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Integrated sample collection and handling for drug discovery bioanalysis

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Abstract

An integrated sample handling process for drug discovery bioanalysis is described. The streamlining of study design, sample collection and automatic bioanalytical sample processing is demonstrated. Specific details for the entire procedure regarding the time saved, ease of automation and integration are defined. Details of sample handling involved a sample collection map, sample collection formatting and volume, dilution schemes for high concentration samples, choice of biological fluid and evaluating the capabilities of two liquid-handling workstations. Numerous comparisons were conducted between the new approaches and the conventional sample handling approaches. The precision and accuracy obtained from the new integrated sample handling process were comparable to those obtained from a conventional approach, as were pharmacokinetic profiles and parameters. This new sampling process greatly improved the efficiency of drug discovery bioanalysis. The integration of pre-clinical protocol design, sample collection and bioanalysis processes was also achieved. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Emerging technologies, such as combinatorial chemistry [1,2] and cassette dosing [3,4], have accelerated the drug discovery process. In response, the demand for bioanalytical practice has resulted in faster analytical techniques and higher sample preparation capacity. At present, one of the most widely used techniques in bioanalytical laboratories supporting pharmacokinetics, drug transport and metabolism is liquid chromatography-tandem mass spectrometry (LC/MS/MS) with multiple-channel-monitoring (MRM) [5,6]. This technique has greatly facilitated bioanalytical work, so that the most time consuming and labor

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intensive steps are still associated with sample handling. Recent studies have reported significant improvements in the sample preparation process. One area showing much progress is the automation of sample preparation by using various liquid-handling workstations. These workstations replace manual liquid transfers and use parallel sample preparation (e.g. 96-well plate) for higher sample throughput [7–9]. Workstations have proven useful by improving the sample preparation process through more effective extractions [10], better automation of extractions [11], reduction in scale [12] and use of automated method development [13]. These advances are reducing method development time and, in some aspects, increasing assay quality. These efforts, however, only focus on the sample preparation step of the bioanalytical process. Other than those sampling approaches utilized for on-line, in vivo microdialysis sampling [14], there appears to be no recent reports describing a comprehensive sample handling for the drug discovery process.

In drug discovery, processes closely associated with bioanalysis (BA) are study design and animal study conduct/sample collection. These three processes are often driven by groups operating sequentially but independently, or by equivalent, separate groups operating in parallel. Lack of integration or standardization in sample collection and information delivery, present in these processes, can result in inefficiency for sample collection/processing and break-down in information flow between the three groups. For example, an analytical chemist could spend considerable time transferring samples to a 96-well format prior to automated processing on a liquid handling workstation. At this point, it seems reasonable that greater process efficiency could be attained if a closer relationship between the separate processes of study design, sample generation, and bioanalytical sample preparation were developed.

In this paper, we report on an integrated sample handling process for discovery bioanalytical, based on biological sample collection directly in a 96-well format, taking into account the front-end preclinical protocol design and sample processing requirements. The purpose of this study is to demonstrate standardized and efficient sample handling procedures for drug discovery. A comparison of different sample collection formats and sample processing options, is reported here. Characteristic pharamacokinetic data obtained from the different approaches are also discussed and compared for four example compounds.

2. Experimental

2.1. Analyte test system, reagents and LC/MS experimental conditions

Analytes and reagents, such as drugs, organic solvents, blank rat plasma and HPLC mobile phase, were purchased or prepared from the same sources as those in the proceeding paper of this volume [15]. Briefly, diphenhydramine, desipramine, chlorpheniramine and trimipramine were the four compounds tested in the study. Lidocaine was added as an internal standard. The same LC/MS apparatus and sample preparation conditions as those in the last report were also used here.

2.2. Apparatus

A MultiProbe II (Packard Instruments, Meriden, CT) was used to transfer plasma and serum in this study. It was equipped with an x-, y-, z-coordinate robotic arm with four sampling tips. It has been optimized for aspiration and dispensation of different liquids with varying viscosities. In this study, small conductive disposable tips were used, together with the liquid sensing function when aspirating and dispensing (3 mm below the liquid surface). A Tomtec Quadra 96 model 320 (Hamden, CT) was used to handle all 96-well parallel-liquid transfers such as internal standard addition, organic solvent addition, organic layer transfers and reconstitution after nitrogen dry down. This semi-automated 96-well liquid extraction approach using a Tomtec workstation was introduced in the last study. An Eppendorf centrifuge (model 5810R, Hamburg, Germany) operating at 3000 rpms was a refrigerated bench-top centrifuge that could accommodate 96-well plates.

2.3. Drug administration and sample collection

Two studies were conducted to evaluate potential strategies for integration of sample collection step with sample processing. In each experiment, six male Wistar rats (three for oral, three for intravenous) were dosed. The animals were fasted for 12 h before drug administration. Drugs **I**–**IV** were dissolved in a 10% ethanol aqueous solution (4 mg/ml) for oral gavage. Intravenous infusion solutions contained 4 mg/ml of each drug, dissolved in a mixture of ethanol:dextrose (5%) aqueous solution (10:90 v:v). The total dose was 10 mg/kg for both oral and intravenous treatments. The time points for collection in each study were pre-dose, 30 min and 1, 2, 4 and 6 h. Whole blood samples were collected in either serum or plasma (with sodium heparin) separator tubes. Samples were placed directly into individual 1.1-ml polypropylene tubes in a 96-well tube-rack format (Costar, Cambridge, MA). The arrangement of the sample tubes in the rack will be discussed later.

For the first study, two plates of plasma samples were collected, with ≈ 400 μl of blood at each time point. In the first plate, exact plasma volumes of 25 μl (30 min and 1 h samples) or 100 μl (predose, 2, 4 and 6 h) were transferred to tubes in the 96-well plate rack. To the second plate, the remaining volumes of ≈ 120–220 μl were transferred and frozen (-20°C).

For the second study, three rats were dosed intravenously with the same four drugs. Both serum and plasma samples were harvested from the same animal, transferred into respective 96-well tube plates and frozen (-20°C) for quantitative comparison at a later time.

Pharmacokinetic parameters were calculated using WinNonlin software (version 2.1, Pharsight Corporation, Palo Alto, CA).

2.4. Experimental overview

2.4.1. Multiprobe precision assessment

Testing the precision of liquid transfer by the MultiProbe II was performed for plasma and serum. Assessment involved gravimetric determinations of transfer volumes.

2.4.2. Parallelism assessment

To evaluate the effect of different dilution approaches on quantitation, blank rat plasma was spiked with the four drugs at 1000 ng/ml. Several dilution approaches were evaluated, including direct assay of 100 μl of plasma, direct assay of 25 μl of plasma (dilution factor of 4) and 25 μl of spiked plasma plus 75 μl of blank plasma (dilution factor of 4). These synthetic samples were transferred into the 1.1-ml polypropylene tubes in 96-rack format by manual transfer and assayed ($n = 3$) for a comparative assessment of parallelism.

3. Results and discussion

3.1. Description of integrated sample handling process

A pharmacokinetic cassette study typically consists of a dosing solution of three to six compounds that are dosed orally in three animals and intravenously in three other animals. The total number of samples generated is 30–60, depending on the number of time points desired. The time spent in labeling, decapping and transferring samples to alternate formats is often significant. Use of the 96-well format can dramatically decrease the need for these steps, so it seems likely that integration of study design, sample collection and bioanalytical sample preparation would improve the efficiency of the preclinical drug discovery process.

Although automatic liquid handling workstations now replace manual transfer [7–9], they required standard sample format such as 96- or 384-well for maximum efficiency [13]. Traditionally in drug disposition studies, biological fluids (plasma, serum, urine, etc.) are collected in individual, capped tubes or bottles. Each tube is labeled individually with the collection time and animal number prior to dosing. The volumes of samples are varied. To work around the transfer of biological fluid samples from individual tubes to 96-well plates it is possible to either: (1) perform manual transferal of samples; (2) use a liquid-handling workstation to transfer samples;

or (3) initially generate and deliver samples directly to a 96-well format. In our laboratories, each of these approaches has been used to some extent, with manual transferal being least desirable. The use of a liquid handling workstation, such as the Packard Multiprobe, has shown some utility in transferring samples from individual vials to a 96-well plate. Most recently, however, we have adopted the latter approach: the initial generation and delivery of samples directly in a 96-well plate. Although not applicable to all types of sample matrices, most notably tissue samples, this approach can streamline the sample preparation approach by eliminating one or more sample transfer steps and by allowing 96-well sample preparation to proceed more efficiently.

Because 96-well is a universal format that fits various automatic workstations, it can be built into those steps preceding bioanalysis, including study design and sample collection. Fig. 1 depicts an integrated sample/information flow for a typical discovery phase ex vivo experiment such as a cassette dosing experiment. In this scheme, a discovery scientist designs a protocol and builds a sample list that is sent electronically to an animals models group. The animal models group executes the dosing protocol, collects and delivers samples to a 96-well plate along a predefined plate map (Fig. 2). At this point, some of the sample locations in the 96-well plate remain vacant to accommodate standards and controls that will be added later. From this point onward, the samples remain in the 96-well format, although they may be frozen, thawed, centrifuged, automatically transferred or otherwise processed in parallel by the bioanalytical chemist. After sample processing, a 96-well plate is delivered to an autosampler and injected into the LC/MS/MS system for separation, detection and quantitation. Quantitation results are reported to a Pharmacokineticist in a format that has been previously defined by the sample list and the study protocol. This approach offers a cogent, streamlined approach to sample collection and data handling for most discovery-phase experiments. Similar processes have been created for the collection of CACO-2, and other in vitro experiments.

The 96-well map (Fig. 2) is recommended here for any study where samples are to be harvested. If designed correctly, this map can provide a blueprint or a plan for study design, sample collection, sample assay, data reporting and data processing structures. The standard 96-well plate has eight rows, containing 12 wells in each row. In

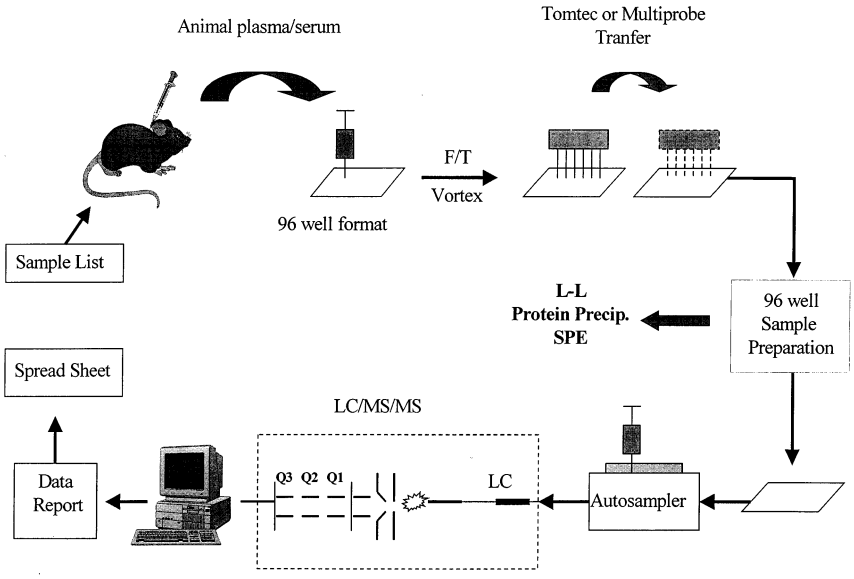

Fig. 1. Conceptual diagram of integrated sample handling process for discovery bioanalysis.

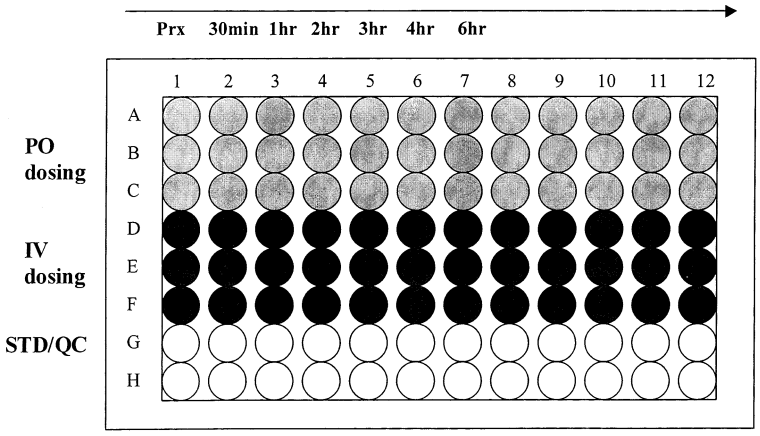

Fig. 2. A predefined 96-well plate map can give details of dosing protocol and sample collection.

our model, samples from orally dosed rats (one to three) were arranged in row A, B and C. Samples from the three intravenously dosed rats (four to six) were arranged in rows D, E and F. In each row, time points were assigned (e.g., 1st well for pre-dose, 2nd well for 15 min collection, 3rd well for 30 min collection, etc.). The collection volume may also be labeled in the map or in an accompanying spreadsheet. The 12 wells in rows G and H are reserved for standards, blanks or quality controls, as needed.

Although the 96-well or similar format is essential to the plan, 1.1-ml tubes were selected for containment of individual samples. The first consideration behind this decision was sample stability: samples can be capped and placed into a freezer in the 96-well format immediately after each time-point is collected. Without individual tubes, an entire plate would need to be put in and pulled out of the freezer repeatedly. Any associated freezing or thawing could cause analyte degradation and clot formation in plasma.

A second consideration, as detailed in the preceeding paper [15], is the ability to do automated or semi-automated extraction directly within the 96-well format. Liquid extraction is especially facilitated by individual tubes containing samples. A 96-well plate containing such tubes is placed in one stage of the Tomtec and a semi-automated liquid–liquid extraction is performed. In this strategy, no sample format conversion, labeling or randomization is necessary so that the time required for bioanalysis is greatly improved. In this way, a higher degree of integration and streamlining of the in vivo portion of the drug discovery process is achieved.

3.2. Parallelism studies

High level samples (15 and 30 min and 1 h) need to be diluted prior to assay. The precision and accuracy of several dilution methods were compared. The standard curve was constructed from 1 to 2000 ng/ml and a relatively high concentration (1000 ng/ml of drugs **I**–**IV**) was chosen for the test. In the first approach, 100 μl of plasma was directly assayed without dilution. In the second approach, 25 μl of plasma was directly extracted and a dilution factor of 4 was used. In the third approach, 75 μl of blank plasma was added to 25 μl of each plasma sample and a dilution factor of 4 was again used. In each case, three replicates were tested. The results, summarized in Table 1, suggest that the three approaches yielded results which were statistically indistinguishable and gave acceptable precision ($<2\%$ RSD except drug **IV** at 100 μl straight) and accuracy (generally $<7.2\%$ relative error, except for drug **IV** at $25+75$ μl blank). The second approach is commonly used in our laboratory because it offers equal performance with a smaller number of dilution steps. The reliability of this

approach appears to be excellent, while volume aliquoting is reduced. This experiment has practical importance in terms of simplifying the analytical procedure and providing guidance in the exact volume of sample necessary for collection. It seems likely that using a reduced sample volume without diluting plasma will be useful for other compounds in similar matrices.

3.3. Quantitative comparison of exact-volume-delivered samples and volume-transferred samples

To automatically convert samples to 96-well format, a MultiProbe II or similar workstation is commonly used because of its flexibility, good precision and accuracy. An evaluation of the performance of transferring biological samples such as plasma and serum was conducted to estimate precision and accuracy. Twelve individual tubes were weighted before and after transfer of 100 μl of plasma or serum. The imprecision associated with these transfers was $<0.5\%$ RSD for rat plasma using the multiprobe after brief centrifugation. The imprecision (RSD%) for manual transfers was found to be 2% RSD.

A comparison study between the collection of exact volumes of plasma and the collection of unknown (total) volumes of plasma was performed. For the exact-volume experiment, volumes of plasma were collected (25 μl at 30 min and 1 h, 100 μl at other time points) into a 96-well tube rack. In the total-volume approach, ≈ 200 μl of plasma was harvested and stored in the 96-well tube rack format. Subsequently, 50 μl of each sample was transferred by MultiProbe from the original collection vessel to a new 96-well tube rack. To compare the results of these different procedures, the post-dose concentrations of drugs **I**–**IV** were determined and are displayed graphically in Fig. 3. For each of the example compounds, the same pharmacokinetic profile was obtained. Differences in plasma levels between each of the four compounds ranged from 0.5 to 40%, but averaged $<12.5\%$. The high degree of similarity in concentrations obtained by the two approaches substantiates the reliability and applicability of the integrated sample handling process.

3.4. Brief comparison of serum and plasma

A comparison of the sample handling properties between serum and plasma was made for human and non-human ex vivo samples. Results of transfers using fresh serum or heparinized plasma were comparable: no clots were visible and none were detected with the clot sensing mechanism. All transfers were successful, with variance of $<1\%$ for 100 μl aliquots. Upon one freeze–thaw (FT) cycle, the sera remained clear, whereas all plasma tubes contained some turbidity. Visual inspection showed that many plasma tubes also contained some clotted material.

Statistical evaluation of 100 μl transfers of all plasma and sera, both fresh and frozen, produced similar precision ($N=47$ each, % RSD of 1.64 and 0.92%, respectively). However, an important factor was the failure rate for transfers. All transfers with sera were successful, regardless of the fresh or frozen history of the samples. Transfers

Table 1
Quantitative comparison ($n=3$) of three dilution approaches for high concentration sample assay[a]

Compound	100 μl, no blank plasma added			25 μl, no blank plasma added			25+75 μl blank plasma		
	Conc.	RSD%	RE%	Conc.	RSD%	RE%	Conc.	RSD%	RE%
I	1023	0.5	2.3	928	1.1	−7.2	975	1.7	−2.5
II	1013	1.7	1.3	1020	1.6	2.0	1049	1.4	4.9
III	1007	0.8	0.7	892	0.6	−10	954	1.4	−4.6
IV	977	5.6	−2.3	1056	1.3	5.6	808	1.1	−19

[a] **I**, diphenhydramine; **II,** desipramine; **III,** chlorpheniramine; **IV,** trimipramine. Samples contained 1000 ng/ml of each compound spiked into blank plasma.

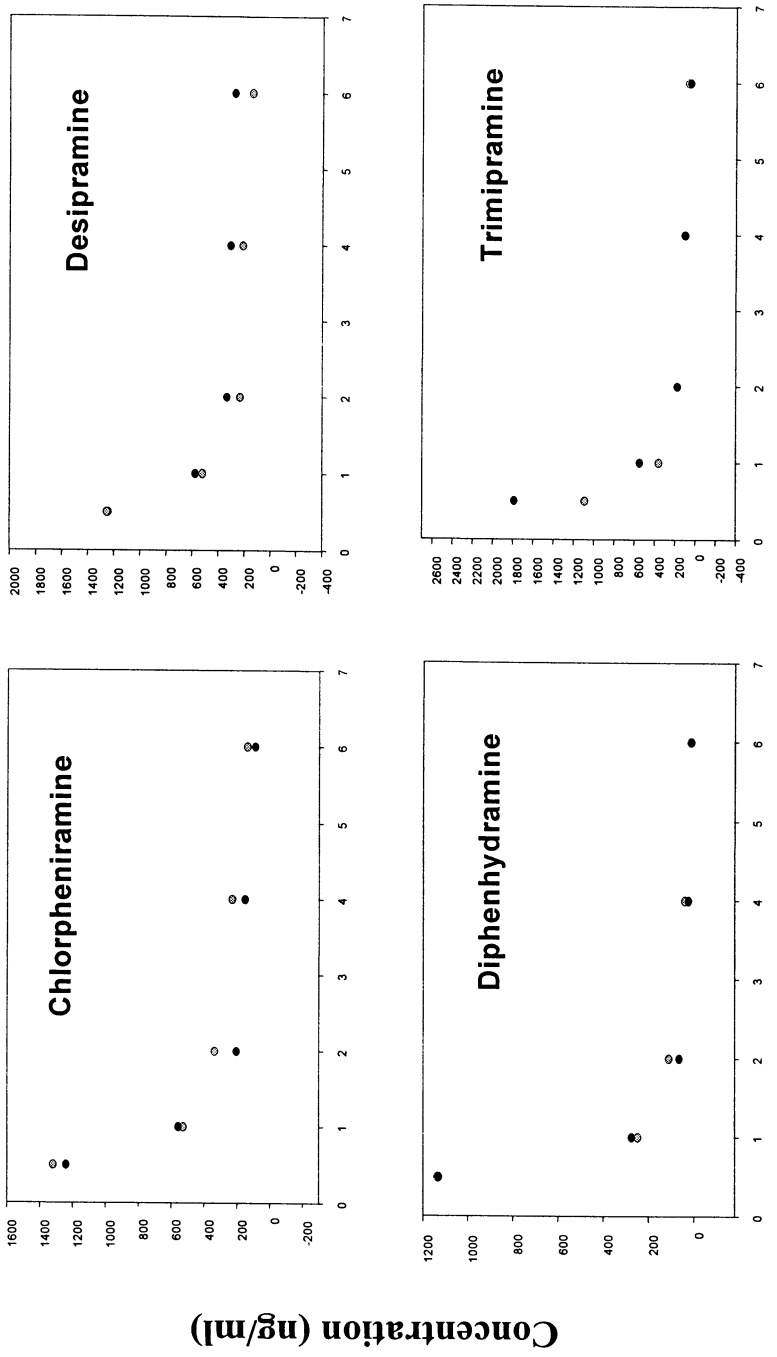

Fig. 3. Comparison of drug concentrations for four example compounds after sample processing by two approaches: (1) Exact volumes of plasma delivered to sample collection tubes in 96-tube rack. (2) Post-collection transfer of plasma to 96-tube rack by MultiProbe.

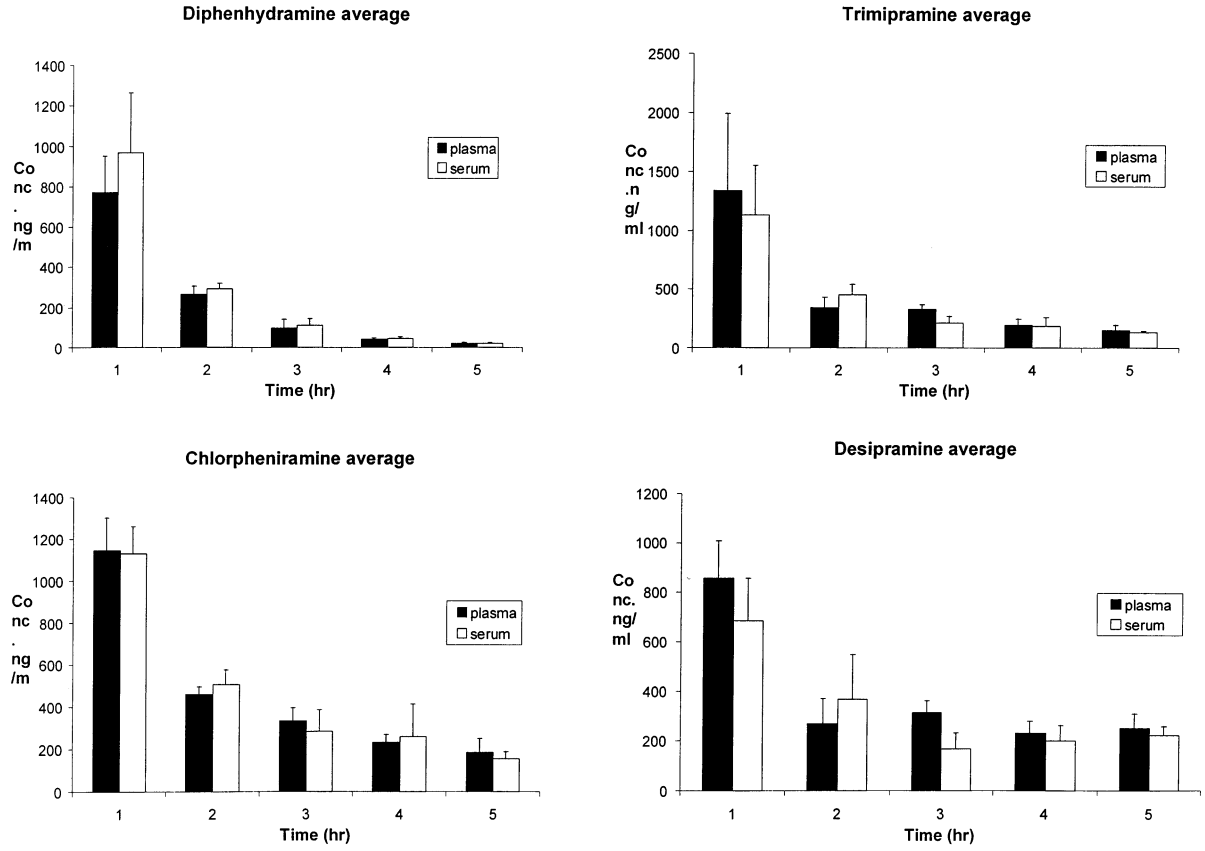

Fig. 4. Comparison of analyte concentrations in plasma and serum samples. The plasma and serum samples were collected from the same animal. The average concentrations of three animals at different time points are shown. The error bars are the S.D. among the three animals.

of frozen plasma samples from five of six patients contained clots that were detected by the clot sensor of the Multiprobe II. In some cases, the clotted samples were transferred successfully after several attempts. However, even when transfers of clotted samples were successful, precision sometimes was adversely affected. One of the most important findings of these studies was that when sample volume was limited, the presence of clots had the potential for a significant effect on precision and accuracy of automated transfers.

In contrast, transfers of all previously frozen plasma samples in which sample volume was not limited were successful when a centrifugation (14 000 rpm) step was performed prior to the transfer. Precision and success rates using centrifuged plasmas were comparable to those of sera. Centrifugation did not provide additional improvement for the transfer of sera.

To evaluate the drug concentrations in serum and plasma, both matrices were collected at all time points of the study protocol. The comparison of serum and plasma concentrations at each time point is shown in Fig. 4. The average differences in the four compounds between serum and plasma were: diphenhydramine 7.2%, desipramine 22%, chlorpheniramine 3.3% and trimipramine 14%. The results indicate that concentrations from serum and plasma were equivalent, to within the limit of experimental variability ($n = 3$). Concentration related pharmacokinetic parameters obtained from serum and plasma, summarized in Table 2, agreed well. Because of the limited time

Table 2
Characteristic pharmacokinetics parameters comparison from plasma and serum[a]

Test compound	Bioavailability (%)		C_{max} (ng/ml)	
	Plasma	Serum	Plasma	Serum
Chlorpheniramine (**I**)	31 ± 4.1	31 ± 4.7	233 ± 12	260 ± 42.7
Desipramine (**II**)	26 ± 10.4	26 ± 5.4	184 ± 54	210 ± 40
Diphenhydramine (**III**)	1.6 ± 0.66	1.7 ± 0.4	16 ± 2.4	16 ± 4.3
Trimipramine (**IV**)	3.9 ± 2.1	3.7 ± 0.3	27 ± 12.9	29 ± 8.6

[a] The data is presented as the average of three rats ± S.D. of three rats. The data for plasma and for serum were obtained from different doses.

points, other pharmacokinetic parameters such as $t_{1/2}$ were not compared.

In addition to the concern regarding clots in plasma, another issue was raised during the study. Lipid layers were frequently observed as an upper layer on samples, which affected the experimental results. This issue was addressed by using the MultiProbe II to control the transfer tip at the surface of the sample. A liquid sensor first identifies contact with the sample surface, then the program controls tip descent an additional specified distance. Plasma samples from some high fat rabbit plasma samples produced a considerable (25–50% of the volume) layer upon standing for several hours at room temperature. Lipids could be handled by directing the tip through the top layer, but this distance would have to be determined by inspection of the operator, as the clot or liquid detection was not effective at differentiating a lipid layer.

Although the issue regarding the lipid layer in centrifuged plasma can be addressed, a caveat appears to be the need to identify the minimum distance for additional tip movement. This is a particular issue in cases where sample volume is limited, because a maximum distance cannot be used by default. If the tip descends too far into the sample after sensing the liquid level, it could disturb a centrifuged clot, thus causing an error.

4. Conclusion

An integrated approach for study design, sample collection and bioanalytical sample preparation has been developed and presented. The integrated sample handling process is more efficient because numerous steps associated with sample transfer have been eliminated or automated. The standardization allowed more efficient information transfer between different study areas. A 96-well format has proved to be compatible with automated liquid–liquid extraction. Exact volume sample collection appears to be most efficient, but transfer from plate to plate can also be effectively accomplished and appears to be more efficient in terms of overall work flow. If the samples are serum, a high success rate can be anticipated whether the samples are fresh or frozen. In contrast, if the samples are heparinized plasma, a high success rate can only be anticipated if the samples are thawed and centrifuged prior to transfer. An additional factor, particularly in the case of heterogeneous high fat samples, is the volume of sample available. Comparison between serum and plasma showed effectively identical drug recoveries and pharmacokinetic properties for four example compounds.

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