Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 1120–1126

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

High-throughput analysis of standardized pharmacokinetic studies in the rat using sample pooling and UPLC–MS/MS

Tjerk Bueters[a,*], Jessie Dahlström[a], Kristine Kvalvågnaes[a], Ingvar Betnér[b], Sveinn Briem[a]

[a] DMPK, CNSP Innovative Medicines, AstraZeneca AB, SE-151 85, Södertälje, Sweden
[b] Waters Corporation, Sollentuna, Sweden

ARTICLE INFO

Article history:
Received 10 January 2011
Received in revised form 2 March 2011
Accepted 25 March 2011
Available online 6 April 2011

Keywords:
Cassette analysis
Sample pooling
High-throughput
Pharmacokinetic
UPLC–MS/MS

ABSTRACT

As a consequence of a continuous demand for increased throughput of pharmacokinetic (PK) studies, industries have introduced strategies to reduce the number of samples such as cassette analysis (pooling of samples after the in-life phase). Here, we have investigated whether relevant PK parameters change as a consequence of cassette analysis, and whether there are circumstances that disqualify this technique from being used.

22 compounds were intravenously and orally administered to parallel groups of 3 rats. Each compound was administered discretely. Equal volumes of three plasma samples corresponding to each time point of three discretely dosed rats with different compounds were pooled (cassette analysis). Samples were prepared by protein precipitation followed by UPLC–MS/MS analysis using pos/neg switching when required.

With cassette analysis, 4 compounds, morphine, phenytoin, rofecoxib and diclofenac, showed high limit of quantification (LOQ) values after pooling, which led to less reliable PK analyses. Of all samples with contents above LOQ, about 5% could not be detected in pool samples compared to single samples. However, an excellent correlation was seen for all PK parameters when comparing the parameters obtained from discrete analysis versus those obtained from cassette analysis, although half life showed somewhat more scatter than the others. When PK parameters were grouped as low–medium–high, clearance, volume of distribution, half life and bioavailability were similar between discrete and cassette analysis for 90%, 86%, 95% and 90% of the total number of compounds tested, respectively. Some additional improvement was achieved if compounds with a low MS response were excluded.

In summary, cassette analysis is an effective strategy to reduce samples without affecting the estimated PK parameters that are important for decision-making.

1. Introduction

In the Lead Identification and Lead Optimization phases in Drug Discovery rapid in vivo pharmacokinetic (PK) profiling of new chemical entities is important to guide medicinal chemists in the optimization process of a chemical series and to assist pharmacologists to design in vivo efficacy studies. In contrast to most in vitro DMPK assays, PK screening is still a labor- and time-intensive process in many pharmaceutical industries. Higher throughput approaches are desired for rapid and cost-effective generation of PK studies. This has led to the development of novel concepts for performing high throughput PK studies, i.e. sample pooling and cassette dosing.

There are three different strategies described in the literature concerning sample pooling. All strategies involve administration of one compound to multiple animals. The first strategy, that has been named rapid rat or "snapshot PK" in the literature [1,2], involves pooling of samples of the same time point per animal. This results in the average concentration of the compound per time point. With the second strategy, all samples from one animal are pooled and analyzed. The final result is one concentration per animal that is proportional to the AUC of this compound, but the time profile is lost [3]. A third strategy, named cassette analysis, involves pooling of samples of different compounds at the same time point and simultaneous bioanalysis [4,5]. This sample pooling technique maintains all information for each animal and each compound. The cassette analysis strategy has been used in this report.

Abbreviations: ACN, acetonitrile; AUC, area under the curve; CL, clearance; DMA, dimethylamine; *F*, bioavailability; LOQ, limit of quantification; PEG400, polyethylene glycol 400; PK, pharmacokinetic; rt, retention time; $T_{1/2}$, half life; UPLC–MS/MS, ultra performance liquid chromatography–mass spectrometry/mass spectrometry; *V*, volume of distribution.

* Corresponding author. Tel.: +46 0855329623; fax: +46 0855321560.
E-mail address: Tjerk.Bueters@astrazeneca.com (T. Bueters).

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2011.03.042

Table 1
Overview of the studied compounds.

	M_w	$C\log P$ (7.4)	pK_A	Vehicle	LOQ discrete (nM)	LOQ cassette (nM)
Phenytoin	252.3	2.4	5.7	Water	32	110
Imipramine	280.4	2.5	9.4	0.3 M gluconic acid	5	10
Cyclobenzaprine	275.4	2.7	9.7	0.3 M gluconic acid	5	10
Lidocaine	234.2	1.6	8.1	Saline	5	5
Haloperidol	375.9	2.9	9.0	10% DMA in saline (pH 5.3)	5	5
Trifluoperazine	407.5	4.3	7.8	5% DMA in saline	5	5
Risperidone	410.5	2.1	8.6	0.3 M gluconic acid	5	10
Thioradazine	370.6	3.6	8.9	5% DMA in saline	5	5
Caffeine	194.2	−0.1	–	5% DMA in saline	5	10
Diazepam	284.7	2.8	3.4	5%DMA, 20%HPbCD in 0.3M gluconic acid	5	10
Citalopram	324.4	1.6	9.7	5% DMA in saline	5	5
Gabapentine	171.2	1.2	10.5	Saline	5	33
Paroxetine	329.4	1.8	10.1	5% DMA in saline	5	5
Morphine	285.3	−0.1	8.3	Saline	45	112
Chloropromazine	318.9	3.3	9.5	Saline	5	5
Fluoxetine	309.3	1.9	10.2	Saline	5	10
Perphenazine	404.0	3.6	8.0; 3.7	5%DMA, 5%1M lactic acid in saline	5	5
Rofecoxib	314.4	1.8	–	5%DMA, 20%HPbCD in water	162	52
Carbamazepine	236.3	1.6	13.4	2% DMA in saline	5	5
Diclofenac	296.2	4.7	4.2	5% DMA in saline	45	160
Dimethyl-sulpiride	369.5	0	9	Saline (pH 3)	5	5

There are several advantages to sample pooling. The number of samples is reduced, which shortens the time required to perform the analysis. Also, the different sample pooling strategies avoid potential complications from in vivo drug–drug interactions as is the case for cassette dosing, in which multiple compounds are administrated to a single animal. Moreover, administration of higher doses is circumvented with cassette dosing to reduce the risk for such interactions [6,7]. No restrictions to dose size are required with the different sample pooling strategies.

Sample pooling of different compounds after the in-life phase is not an entirely novel approach. In the late nineties, Kuo et al. [5] developed a HPLC method for pooled analysis of dopamine D_4 receptor antagonists in rats. Olah et al. [8] described an LC/MS/MS method to combine up to 10 compounds from in vivo studies for screening purposes, and Cai et al. [9] presented a LC/MS method for pooled analysis of α-1a antagonists and their metabolites in mice. Since then, however, the analytical and sample work-up hardware have been improved tremendously, creating possibilities to industrialize the sample pooling methods and make them more suitable for a Drug Discovery environment. From an implementation perspective it is essential to consider how many additional compounds are wrongfully characterized for their PK properties using a high-throughput method combined with sample pooling compared to traditional discrete bioanalysis, and if there are limitations in the experimental setup. If the reduction in quality is too high, it might not be worthwhile implementing. To our knowledge, literature that compares cassette analysis versus discrete analysis with a diverse data-set using state-of-the-art technology with regard to the analytical performance and the calculated PK parameters is currently unavailable.

Here, we describe an evaluation of sample pooling according to the cassette analysis strategy of 3 compounds run in standardized PK studies in the rat using high-throughput UPLC–MS/MS analysis. For this, 22 known drugs were selected with different physicochemical properties (Table 1). We deliberately included some compounds that we knew our standard UPLC/MS/MS set-up has difficulties with. All compounds were administered intravenously and orally to parallel groups of 3 rats. Collected samples were subjected to both discrete and cassette analysis and compared with respect to sample concentrations and PK parameters. We have also studied possible limitations that could disqualify a compound from being pooled, such as a low response or ion suppression of different commonly used vehicles.

2. Materials and methods

2.1. Chemicals

Compounds tested for PK studies were supplied by AstraZeneca's internal Compound Management. All chemicals used for in vivo studies were purchased from reputable companies and of standard purity. Solvents used for UPLC were of gradient grade purity and bought from Merck (Darmstadt, Germany). Deionized water was purified using a Milli-Q water purification system (Millipore, Bedfrod, MA, USA). Blank plasma from Sprague Dawley rats was provided by the Laboratory Animal Science Group within AstraZenca, Södertälje.

2.2. Liquid chromatography and mass spectrometry

An Ultra Performance Liquid Chromatography system (UPLC) equipped with a BEH C-18 column (1.7 μm, 2.1 mm × 30 mm) from Waters Corporation (Sollentuna) was used for chromatographic separation. The mobile phase A and B consisted either of 10:90 and 99:1 MeOH:H_2O in ammonium acetate (10 mM) and 1% isopropanol or 2:98 and 95:5 of acetonitrile (ACN):H_2O in 0.1% acetic acid. A generic 1.1 min gradient with the following conditions was applied: 0–0.2 min 2%B, 0.2–0.5 min 2–100%B, 0.5–0.8 min 100%B, 0.8–0.81 min 100–2%B, 0.81–1.1 min 2% B. The flow rate was 0.6 ml/min. The injection volume was set to 10 μl.

The mass spectrometer was a triple quadrupole Acquity Quattro Premier XE system (Waters Corp.). Electrospray ionization in multiple reaction monitoring mode was used and pos/neg switching was applied whenever required. The following setup of the analyzers was used: the low and high mass resolutions were both set at 13.0 with ion energy of 1 V. The collision entrance and exit lenses were set to −5 and 1 V, respectively. High purity argon was used to enable collision-induced dissociation. The cone and desolvation gas was set to 50 and 900 l/h (high purity nitrogen gas), respectively. The masslynx software package version 4.1 (Waters Corp.) was used to control the UPLC–MS/MS system and optimize MS conditions.

2.3. Sample and standard series preparation

The following criteria were used to avoid complications during bioanalysis: molecule weight should differ at least 3 units, weight differences between 2 analytes should not be exactly the

weight of an obvious metabolite (e.g. +16, −14), and compatibility with one mobile phase. In vivo plasma samples were vortexed and centrifuged at 3500 rpm for 15 min (Sigma Laborzentrifugen, Osterode, Germany) prior to sample preparation. Single sample preparation was achieved by adding 150 μl ice-cold acetonitrile containing 200 nM of warfarin (internal standard) to a 25 μl plasma sample. When pooling, equal volumes (25 μl) of plasma samples from three rats dosed with different compounds were brought together into 1 ml 96-well plate for each time point. There were always three compounds analyzed in one cassette. Subsequently, 200 μl of cold acetonitrile with internal standard was added. The amount of organic solvent utilized for precipitation was optimized with regard to sample clean up and LOQ [10]. Precipitated samples were stirred and centrifuged (4 °C, 4000 rpm, 20 min) and 120 μl of the supernatant was transferred into 2 ml 96 well plates. 300 μl of mobile phase A was added prior to analysis. Plasma precipitation, supernatant transfer and buffer addition was all performed by a Biomek FX robot using a 96 channel pipetting head.

2.4. Calibration curves

Calibration series were prepared to perform ion suppression check and to quantify the 22 compounds in the evaluation study. Stock solutions were prepared in DMSO (10 mM) and working standard solutions were prepared in acetonitrile:water (50:50). Calibration standard series were constructed by spiking working solutions to blank plasma in a concentration range of approximately 5–10,000 nM. The accuracy and within day repeatability of the method was examined by preparing and analysing quality control samples containing diazepam, diclofenac and propranolol at three concentration levels (113–10,000 nM).

The most suitable curve fit (generally quadratic regression) and weighting function was used to fit the calibration curve after analysis. The lower limit of quantification was defined as the lowest concentration that had accuracy within 25% of the theoretical concentration.

2.5. In vitro ion suppression check of common vehicles

To examine possible matrix effects of commonly utilized formulations, cyclodextrin (HPbCD), glyconic acid, meglumine, dimethylamine (DMA), polyethylene glycol 400 (PEG400), hydroxypropylene methylcellulose/tween 80 or MCC/NaCMC (micro crystalline cellulose, carboxyl methyl cellulose) + lipoid S100 were added to plasma samples to a final concentration of 5%. The effect of the presence of these vehicles was examined for six reference compounds; propranolol, imipramine, diazepam, diclofenac, naproxen and rofecoxib in the concentration range 5–10,000 nM. Chromatographic separation of the analytes and the components in the administration solution was checked to detect ion suppression or enhancement (>30% difference in peak area between blank plasma and vehicle-spiked plasma).

2.6. PK screening study in the rat

Male Sprague–Dawley rats (Scanbur B&K AB, Sollentuna, Sweden) were housed with up to 5 animals per cage and were allowed to acclimatize to the new environment for at least 1 week upon arrival. In the conditioned animal facility, room temperature was kept at 20 ± 2 °C, relative humidity at 60 ± 20% and a 12 h light–dark cycle was maintained including a 0.5 h dusk or dawn period (lights on at 6.30 a.m.). Water and standard rodent diet (R70 Lactamin, Stockholm, Sweden) were freely accessible. All animal handling and experiments were performed in full compliance with authorial and ethical guidelines (Ethical Board of South Stockholm).

Diet was removed from the animals 1 h prior to dose administration and replaced 6 h after dose administration. Rats (~300 g) were weighed and divided into two groups; one group ($n = 3$) received 3 μmol/kg (0.5–1.2 mg/kg) of the test compound (Table 1) intravenously as a bolus injection in the tail vein, the other group received 10 μmol/kg (1.7–4.1 mg/kg) orally via gavage in a parallel study design. All compounds were given discretely. The dose volume was 4 ml/kg for both dose routes. The respective intravenous and *per os* formulations depended on the physicochemical properties of the compounds (Table 1). For each dose occasion a fresh formulation was prepared. To verify the concentration of the administered dose, an aliquot of each formulation was taken, transferred to a polypropylene tube (Cryotubes, A/S Nunc, Denmark), and diluted 10 times with ACN before analysis.

Blood samples (400 μl) from the tail vein were collected in microtainer tubes (BD, Plymouth, UK) containing EDTA at 0.02, 0.08, 0.33, 0.67, 1, 3, 6 and 24 h following iv administration, or at 0.25, 0.5, 0.75, 1, 1.5, 2.5, 6 and 24 h following po administration. The blood samples were centrifuged for 5 min at 2000 × *g* (4 °C), the

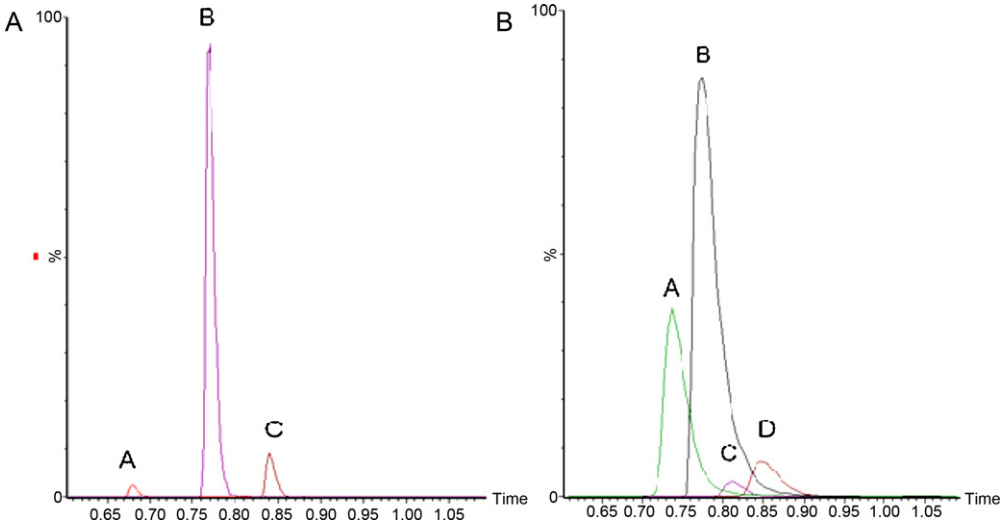

Fig. 1. Typical UPLC chromatograms using a 1.1 min H_2O/ACN gradient. The chromatograms are overlaid. (A) propranolol (A; rt = 0.68 min), imipramine (B; rt = 0.77 min) and diazepam (C; rt = 0.84 min) in rat plasma were spiked with various vehicles to look at ion suppression. Propanolol and imipramine eluated early and were affected by PEG400, while diazepam was not. (B) An UPLC chromatogram of lidocaine (A; rt = 0.72 min), haloperidol (B; rt = 0.77 min), perphenazine (D; rt = 0.85 min) and warfarin (C; internal standard; rt = 0.81 min) in rat plasma.

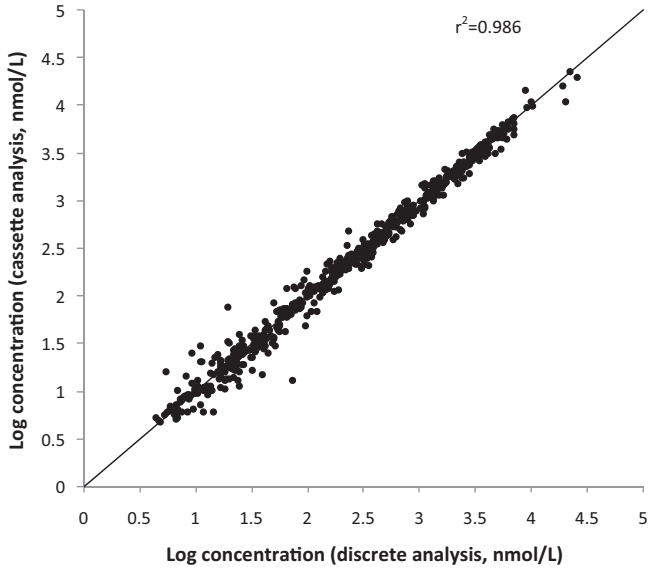

Fig. 2. Relationship between all plasma concentrations from discrete versus pooled sample analysis. Line is line of unity ($N = 594$).

supernatant was transferred to polypropylene tubes and stored at −20 °C until analysis. Polypropylene tubes (1.5 ml per tube) in 96-well format used for storage of plasma samples were obtained from Biotech Solutions (Vineland, NJ, USA).

2.7. Data analysis

The Masslynx software package version 4.1 (Waters Corp.) was used for automatic quantification of all reference compounds. Peak area was used for quantification.

The concentration–time profiles from PK screening studies were non-compartmentally analyzed using the appropriate library model included in the WinNonlin 4.0 software (Pharsight Corporation, Mountain View, CA, USA). In case of discrete sample MS analysis the PK parameters for each individual rat were calculated, and in case of cassette MS analysis the PK parameters for each individual compound were calculated. Non-compartmental analysis is based on the integration of the raw concentration–time profile. The linear trapezoidal rule was used any time that the concentration data was increasing, and the logarithmic trapezoidal rule was used any time that the concentration data was decreasing. Data is reported as mean ± SD.

3. Results

3.1. Effect of multiple vehicles on chromatography

The results from the in vitro ion suppression check showed that addition of PEG 400 resulted in a response difference > 30% for imipramine and propranolol. Peak areas for these two analytes were reduced with 50%. This could be explained by their short retention time on the column resulting in co-elution with PEG 400 (Fig. 1A). None of the other formulations affected the response more than 20%.

3.2. Comparison of cassette and discrete analysis of PK curves

Pooling reduced the number of samples from 144 (3 discrete PK studies) to 48 (1 cassette set). Rapid UPLC–MS/MS using a generic 1.1 min gradient was used for the bioanalysis. This resulted in a final analysis time of 130 min for 3 studies including calibration curves.

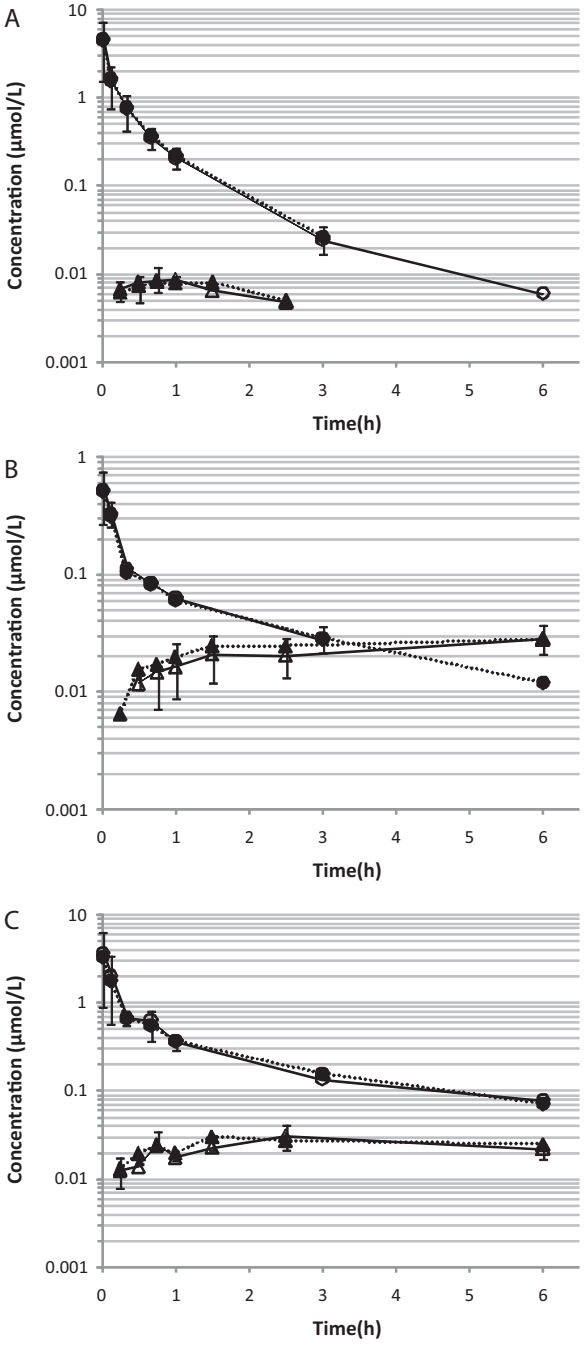

Fig. 3. The intravenous (circles) and oral (triangles) PK curves from lidocaine (A), haloperidol (B) and perphenazine (C) that were analyzed as one cassette (open symbols) and as single compounds (closed symbols). Note that with lidocaine the 6 h point of the iv curve is missing with discrete analysis, and that with haloperidol the 15 min point of the oral curve and the 6 h point of the iv curve are missing with pool analysis.

The UPLC method was successfully used for chromatographic separation and quantification of all compounds except naproxen. Fig. 1B shows a typical chromatogram with lidocaine, haloperidol, perphenazine and warfarin (internal standard). With naproxen the individual PK curves were highly variable, and similar points in cassette and discrete analysis could vary over 1000 times. This is likely to be due to a technical issue and the compound has been excluded from further analyses.

In general excellent correlation was obtained between the concentrations in the samples determined by discrete and cassette

analysis (r^2 = 0.986; Fig. 2). 1008 PK samples were analyzed and 630 of those showed concentrations above the respective LOQ. Of these, 100 samples (16%) deviated more than 25% between cassette and discrete analysis. A closer examination of these samples showed that 56 samples were close to the LOQ (within fivefold). 5% (32 samples) of the analyzed samples were observed when analyzed discrete, but were lost when analyzed in cassette format. 2% (11 samples) of the analyzed samples was detected when analyzed as cassette, but not when analyzed discrete. The LOQ with discrete and cassette analysis are listed in Table 1 for all compounds. For most compounds the LOQ was 5 nM with discrete analysis and 5 or 10 nM with cassette analysis. For phenytoin, morphine and diclofenac the LOQ increased threefold from 30–45 to 110–160 nM after sample pooling. The lower standards for rofe-

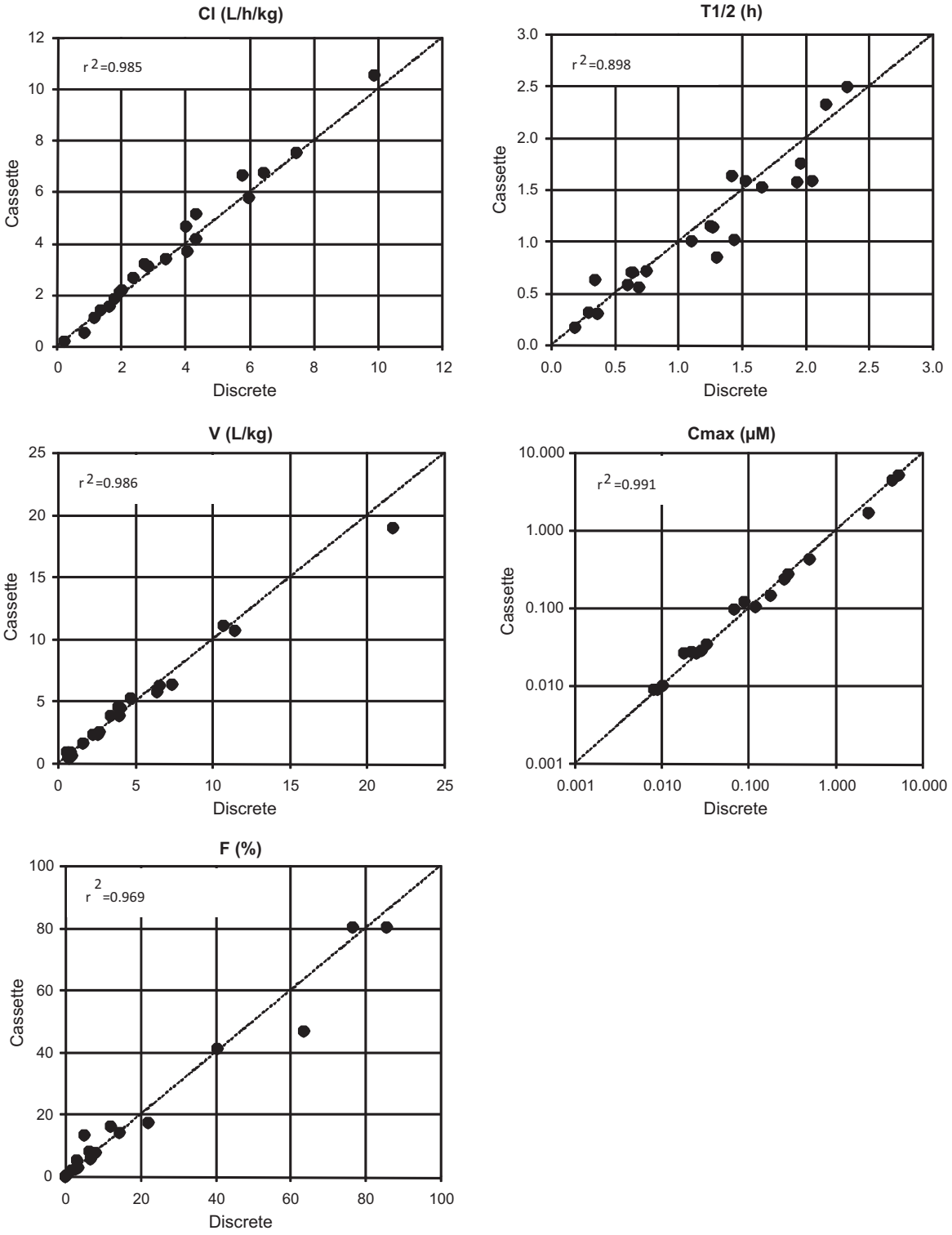

Fig. 4. Correlation between the average PK parameters calculated from the PK curves obtained after cassette and discrete analysis. The compounds were dosed discretely. Each data point represents the average parameter estimate per compound (N = 22). The r^2 values were 0.985; 0.898; 0.986; 0.991 and 0.969 for CL, $T_{1/2}$, V, C_{max} and F, respectively.

Table 2
Criteria used to group the PK parameters.

	Low	Medium	High
Cl (L/h/kg)	<1.3	1.3–2.9	>2.9
VD (L/kg)	<0.7	0.7–3.0	>3.0
$T_{1/2}$ (h)	<0.5	0.5–5.0	>5.0
F (%)	<10	10–50	>50

coxib analyzed in discrete mode did not meet the accuracy criteria and were therefore excluded. These four compounds were responsible for the majority of the deviations described above: 31 samples deviated over 25% between cassette and discrete analysis and 16 samples were below the LOQ in cassette but not in discrete analysis.

PK curves of a typical cassette analysis together with the PK curve of each compound obtained from discrete MS analysis are displayed in Fig. 3. The average curves analyzed discrete and as cassette showed good agreement with each other, as well as the calculated PK parameters, even though 6 samples of perphenazine, 3 samples of lidocaine and 5 samples of haloperidol deviated more than 25% between the cassette and discrete analysis. Moreover, in case of lidocaine, one sample was present with cassette MS analysis only and for haloperidol, two samples were only detected with discrete MS analysis.

Fig. 4 shows the correlation including the correlation coefficients of the different PK parameters calculated from the curves obtained after discrete and cassette analysis. Each point represents the PK parameter estimate for one compound. An excellent correlation was seen for all parameters, although half life showed somewhat more scatter than the others. Another way of analyzing PK data is so-called box-analysis. In this analysis PK parameters are binned in intervals rather than looking at the exact number (Table 2). This type of analysis is often used in early Drug Discovery when a general impression of the PK properties of a chemical series is desired, rather than on a compound level. The binned analysis method is also recommended for cassette dosing [7]. In Fig. 5A can be seen that CL, V, $T_{1/2}$ and F were similar between discrete and cassette analysis for 90%, 86%, 95% and 90% of the total number of compounds tested, respectively. Some additional improvement was achieved if compounds with poor mass response were removed from the analysis (Fig. 5B). Thus, although relatively many individual samples (16%) deviated over 25% between discrete and cassette analysis, this did not translate into a large erroneous characterization of PK parameters, which is important for decision making.

4. Discussion

Industries have introduced strategies to reduce the number of samples such as cassette analysis to meet the continuous demand for increased throughput of pharmacokinetic (PK) studies. However, although used as a screening method, it is important to verify that the quality of these PK studies during the late Lead Identification and Optimization phase is maintained in order to secure effective decision making. These PK studies serve many purposes. It is a first reality check for a potential candidate after a battery of in vitro tests and poor PK properties may lead to a decision to stop the compound from progressing. It guides medicinal chemists in the optimization process and assists pharmacologists to set doses and optimize timing for measurement in efficacy studies. It is used for simulation purposes and for early pharmacokinetic/pharmacodynamic modelling. Moreover, the PK study is used for interspecies scaling. The current findings show that the curves obtained after pooling are identical to the discrete curves, and thus pooling of 3 samples does not seem to affect the usage and decisions based on the PK studies.

To increase the throughput of standard PK studies, it was decided to investigate the possibility to analyse the samples by pooling 3 similar time points from animals dosed with different compounds. This pooling approach does perhaps not give the greatest sample reduction, but it limits the time required for the mass spectrometry analyst for checking and possibly setting up appropriate system conditions. This has previously been reported to be problematic for the analysis of large cassette dose studies [4,7]. In combination with rapid sample preparation, 3×3 studies could be worked up and analyzed per day by one person. This way of pooling also conserves all the features from a PK study as obtained by traditional bioanalysis. There is no risk for in vivo drug–drug interactions nor are there dose restrictions as is the case with cassette dosing. Besides this, individual PK curves per animal are determined, which allows for identification of outliers, experimental errors, e.g. wrongful dosing or mixed-up samples, and a feeling for the spread in the data. This information is lost when only an average curve is constructed by pooling all animals that received the same dose regimen. A disadvantage of the presented method is that compounds with similar molecular weight or expected metabolites with molecular weight of the analytes cannot be pooled together. In this high-throughput method the peaks are not separated, and thus multiple peaks with nearly similar mass may lead to analytical complications. In reality, however, multiple programs are run that utilize different chemistry, and thus the above-mentioned drawback can easily be avoided.

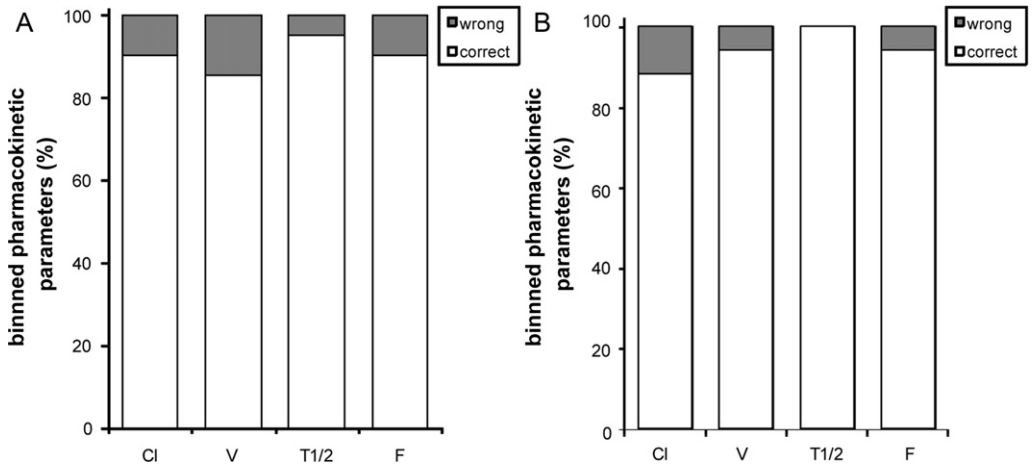

Fig. 5. PK parameters were binned according to the criteria summarized in Table 2. 'correct' was assigned if the PK parameter after discrete and cassette analysis was assigned to the same group. (A) All compounds included; (B) compounds with high LOQ excluded (morphine, phenytoin, rofecoxib and diclofenac).

The presented method appears to be sufficiently robust to guarantee reliable PK parameters. Examination of the method using spiked samples of plasma generated an accuracy and repeatability within 25% for all tested analytes (data not shown), which is the criterion used in our lab in our screening assays [11]. Other industrial screening facilities apply similar criteria [4,8,12]. Although these criteria are not nearly as stringent as those applied to bioanalytical data intended for regulatory agencies, their main purpose is to provide confidence in the results reported. We observed, however, sometimes deviations larger than 25% between single and cassette analysis. Half of the samples that showed too large deviations were around the LOQ value, i.e. low nanomolar range, where small differences in absolute numbers result into large relative deviations (Fig. 2). Still, the other deviating samples contained higher concentrations, which is a problem from an analytical perspective. One might speculate that these discrepancies are caused by clogging of the pipet tip or lack of plasma during the robotized pipeting. If so, the method could be further improved by using sensitive tips. The impact of these occasional individual samples, however, that do not pass the criterion is little on the average PK parameters of 3 rats. As an example, 9 samples did not reach the criterion for the oral curve of trifluoperazine but quite similar C_{max} and bioavailability values were produced, 20 nM and 3% after discrete and 30 nM and 5% after pooling. The differences between cassette and discrete analysis around LOQ, however, contribute to the increased scatter in the terminal half life. The deviations observed here are comparable with those reported previously [4].

We identified a few circumstances when cassette analysis combined with rapid UPLC–MS/MS might be less suitable. PEG400 showed ion suppression of a few test compounds, which is in agreement with previous findings [13]. We recommend not to pool compounds when PEG400 has been used in the formulations, but many other vehicles did not cause analytical challenges. Moreover, a few compounds showed poor response in the mass spectrometer leading to a high LOQ when analyzed as a cassette. This increases the risk that unnecessary many samples get values below LOQ, resulting in unreliable PK curves. Indeed, it was shown that some improvement in the accuracy of the PK estimates was achieved when these compounds were omitted from the box analysis. Theoretically, increasing the injection volume into the MS instrument could help to reduce the LOQ, but this was not investigated.

In summary, cassette analysis is an effective strategy to reduce samples without affecting the estimated PK parameters that are important for decision-making. Caution should be taken in including compounds that show reduced response in the mass spectrometer.

Acknowledgments

The authors would like to express their gratitude to Ulrika Määttä, Ingemo Sjögren, Annica Rask and Sofia Martinsson for their formidable biotechnical contributions.

References

[1] W.A. Korfmacher, K.A. Cox, K.J. Ng, J. Veals, Y. Hsieh, S. Wainhaus, L. Broske, D. Prelusky, A. Nomeir, R.E. White, Cassette-accelerated rapid rat screen: a systematic procedure for the dosing and liquid chromatography/atmospheric pressure ionization tandem mass spectrometric analysis of new chemical entities as part of new drug discovery, Rapid Commun. Mass Spectrom. 15 (2001) 335–340.
[2] B. Liu, J. Chang, W.P. Gordon, J. Isbell, Y. Zhou, T. Tuntland, Snapshot PK: a rapid rodent in vivo preclinical screening approach, Drug Disc. Today 13 (2008) 360–367.
[3] C.E. Hop, Z. Wang, Q. Chen, G. Kwei, Plasma-pooling methods to increase throughput for in vivo pharmacokinetic screening, J. Pharm. Sci. 87 (1998) 901–903.
[4] H.K. Han, N. Sadagopan, G.A. Reichard, U. Yapa, T. Zhu, A. Hubbel, K. Johnson, J. Brodfuehrer, An efficient approach for the rapid assessment of oral rat exposures for new chemical entities in drug discovery, J. Pharm. Sci. 95 (2006) 1684–1692.
[5] B.S. Kuo, T. Van Noord, M.R. Feng, D.S. Wright, Sample pooling to expedite bioanalysis and pharmacokinetic research, J. Pharm. Biomed. Anal. 16 (1998) 837–846.
[6] P. Manitpisitkul, R.E. White, Whatever happened to cassette dosing pharmacokinetics? Drug Disc. Today 9 (2004) 652–658.
[7] R.E. White, P. Manitpisitkul, Pharmacokinetic theory of cassette dosing in drug discovery screening, Drug Metab. Disp. 29 (2001) 957–966.
[8] T.V. Olah, D.A. McLoughlin, J.D. Gilbert, The simultaneous determination of mixtures of drug candidates by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry as an *In Vivo* drug screening procedure, Rapid Commun. Mass Spectrom. 11 (1997) 17–23.
[9] Z. Cai, C. Han, S. Harrelson, E. Fung, A.K. Sinhababu, High-throughput analysis in drug discovery: application of liquid chromatography/ion-trap mass spectrometry for simultaneous cassette analysis of alpha-1a antagonists and their metabolites in mouse plasma, Rapid Commun. Mass Spectrom. 15 (2001) 546–550.
[10] C. Polson, P. Sarkar, B. Incledon, V. Raguvaran, R. Grant, Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 785 (2003) 263–275.
[11] S. Briem, S. Martinsson, T. Bueters, E. Skoglund, Combined approach for high-throughput preparation and analysis of plasma samples from exposure studies, Rapid Commun. Mass Spectrom. 21 (2007) 1965–1972.
[12] N. Sadagopan, B. Pabst, L. Cohen, Evaluation of online extraction/mass spectrometry for in vivo cassette analysis, J. Chromatogr. B 820 (2005) 59–67.
[13] X. Xu, H. Mei, S. Wang, Q. Zhou, G. Wang, L. Broske, A. Pena, W.A. Korfmacher, A study of common discovery dosing formulation components and their potential for causing time-dependent matrix effects in high-performance liquid chromatography tandem mass spectrometry assays, Rapid Commun. Mass Spectrom. 19 (2005) 2643–2650.