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Collection, Storage, and Filtration of in Vivo Study Samples Using 96-Well Filter Plates To Facilitate Automated Sample Preparation and LC/MS/MS Analysis

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The benefits of high-throughput bioanalysis within the pharmaceutical industry are well established. One of the most significant bottlenecks in bioanalysis is transferring in vivo-generated study samples from their collection tubes during sample preparation and extraction. In most cases, the plasma samples must be stored frozen prior to analysis, and the freeze/thaw (F/T) process introduces thrombin clots that are capable of plugging pipets and automated liquid-transfer systems. A new approach to dealing with this problem involves the use of Ansys Captiva 96-well 20-μm polypropylene filter plates to collect, store frozen, and filter plasma samples prior to bioanalysis. The samples are collected from the test subjects, and the corresponding plasma samples are placed directly into the wells of the filter plate. Two Duo-seal (patent pending) covers are used to seal the top and bottom of the plate, and the plate is stored at down to −70 °C. Prior to sample analysis, the seals are removed and the plate is placed in a 96-well SPE manifold. As the plasma thaws, it passes (by gravity or mild vacuum) through the polypropylene filter into a 96-well collection plate. A multichannel pipet or automated liquid-transfer system is used to transfer sample aliquots without fear of plugging. A significant advantage of this approach is that, unlike other methods, issues related to incomplete pipetting are virtually eliminated. The entire process is rapid since thawing and filtering take place simultaneously, and if a second F/T cycle is required for reanalysis, it is not necessary to refilter the samples (additional clotting was not observed after three F/T cycles). This technique was tested using monkey, rat, and dog plasma and sodium heparin and EDTA anticoagulants. To assess the possibility of nonspecific binding to the polypropylene filter, a variety of drug candidates from diverse drug classes were studied. Validation data generated for two Lilly compounds from distinct classes, before and after filtering, are presented in this paper as practical examples of this technique. While LC/MS/MS is the primary method of bioanalysis in our laboratory, the technique presented in this paper is applicable to other forms of detection as well.

Owing to the competitive nature of pharmaceutical research and development, much time and effort have been invested in limiting the time required to perform bioanalysis, thereby decreasing the time required for a drug to reach the market. Because drug analysis is required during all aspects of drug discovery and development, gains in bioanalytical efficiency are applicable from early drug discovery through clinical evaluation. Liquid chromatography tandem mass spectrometry has proven to be a powerful tool for rapidly determining drug concentrations in biological matrixes[1,2] and in most cases has made sample preparation the rate-limiting step. Consequently, approaches are continuously being developed to minimize the time required to prepare samples for analysis. These approaches include handling plasma samples and their extracts in 96-well format,[3−6] the use of robotic workstations,[7,8] and column switching techniques.[9−14] These new techniques are themselves limited by the presence of thrombin clots in study samples and make the efficient transfer of plasma samples from their collection tubes one of the most rate-limiting steps during sample preparation.

While the advent of 96-well technology, multichannel pipets, and robotic workstations has made sample transfer more efficient, these techniques are limited by the presence of thrombin clots in the plasma samples. Depending on the species, storage temperature, and number of freeze/thaw cycles,[15] these clots can

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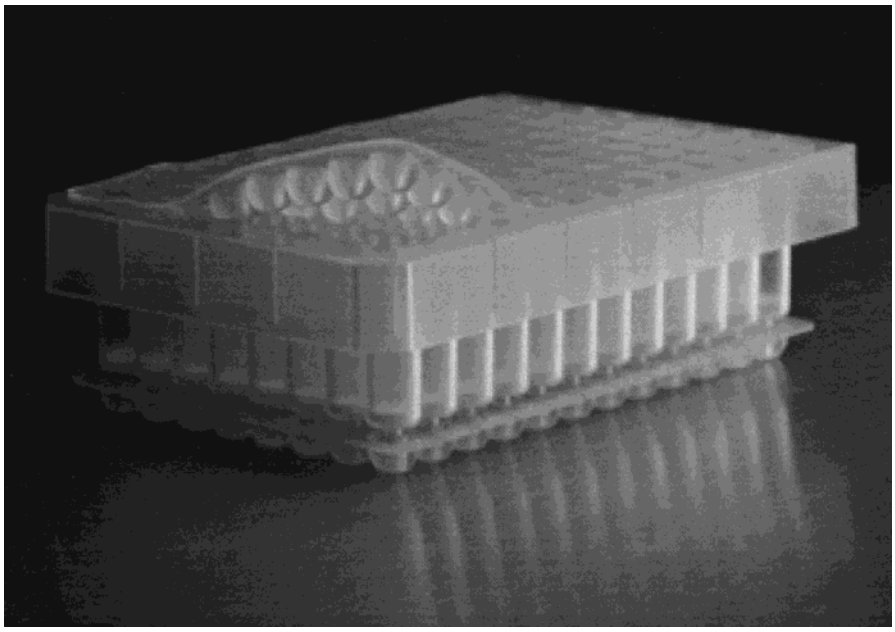

Figure 1. Captiva 20-μm polypropylene 96-well filter plate with top and bottom Duo-seals.

cause a pipet failure rate due to plugging that can approach 100%. In these cases, multiple transfer attempts are required to transfer study sample aliquots during preparation. This greatly reduces the overall efficiency of sample preparation and usually requires manual intervention when robotic systems are used. The use of the anticoagulant EDTA has been investigated to minimize the appearance of thrombin clots, but the results are mixed as to whether EDTA is more effective than the more commonly used anticoagulant, sodium heparin.[16,17] In our laboratory, we have found that EDTA reduces the pipet failure rate by reducing the number and severity of thrombin clots; however, the presence of clots in even a small percentage of the study samples remains problematic (unpublished data).

Our approach to dealing with thrombin clot formation is to collect, store frozen, and filter study samples prior to analysis using Captiva 96-well 20-μm polypropylene filter plates. Using this approach, in vivo samples are collected using Vacutainer tubes, and after a centrifugation step, the resulting plasma is placed in the filter plate and stored in the freezer. Duo-seals are used to seal both ends of the filter plate (Figure 1). Prior to sample analysis, the filter plate is allowed to thaw over a 96-well collection plate; the plasma passes the polypropylene filter as it thaws. Next, an octet pipet or robotic system is used to transfer the samples with 100% success with respect to thrombin clot plugging. In addition, we have found that additional freeze/thaw cycles necessary for reanalysis do not reintroduce clots to the samples, thereby eliminating the need to refilter.

When evaluating this technique, we were cognizant of the potential for nonspecific binding of analytes to the filter media. Polypropylene was chosen as the filter media due to its inertness and low incidence of this type of binding, and it is often used during all stages of sample preparation including sample tubes, 96-well plates, pipet tips, etc. Nonetheless, we evaluated the possibility of nonspecific binding using a variety of drug candidates from diverse chemical classes, and two examples are presented in this paper. Another potential problem is partitioning of the analyte into the thrombin clot; in this case, eliminating the clot during sample preparation would bias the resulting data. This phenomenon requires investigation on a case-by-case basis and has the potential to be a source of bias whether the samples are being filtered during sample analysis (due to the heterogeneity of the matrix).

By collecting the samples in 96-well format, we have greatly improved the efficiency of sample transfer by providing for the use of multichannel pipets and robotic liquid handlers. In addition, by concomitantly filtering and thawing the samples prior to sample preparation, we have eliminated time-consuming redundancy during sample transfer and have reduced the requirement of manual intervention during automated sample preparation. We have found the use of this technique to greatly improve the overall efficiency of bioanalysis during drug discovery and development.

EXPERIMENTAL SECTION

Solvents and Chemicals. Compounds EL-A, EL-B, EL-ISA, and EL-ISB were obtained from Eli Lilly and Co. (Indianapolis, IN). EL-A and EL-B are drug candidates from diverse therapeutic classes, and EL-ISA and EL-ISB are structural analogue internal standards of EL-A and EL-B, respectively. Due to the proprietary nature of these compounds, their chemical structures and proposed indications are not presented. Control plasma was obtained from animal services at Eli Lilly and Co. HPLC grade methanol, acetonitrile, and 2-propanol were obtained from Burdick & Jackson (Muskegon, MI). Analytical reagent grade formic acid (88%) was obtained from Mallinckrodt (Paris, KY), and water was from a Waters Milli-Q system (Bedford, MA).

Equipment and Methods. The HPLC system consisted of two Shimadzu (Kyoto, Japan) model LC-10AD pumps, a Shimadzu model SCL-10A VP system controller, a Shimadzu model DGU-14A vacuum degasser, and a LEAP (Carrboro, NC) HTS PAL autosampler. Chromatographic separations were performed on a MetaChem (Torrance, CA) monochrom C-18 column (2.1 $\times$ 50 mm, dp 5 μm). Mobile phase A consisted of 0.1% formic acid AQ,

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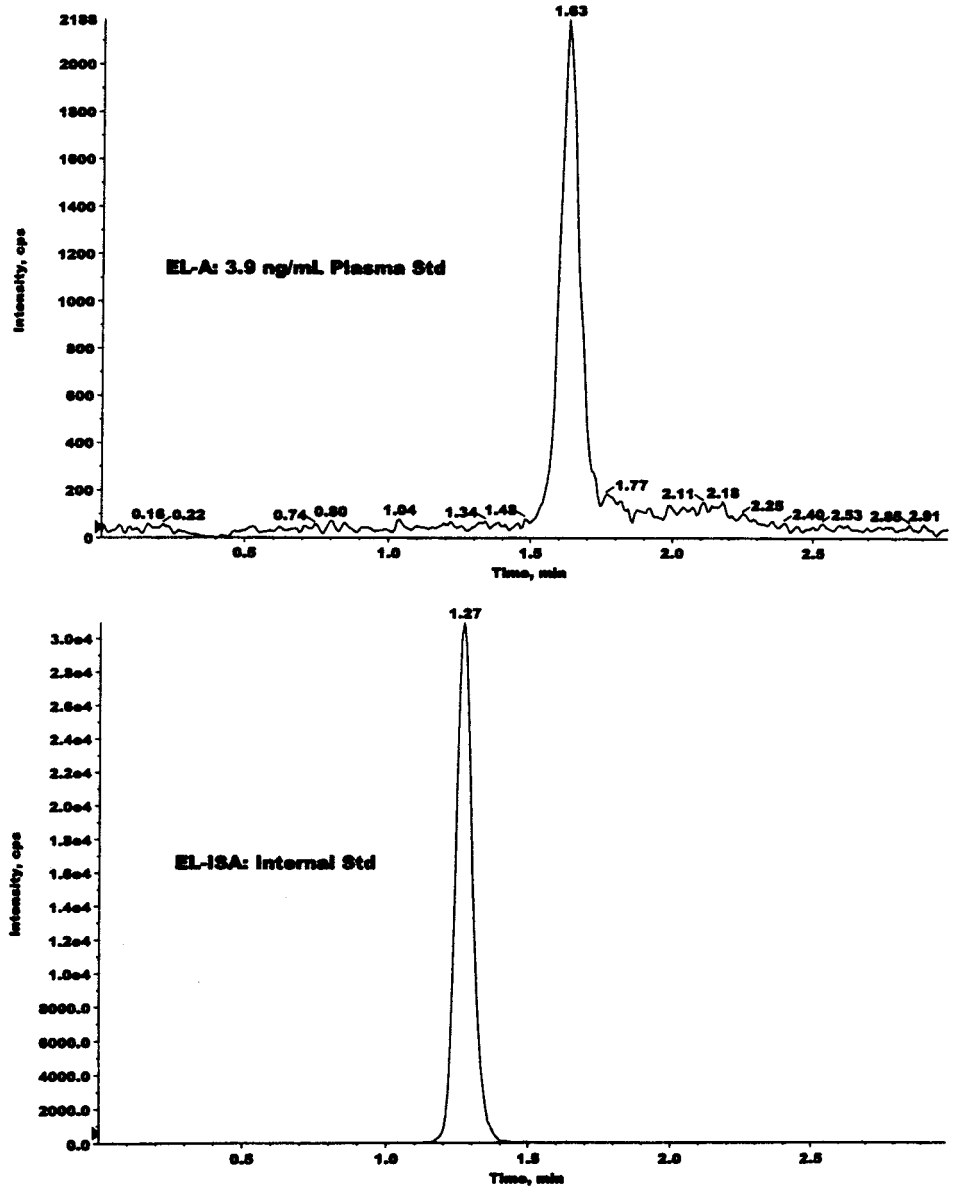

Figure 2. Extracted ion chromatograms of EL-A and EL-ISA from a 3.9 ng/mL extracted plasma standard.

and mobile phase B consisted of 0.1% formic acid in methanol. For EL-A and EL-ISA, a flow rate of 0.35 mL/min was used to deliver the mobile phase A and B gradient: (min/% B) 0.0/20, 1.7/60, 1.8/20, 2.75/20. For EL-B and EL-ISB, a flow rate of 0.35 mL/min was used to deliver the mobile phase A and B gradient: (min/% B) 0.0/20, 1.0/70, 1.1/20, 1.75/20. The injection volume was 20 μL for both sets of compounds.

Analyte detection and data acquisition were accomplished using a Sciex API 3000 LC/MS/MS system using PE Sciex Analyst version 1.1 software. The LC/MS/MS was setup in the positive turbo ion spray mode, and multiple reaction monitoring was used to detect the following analyte transitions: EL-A 416.4 → 183.4, EL-B 501.3 → 225.1, EL-ISA 468.3 → 99.1, and EL-ISB 518.2 → 268.0. Representative ion chromatograms of EL-A, EL-B, EL-ISA, and EL-ISB are presented in Figures 2 and 3.

Standard Solutions. Primary stock solutions of each of the analytes and the internal standard were prepared by dissolving 10 mg of each compound into 100 mL of methanol. A 400 ng/mL internal standard working solution was prepared by placing 80 μL of the 100 μg/mL primary stock into 20 mL of acetonitrile QS. A 4 μg/mL plasma standard was prepared for each analyte by placing 20 μL of the corresponding 100 μg/mL stock solution into 480 μL of control plasma. Additional 1:4 serial dilutions were performed by placing 100 μL of each standard into 300 μL of control plasma covering the range of 3.9−4000 ng/mL.

Sample Preparation. Study samples were placed into a Captiva 96-well filter plate after collection and were stored frozen at −70 °C. Prior to analysis, the samples were allowed to thaw and pass the filter into a 96-deep well collection plate. Depending on the application, an octet pipet or robotic system (Packard MultiProbe II or Tomtec Quadra 96) was used to transfer aliquots for sample preparation. For the applications presented in this paper, 50-μL aliquots were transferred using a Quadra 96 (model 320) into a 96-shallow well plate. The plasma proteins were precipitated by the addition of 100 μL of internal standard working solution. The samples were centrifuged at 4000 rpm (Jouan GR 4 22 centrifuge) for 10 min at 4 °C. Sample supernatants (50 μL) were transferred to a second 96-shallow well plate and were diluted by the addition of 200 μL of 0.1% formic acid AQ. The diluted supernatants were injected onto the LC/MS/MS for analysis.

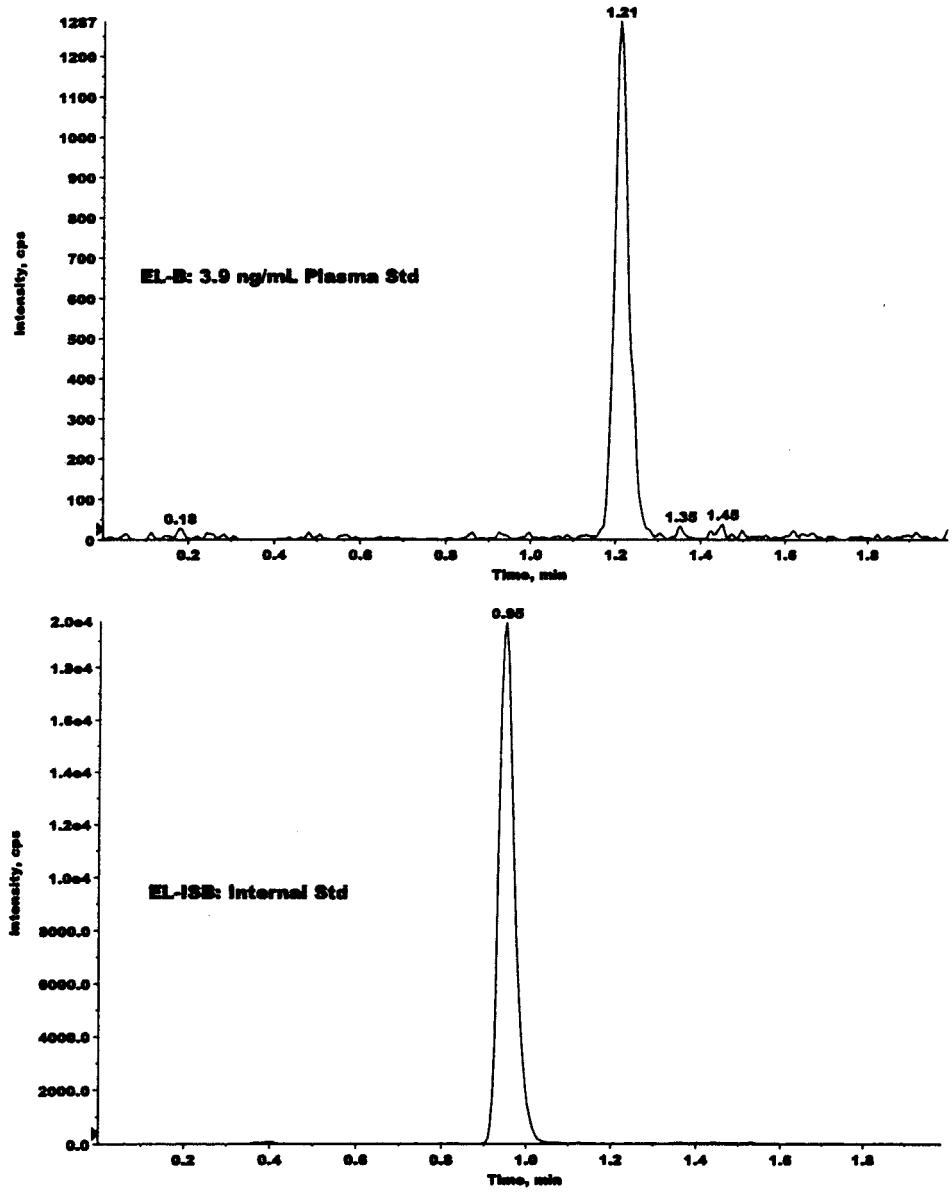

Figure 3. Extracted ion chromatograms of EL-B and EL-ISB from a 3.9 ng/mL extracted plasma standard.

Nonspecific Binding Analysis. A potential problem with filtering study samples prior to analysis is the issue of nonspecific binding to the filter media. To investigate the potential of this problem, we decided to test this technique using a variety of drug candidates from diverse chemical classes. Two sets of experiments were conducted. One, a series of standard samples was prepared (see Standard Solutions) using analytes EL-A or EL-B. The samples were split into two groups with each group containing two standard curves: one group was processed without the use of the filter plate, and the second set was processed after filtration using the Captiva plate. Next, the internal standard working solution was added to both groups, and the samples were prepared as described under Sample Preparation. The two groups of standards were analyzed along with a set of study samples, and the residual errors of the standard points were used to determine differences between the two groups of standards with respect to nonspecific binding. In the second experiment, animal studies were conducted, and the resulting plasma samples were stored frozen in the filter plate. On the day of analysis, aliquots of each sample were taken off the top of the plate (prior to filtering) and off the bottom after filtering. Both sets of samples were prepared and analyzed along with standard samples that were not filtered. The data from the two sets of samples were evaluated in terms of their percent difference and their pharmacokinetic properties. Due to the proprietary nature of the test compounds, only the results of the first experiment are presented here.

RESULTS AND DISCUSSION

We have identified the transfer of study samples from their collection tubes during sample preparation as a significant bottleneck during the bioanalysis of in vivo study samples. The inefficiencies are twofold: one, it is time-consuming to use single-channel pipets to transfer samples from collection tubes, and two, the appearance of thrombin clots after freezer storage dramatically increases the pipet failure rate. The use of 96-well plates and multichannel pipets has improved the efficiency of sample transfer but fails to address the issue of thrombin clot formation. Initially, we investigated the affect of anticoagulants and storage conditions on clot formation. We found that using EDTA instead of heparin helped reduce clotting in plasma samples, but the reduction was

Table 1. Statistics from the Determination of EL-A Pre- and Postfiltration Using a 96-Well Polypropylene Filter Plate

nominal, ng/mL	prefilter, average ($N = 2$)	difference, %	postfilter, average ($N = 2$)	difference, %	mean ($N = 4$)	standard deviation	% CV	accuracy
3.9	4.15	6.4	3.695	−5.3	3.9	0.48	12.3	100.0
15.6	16.55	6.1	14.3	−8.3	15.4	1.36	8.8	98.9
62.5	65.5	4.8	65.05	4.1	65.3	3.12	4.8	104.0
250	212.5	−15.0	252	0.8	232.3	25.40	10.9	92.9
1000	972	−2.8	908.5	−9.2	940.3	64.60	6.9	94.0
4000	4685	17.1	4095	2.4	4390.0	412.10	9.4	109.8

Table 2. Statistics from the Determination of EL-B Pre- and Postfiltration Using a 96-Well Polypropylene Filter Plate

nominal, ng/mL	prefilter, average ($N = 2$)	difference, %	postfilter, average ($N = 2$)	difference, %	mean ($N = 4$)	standard deviation	% CV	accuracy
3.9	3.7	−5.1	4.14	6.2	4.0	0.49	12.3	102.6
15.6	14.9	−4.5	14.6	−6.4	14.5	1.20	8.3	92.9
62.5	65.85	5.4	67.4	7.8	66.6	5.18	7.8	106.6
250	278	11.2	288	15.2	284.0	8.16	2.9	113.6
1000	1025	2.5	1015	1.5	1022.5	26.30	2.6	102.3
4000	3220	−19.5	3405	−14.9	3325.0	308.17	9.3	83.1

not sufficient enough to allow reliable sample transfer using octet pipets or robotics. Additionally, we found that storing samples at −70 versus −20 °C resulted in a positive but insufficient improvement in sample transfer. Therefore, we tested the use of 96-well filter plates to remove thrombin clots from the study samples prior to analysis. Using the filter plates, we were able to decrease the pipet failure rate from a worst case of near 100% (depending on the matrix, anticoagulant, and storage temperature) to 0%, resulting in a dramatic decrease in the time required for sample preparation. To eliminate another step, transfer to the filter plate, we developed a Duo-seal (patent pending) with Ansys Technologies to allow the samples to be stored frozen in the filter plate prior to analysis. Finally, we demonstrated that the plates would allow robotic liquid handlers to perform without the need for manual intervention to address pipet plugging, and when a freeze/thaw cycle was necessary due to reanalysis, we found that thrombin clots did not reappear (the samples did not require refiltering).

We had two primary concerns with using the technique outlined in this paper: one, would the analytes undergo nonspecific binding to the filter media, and two, what would be the impact on the accuracy and precision of the resulting determinations (even in the absence of nonspecific binding). The issues of nonspecific binding and accuracy/precision were evaluated as described in the section Nonspecific Binding Analysis. The results of the determinations are presented in Tables 1 and 2. For both EL-A and EL-B, there were no significant differences in analyte response, accuracy, or precision when comparing drug concentrations pre- and postfiltration. The precision and accuracy of EL-A ranged from 4.8% to 12.3% and 92.9% to 109.8%, respectively; and the precision and accuracy of EL-B ranged from 2.6% to 12.3% and 83.1% to 113.6%, respectively. In addition to the representative drug candidates presented here, similar comparisons were made using candidates from a variety therapeutic areas, and the data from these experiments corroborated the results presented here. In conclusion, nonspecific binding has not presented a source of error in determining drug concentrations when 96-well polypropylene filter plates are used, and the accuracy and precision of those determinations are well within the limits of acceptability (relative error and precision not exceeding ±25%) governing discovery mode bioanalysis.

The procedure outlined here is both fast and simple, and the success rate for transferring samples during preparation and extraction is increased to 100%. This translates into a dramatic improvement in sample preparation efficiency, especially when a robotic 96-well pipet such as the Tomtec Quadra-96 is used; when a robotic system capable of unattended operation is used (e.g., Packard MultiProbe II), sample preparation can occur without additional user intervention to address error messages related to pipet failure. There are two additional benefits when the filter plates are used for freezer storage: one, a time-consuming transfer step is eliminated, and two, the samples are filtered as they thaw, thus streamlining the process. With the emergence of LC/MS/MS, sample preparation is often the rate-limiting step. This technique should find broad applicability in bioanalysis by allowing bioanalytical chemists to take full advantage of plasma collection and storage schemes employing a 96-well format.

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