Salminen, T. et al., 1997, Krause, E. et al., 1999, Valko, K. et al., 2000), the retention factors (log k_{IAM}) change linearly. As can be seen from Table 13, the log k_{IAM} values obtained with phosphate buffer and ammonium acetate buffer are very similar. Correlation analysis of the log k_{IAM} values obtained with method I and II using different buffers gave an r^2 of 0.970. The slight difference between the results may be explained as a salt effect. In the buffer of method I, the inorganic salts of potassium chloride and sodium chloride were included. The research group of Haroun (Haroun, M. et al., 2002) tested different salts against the retention of IAM chromatography and noticed some changes. They found that, in addition to lipophilic and hydrophobic effects, the properties of the amphiphilic solute with polar interfacial sites on the phospholipid monolayer may explain some of the differences.

5.2.4 UV versus MS

Positive and negative ion APCI mass spectra were recorded for the compounds in method development. The APCI spectra showed protonated molecules for indomethacin, ketoprofen, midazolam, procaine, propranolol, thioridazine and timolol, and deprotonated molecules for ibuprofen, indomethacin, ketoprofen and phenobarbital. Indomethacin was the only compound for which significant fragmentation was observed in positive and negative ion spectra. However, the deprotonated ion was intense enough for the single ion chromatography experiments performed in the log k_{IAM} determinations. In accordance with the ionization efficiencies of the compounds,

midazolam, procaine, propranolol, thioridazine and timolol were monitored with $[M+H]^+$ ions and ibuprofen, indomethacin, ketoprofen and phenobarbital with $[M-H]^-$ ions.

The log k_{IAM} values determined using 10mM ammonium acetate buffer and APCI ionization are reported in Table 13. Note that in IAM–APCI-MS, instead of the injection of each compound separately, the nine compounds were mixed together (100 µM each) and the mixture was directly injected onto the IAM column. The log k_{IAM} values obtained by the n-in-one (III) method using IAM–APCI-MS correlate well with both the UV methods; correlation analysis gave an r^2 value of 0.991 with the UV ammonium acetate buffer method (II) and a value of 0.967 with the UV phosphate buffer method (I).

5.2.5 Single versus n-in-one

The disadvantage of the isocratic method is that several runs with increasing amounts of organic modifier must be made if the mixture includes lipophilic compounds. As demonstrated by Valko et al. (Valko, K. et al., 2000), the extra runs can be avoided by using gradient elution and reference compounds with known log k_{IAM} values for calculation of the unknown log k_{IAM} values. Figure 9 illustrates selected ion chromatograms of the $[M-H]^-$ and $[M+H]^+$ molecular ions obtained with the use of gradient elution (100% buffer to 100% acetonitrile in 10 min). All compounds eluted rapidly within 8 minutes. The ion chromatograms show good stability of the system; all compounds were clearly detected and the background ions did not disturb the detection. The elution order of the compounds in the gradient method was the same as the order of the log k_{IAM} values calculated in isocratic runs, with the exception of midazolam and propranolol, which were in reversed order. The log k_{IAM} values of midazolam and propranolol (2.49 and 2.23, Table 13) are in any event very similar. Our results demonstrate that gradient elution is suitable for lipophilicity ranking with the IAM–APCI-MS method. If log k_{IAM} results are needed, the gradient run method should be calibrated with use of a homologous series appropriate for the studied compounds (Valko, K. et al., 2000). Measurement in parallel artificial membrane permeation assay (PAMPA) screening methods often involves a well plate format and an automatic UV reader. Similarly, the n-in-one strategy for immobilised artificial membrane (IAM) chromatography with a multiplexed electrospray interface (MUX) could be evaluated. If the MUX technology includes four or eight channels of IAM columns for one MS detector, it increases the throughput. The computerised protocol to calculate retention factors should be easy to automate.

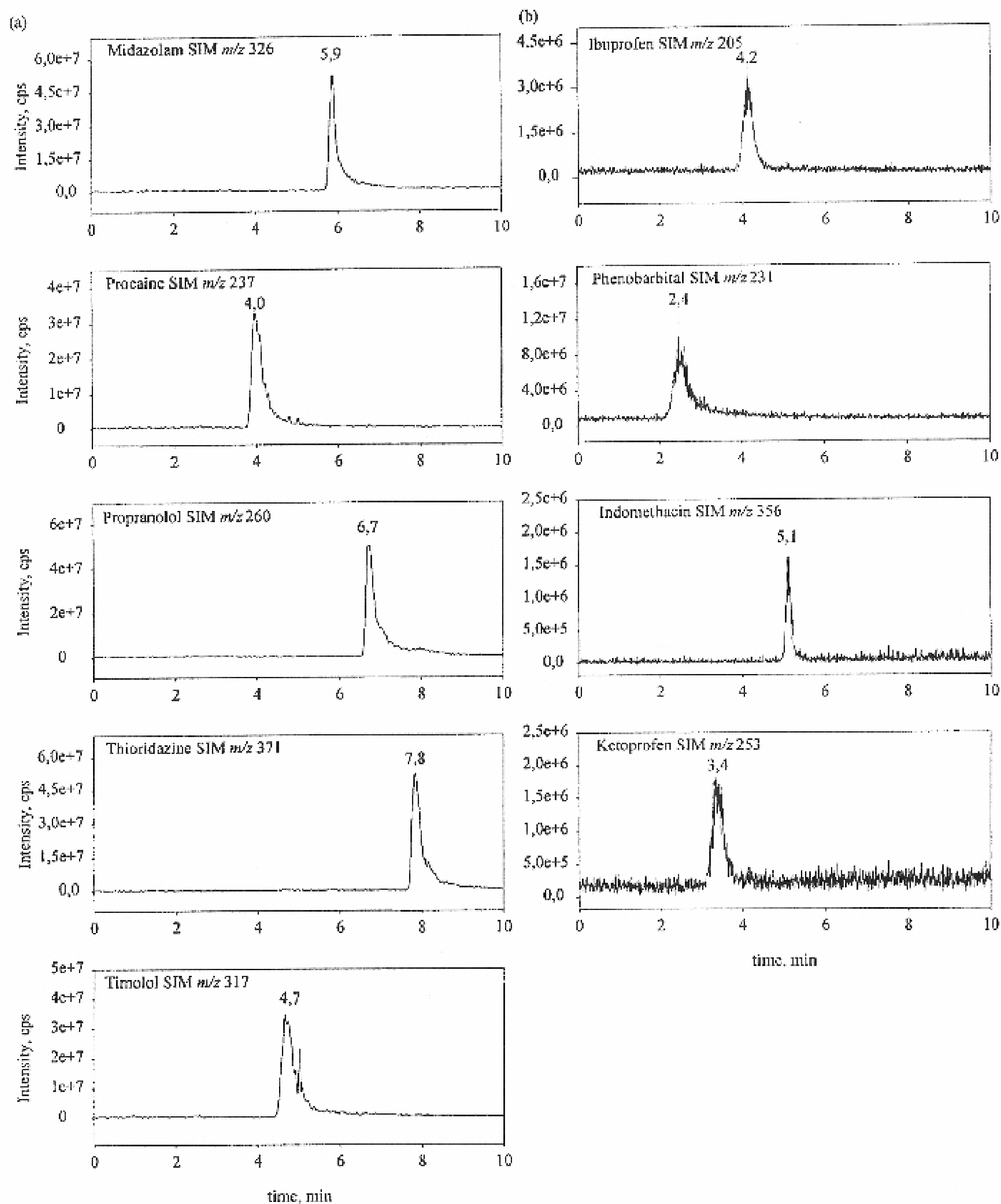


Figure 9. Extracted ion chromatograms of (a) $[M+H]^+$ ions and (b) $[M-H]^-$ ions in immobilised artificial membrane chromatography (IAM) of nine compounds and gradient elution (100% buffer to 100% acetonitrile in 10 min).

5.3 Caco-2 study

5.3.1 LC-MS/MS method

The purpose of the Caco-2 study was to investigate cocktail dosing in the cell test. The role of the author was to develop a new analytical method based on LC-MS/MS. The Caco-2 tests are only discussed in literature survey, because the interpretation of the cell test results using n-in-one was performed by Leena Laitinen.

The mass spectrometric separation allowed the use of a fast gradient in LC separation. All the compounds presented in Table 4, compounds C, were eluted within 8 minutes with good enough resolution (Figure 10). A small particle column (3µm) was selected because of its good capability in chromatographic separation. The most polar compound, procaine, eluted first in 2.9 minutes before all the heterocyclic compounds. Because of the acidic mobile phase (pH 3.5), ketoprofen (pKa 4.8) was in its unionised form. Therefore, ketoprofen was the most lipophilic compound and also the last to elute, with a retention time of 7.5 minutes.

To avoid instrument contamination and consequently poor reproducibility and decreased sensitivity, it was necessary to use column switching. Salts and other possible non-volatile materials in Caco-2 samples were flushed to waste for 1.5 min, after which the eluent was directed to MS. Without column switching, the sensitivity decreased significantly due to contamination of the sampling orifice of the API source after 10-20 runs. The use of column switching also ensured that no suppression in the ionisation process was observed and good analytical reproducibility was achieved, including for co-eluting compounds.

The ionisation efficiency in the positive ion mode for all the compounds studied was good and the spectra showed very abundant $[M+H]^+$ ions with minimal fragmentation. However, the addition of formic acid was necessary to also achieve a good ionisation efficiency for acids. The protonated molecule was chosen for the precursor ion and two specific and intense product ions were chosen for each compound for multiple reaction monitoring. One product ion was used for quantitation and the other was a qualifier (Table 14). An external standard method was used in quantitative analysis instead of an internal standard method because of the heterogeneous characters of the compounds studied.

The linearity of the method was studied between 50 and 2000 ng/ml (Table 14). Calibration graphs were constructed using spiked samples of at least six different concentrations in each series to cover the quantitative range. The calibration curves for all the compounds showed good linearity with correlation coefficients (r^2) better than 0.990. The within-day precision of the method was evaluated with seven samples (250 ng/ml) and between-day precision with eight samples within 4 days. Relative standard deviations (RSD) for within-day and between-day experiments were below 10%, indicating acceptable precision of the analytical method. Procaine was an exception to the high within-day precision, with a value of 16%. The use of column switching allowed injection volumes of 50 µl that improved the limits of detection. Without column switching, injection volumes of 50 µl rapidly contaminated the instrument. The sensitivity of the method was perhaps surprisingly good, because all too often the samples had to be diluted. If this had been known from the start, dilution might have been one of the sample pre-treatments itself. However, as carried out here, the method offered the promise of even more dilute Caco-2 cocktail analysis. In addition, it would be

better to continue the validation of the method. Selectivity was tested using blank samples and at the lower limit of quantitation (LLOQ) during sample analysis. In the future, accuracy should be validated with different calibration models and sample stability should be examined.

The development of faster chromatographic separation could also be continued by using smaller diameter particles, but there is a risk of higher pack pressure. Different column materials, such as monolith phases, could also shorten the analysis time. As a follow-up study, with a similar set of compounds and a monolith column (Hakala, K. et al., 2003), the run was slightly shorter. Using the same ionisation as here, i.e. ESI (electrospray ionization), the chromatographic separation took 7 minutes. A more important and substantial difference in the study of Hakala and co-workers was achieved by changing the ionisation to an APPI (atmospheric pressure photo-ionisation) detector, which resulted in an elution time of only 5.5 minutes.

Table 14. The selected reaction monitored for quantitation (and qualifier ions) of ten compounds in Caco-2 sample; within-day and between-day (4 days) repeatability, LLOQ and linearity.

	Repeatability, RSD		LLOQ	Linearity
	Within day % (n=7)	Between 4 days % (n=8)	(ng/ml)	r^2
Phenazone 189/104	7.5	8.5	10	0.994
(Phenazone 189/147)	5.2	7.8	25	0.994
Naproxen 231/170	5.0	14	25	0.990
(Naproxen 231/185)	5.7	12	25	0.990
Ketoprofen 255/105	4.9	4.7	5	0.994
(Ketoprofen 255/209)	4.4	7.1	5	0.990
Propranolol 260/116	3.1	3.1	5	0.996
(Propranolol 260/183)	3.7	7.1	5	0.994
Procaine 237/100	16	24	50	0.990
(Procaine 237/120)	20	19	25	0.990
Timolol 317/244	4.0	6.0	25	0.992
(Timolol 317/261)	5.2	5.4	25	0.994
Midazolam 326/291	6.8	9.1	10	0.994
Hydroxyzine 375/201	7.0	8.8	10	0.990
Buspirone 386/122	5.8	12	5	0.992
(Buspirone 386/265)	2.6	8.2	25	0.990
Verapamile 455/165	3.0	3.5	25	0.998
(Verapamile 455/303)	2.6	6.4	25	0.994

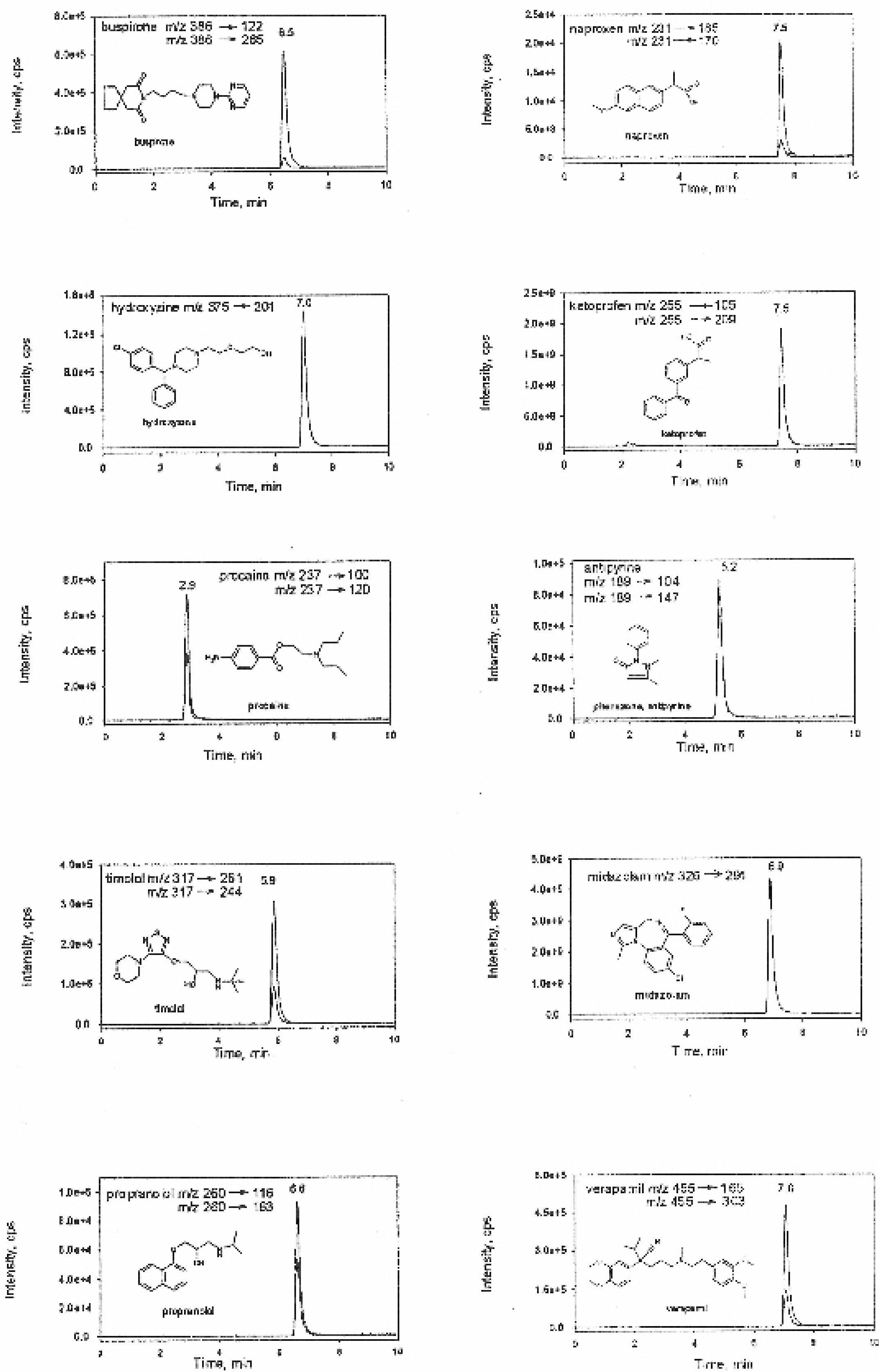


Figure 10. Representative chromatograms for ten compounds in Caco-2 quantitation.

6. Conclusions

Drug discovery has used liquid chromatography - mass spectrometric (LC-MS) methods because of their good sensitivity and selectivity. Combinatorial libraries need to be evaluated while new compounds are being rapidly synthesised and screened for their activity and other drug properties, such as lipophilicity and absorption. In first method described here, no chromatographic separation was carried out, but there was direct infusion to mass spectrometry. Ten nitrobentzoate compounds produced by combinatorial chemical synthesis were characterised, their fragmentation studied and the presence of the compounds determined in a combinatorial library.

The CID fragmentation of ten nitrophenoxy benzoate compounds was studied by product and precursor ion scanning. Because of the serial homology in these chemicals, interesting structure-fragmentation relationships were noticed. Two nitro group compounds increases fragmentation compared to one nitro group, whereas a longer alkyl chain stabilised the ion structure and less fragmentation was noticed from methyl to pentyl in both series. The most abundant product ions were nitrophenoxy ions. Common fragmentation made it possible to employ precursor ion scans to identify compounds in combinatorial mixtures. A precursor ion scan filters compounds from the matrix with better signal-to-noise ratios.

Secondly, immobilized artificial membrane (IAM) chromatography was tested. This study was a method transfer and development study, where the traditional IAM chromatographic method using phosphate buffer and ultraviolet detection (UV) was changed to ammonium acetate buffer and mass spectrometric (MS) detection. Mass spectrometric detection scanned the mass spectrum separately in positive and negative modes, and the chromatographic retention of compounds was analysed using extract ion chromatograms. When changing detectors and buffers and including n-in-one in the method, the results showed good correlation. We demonstrated that the log k_{IAM} values produced using ammonium acetate buffer are as reliable as those produced in phosphate buffer. Moreover, we also showed that analysis with MS gives as good results as with UV. Finally, the results demonstrated that mass spectrometric detection with gradient elution can provide a rapid and convenient n-in-one method for ranking the lipophilic properties of small organic drugs, including both acidic and basic compounds.

The last study was set up in collaboration between research groups in pharmaceutical technology and pharmaceutical chemistry at the University of Helsinki. There was interest in determining whether drug-drug interaction occurred in the Caco-2 absorption cocktails. The analytical work aimed to develop a liquid chromatography mass spectrometric method for Caco-2 cocktail samples. All ten compounds required some chromatographic separation and mass spectrometric detection, using selective reaction monitoring, for quantitative analysis.

The LC-MS/MS method offered ideal sensitivity, selectivity and sample capacity, especially for low sample concentrations and n-in-one cocktails. The absorption of ten chemically and physiologically different compounds was screened using both single and n-in-one approaches. The analysis was speeded up using the n-in-one cocktail, but the development of the method was also boosted by making it for several compounds at once.

These three studies used mass spectrometry for compound identification, method transfer and quantitation in the area of mixture analysis. The selectivity of the mass spectrometric method is appreciated when analysing combinatorial synthesis, where possible side reactions of the synthesis could disturb the identification. Lipophilicity can rapidly be measured with multiple analytes at

once using immobilized artificial membrane (IAM) chromatography. With other *in vitro* or *in silico* methods, IAM chromatography has promising capabilities to estimate drug properties such as the volume of distribution and intestinal absorption or blood-brain penetration. In cocktail dosing studies, the concentrations are recommended to be kept at a minimum because of the risk of compound interactions. In the analysis of Caco-2 sample mixtures, especially with small concentrations, sensitive methods such as mass spectrometry are required. Different mass spectrometric scanning modes for the triple quadrupole instrument were used in each method. Early drug discovery with n-in-one is area where mass spectrometric analysis, its possibilities and proper use, is especially important. Past failures, such as unexpected compound interactions, have generally discouraged research into n-in-one. However, more information is needed, especially if the suitability of n-in-one is method-dependent.

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