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Semi-automated 96-well liquid–liquid extraction for quantitation of drugs in biological fluids

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Abstract

A semi-automated liquid–liquid extraction (LLE) technique for biological fluid sample preparation was introduced for the quantitation of four drugs in rat plasma. All liquid transferring during the sample preparation was automated using a Tomtec Quadra 96 Model 320 liquid handling robot, which processed up to 96 samples in parallel. The samples were either in 96-deep-well plate or tube-rack format. One plate of samples can be prepared in approximately 1.5 h, and the 96-well plate is directly compatible with the autosampler of an LC/MS system. Selection of organic solvents and recoveries are discussed. Also, precision, relative error, linearity and quantitation of the semi automated LLE method are estimated for four example drugs using LC/MS/MS with a multiple reaction monitoring (MRM) approach. The applicability of this method and future directions are evaluated. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: 96-Well format; LC/MS/MS; Plasma; Parallel sample processing

1. Introduction

A number of recent papers have demonstrated the effectiveness of automated solid-phase extraction (SPE), in either 96-well or tube-based formats, as a practical and worthwhile application of laboratory automation to bioanalytical chemistry [1–3]. Until recently, major disadvantages of automated SPE were the extensive time required to develop procedures and the absence of commercially available systems which could process samples in parallel [2–4]. These barriers have been largely overcome by the introduction of workstations which are dedicated to SPE and which employ parallel sample processing approaches [5–7].

Liquid–liquid extraction (LLE) has long been popular as an alternative to SPE because reasonably effective bioanalytical methods could be developed quickly and the lot-to-lot variability associated with packed-bed or membrane media is not an issue. Liquid–liquid extraction (LLE) has been a favorite sample preparation approach among bioanalytical chemists doing liquid chromatography–tandem mass spectrometry (LC/MS/

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MS) because it desalts samples very well and has a little tendency to foul the mass spectrometer ion source. One major issue associated with LLE has been the difficulty in conveniently automating the phase separation process. The introduction of several versatile liquids handling workstations for the parallel processing of samples has greatly facilitated this task [8,9].

This work demonstrates the utility of such a workstation, a commercially available system applied to LLE which uses a parallel processing algorithm to improve sample throughput relative to manual serial processing. Extracted samples were subsequently separated by analytical scale liquid chromatography system prior to tandem mass spectrometry (LC/MS/MS). The capability of such a system for enabling a semi-automated LLE on biological fluid samples has been examined and characterized. Organic solvent selection, precision, recovery, linearity and quantitation limits for the extraction of four example compounds from plasma have been assessed and are reported here.

2. Materials and methods

2.1. Analyte test systems

A four-compound test system consisting of diphenhydramine (I), desipramine (II), chlorpheniramine (III) and trimipramine (IV) was used to evaluate the liquid extraction process. Lidocaine (V) was chosen as an internal standard. Structures for test compounds are given in Fig. 1. Compounds I–V were purchased from Sigma (St. Louis, MO). All compounds were used as received.

2.2. Reagents

Sodium hydroxide, ammonium acetate, potassium carbonate and acetic acid were obtained from EM Science (Gibbstown, NJ). Reagent grade chloroform and methyl alcohol were obtained from Mallinckrodt Baker, Inc. (Paris, KT). Reagent grade water was prepared from in-house deionized water using a Milli-Q system (Millipore, Millford, MA). HPLC grade ethyl acetate and methyl *t*-butyl ether were purchased from Burdick & Jackson (Muskegon, MI). Absolute alcohol was from AAPER Alcohol & Chemical Co. (Shelbvville, KT). Rat plasma (heparinized) was from Pel-Freeze Biologicals (Rogers, AK). All reagents were used as received, without further purification.

2.3. LC/MS/MS conditions

Compounds I–V were separated isocratically, using a mixture of 50% methanol, 50% 10 mM ammonium acetate (pH 4) as the mobile phase at room temperature and a flow rate 200 μl/min. The separation columns used were either YMC basic (3 μm, 2 × 50 mm, Wilmington, NC) or Phenomenex C-18 (5 μm, 2 × 50 mm, Torrance, CA). Liquid chromatographic separations were performed by using a quaternary solvent delivery system and autosampler (series 200, Perkin-Elmer, Norwalk CT). Injection volumes of 5 μl were used.

A Quattro II triple-quadrupole LC/MS system (Micromass, Beverly, MA) operating under MASSLYNX 3.1 software was used for all experiments. The Z-electrospray ion source was run in a positive ionization mode for all experiments, with parameters typically set as follows: Capillary 3.5 kV, skimmer 1.5 kV, RF lens 0.2 kV, source temperature 100°C, desolvation temperature 250°C. Quadrupole 1 (Q1) parameters were: low mass resolution 14 V, high mass resolution 14 V, ion energy 2 V, ramp 0 V, and lens 6.5 V. Quadrupole 3 (Q3) parameters were: low mass resolution 14 V, high mass resolution 14 V, ion energy 1 V, ramp 0 V, lens 8 40 V and lens 9 V. Multiplier 1 and 2 were set at 650 V, respectively. Test compounds were detected by mass spectrometry, using parent–daughter ion combinations. The multiple reaction monitoring (MRM) scan functions for each compound are given in Table 1. An interchannel delay of 0.03 s was used for all MRM experiments.

2.4. Semi-automated LLE procedure

Tomtec Quadra 96 model 320 workstation (Hamden, CT) was equipped with a 96-well pipetting head, using compatible 96 disposable tips in a

rack. It aspirates and dispenses liquid (0.5–450 μl) to 96 wells simultaneously. The six-station shuttle facilitates complete automatic pippetting sequences with the operator simply changing plates and reservoirs. In this study, the controlling program was written on a personal computer, then

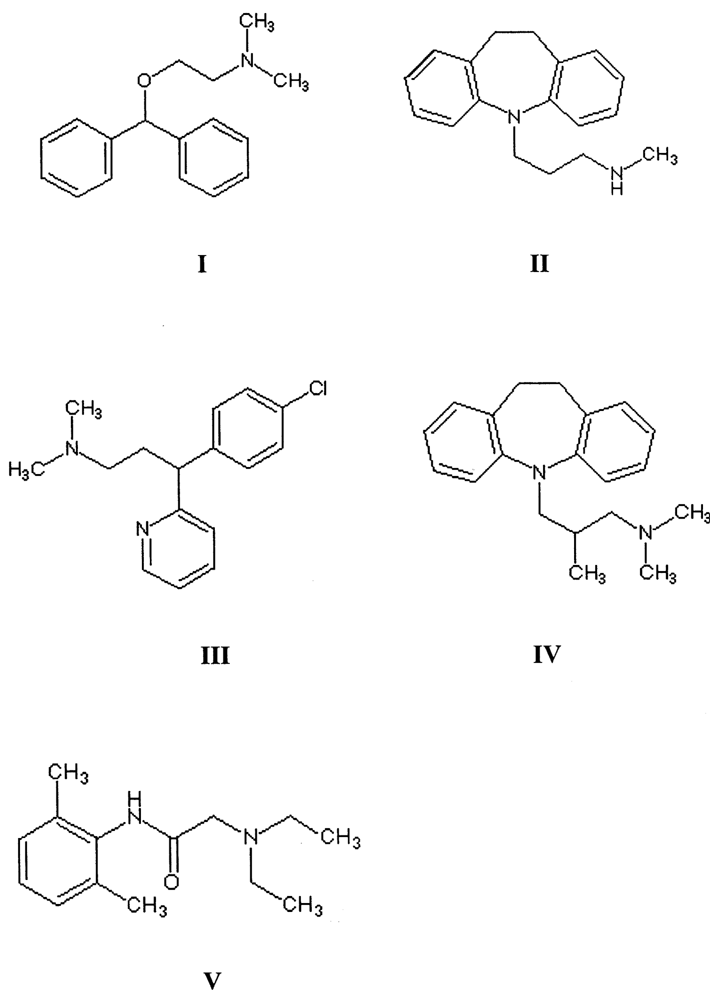

Fig. 1. Chemical structures for test analytes used in this work: (a) diphenhydramine (I), (b) desipramine (II), (c) chlorpheniramine (III) (d) trimipramine (IV), (e) lidocaine (V).

Table 1
MRM scan functions for diphenhydramine (I), desipramine (II), chlorpheniramine (III), trimipramine (IV) and lidocaine (V)

	Parent (*m/z*)	Daughter (*m/z*)	Dwell (s)	Coll energy (eV)	Cone energy (eV)
I	253.0	86.0	0.08	15	30
II	256.0	166.7	0.08	10	25
III	267.0	72.0	0.08	15	45
IV	275.0	229.9	0.08	15	35
V	295.1	100.0	0.08	15	50

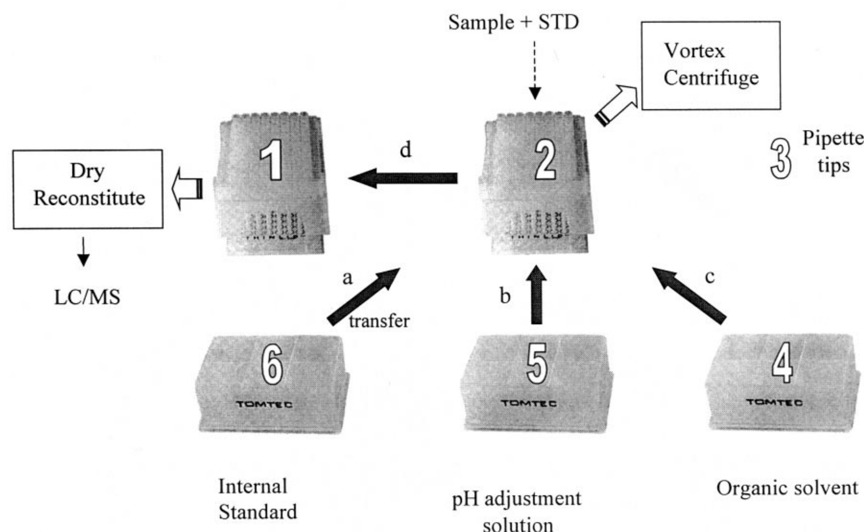

Fig. 2. Conceptualized semi-automated 96-well LLE procedure using Tomtec Quadra-96 liquid handling workstation. Refer to text for a stepwise description of the operation.

downloaded to the workstation by a serial RS-232 interface.

Drugs I–IV were spiked into blank rat plasma to form standards with the following concentrations: 2000, 1000, 500, 250, 100, 50, 25, 10, 5, 2.5 and 1 ng/ml in glass test tubes. An aliquot (100 μl) of each standard was added to a rack containing 96 miniature plastic tubes (1.1 ml) in a rack (Costar, Cambridge, MA). The rack was placed in stage 2 of the workstation, as shown in Fig. 2. The workstation contained reservoirs for 10 ml of 200 ng/ml of internal standard (compound V, prepared in 70% water–30% acetonitrile), 20 ml of pH adjustment solution (0.1 M KOH/K_2CO_3, pH 12), 50 ml of organic solvent for extraction and a clean 96-deep-well plate in stages 6, 5, 4 and 1, respectively. For liquid transfers, air gaps (25 or 50 μl) were programmed before and after each aspiration so that all the liquid would be blown out when dispensing. The air gaps also prevented the liquid from seeping out of the transfer tip before dispensing.

Aliquots (25 μl) of internal standard were transferred first from the reservoir at stage 6 to the sample plate at stage 2(a), then 100 μl of pH adjustment solution was transferred from stage 5 to 2(b). Two aliquots of organic solvent, 400 μl each, were transferred from stage 4 to 2(c). The 96 well-rack on stage 2 was then manually capped and shaken for 10 min, followed by centrifugation at 4000 rpm for 5 min. The rack containing extracted samples was then returned to stage 2. The organic layer was transferred (2 × 350 μl) from the 96-well tube rack to a clean 96-well deep-well plate (Beckman, Fullerton, CA) on stage 1 by carefully choosing the height of the transfer stage so that only organic layer was transferred (d). If a denser than water organic solvent such as chloroform was used, the stage height was adjusted so that the disposable tips were close to the bottom of the tube to siphon chloroform gently away from beneath the aqueous layer. The organic solvent was dried gently by passing nitrogen gas to each of the wells, using a 96-well drying block. The drying time varied among different solvents, usually ranging between 15 and 30 min. Reconstitution of sample residues was finished by repeating the second transfer process (stage 5 to 2), and simply switching the reservoir on stage 5 with 50:50 methanol:H_2O, as well as the reservoir on stage 2 with the 96-deep well plate containing sample

residues. A fully processed plate was now autosampler compatible and ready for LC/MS/MS injection.

3. Results and discussion

3.1. Chromatographic performance

The extraordinarily high selectivity of tandem mass spectrometry operated under multiple reaction monitoring mode permits the use of fast LC columns for the quantitation of drugs from biological samples [9,10]. The major role of the LC system is automated sample introduction. Moderate capacity factors ($k' = 1-5$) are, however, still necessary to prevent ion suppression caused by salts or matrix components eluting at the void volume. Several mobile phase systems were tested: combining different percentage of (a) Methanol and 0.1% formic acid in water, (b) acetonitrile and 0.1% acetic acid in water or (c) methanol and 10 mM ammonium acetate at pH 4. Compounds I–V had almost no retention using (a) and (b) on various 5-cm long HPLC columns. Mobile phase (c) provided appropriate separation of drugs I–V when an organic/aqueous composition of 50:50 (v:v) was chosen. The representative MRM chromatograms are shown in Fig. 3. Using this chromatographic system, the capacity factors for the four model drugs (from top to bottom traces) were 8.2, 2.4, 6.9, 3.0 and 1.1, respectively, with retention time ranging from 0.8–5 min. Chromatographic peaks displayed excellent symmetry (tailing factors between 1.0 and 1.5 [11] and relatively high efficiency (N ranging from 383 to 2168 plates). Chromatographic selectivity was characterized by an absence of channel cross talk, and no endogenous matrix components were detected.

3.2. Solvent evaluation and analyte recovery

Using LLE for sample preparation, general rules must be followed. These rules include the immiscibility of the aqueous and organic phases, acceptable analyte recovery and minimal interference from matrix components, as well as high volatility for easy dry down. For automation and cassette dosing [12], the optimal organic solvent should provide good recoveries for all drugs in the cassette. A solvent should also have moderate viscosity for reliable automated pipetting, yet be easy to evaporate to dryness. Four different solvents were tested. The polarities of these solvents increase in the order from methyl *t*-butyl ether (2.5), 95% methyl *t*-butyl ether with 5% ethanol (3.2), chloroform (4.1) and ethyl acetate (4.4). A graphical representation of recoveries for the four drugs from rat plasma included in this study is shown in Fig. 4. Ethyl acetate was chosen over the other solvents because it gave relatively good recoveries (45–60%) for all four compounds, also it was more convenient to pipette than chloroform because it occupied the top layer of the biphasic system.

The ratio of organic to aqueous solvent in the 96-deep well extraction vessels was 3.6:1. The extraction recovery could be improved if this model ratio were to be increased. Also, as a compromise to the automated aspects of the phase separation, approximately 100 μl (13%) of the organic layer is wasted during automated transfer. It could be possible to increase the recovery of the extraction step by further refining the phase separation step. One possible way to do this without the loss of ruggedness is to freeze the aqueous layer, then more aggressively position the workstation tips close to the solid–liquid interface. The trade-off here was between analyte recovery and keeping the extract free from matrix particles that reside near the phase interface. It seemed prudent to err on the side of caution when automatically performing the phase separation, especially when other method performance benchmarks, such as precision and quantitation limit were acceptable.

3.3. Method performance

A summary of the method performance, including precision, accuracy and detection limits is shown in Table 2. All parameters were acceptable. Standard curves ranging from 1 to 2000 ng/ml of compounds I–IV provided back-calculated standard concentrations. Analyte carry-over, assessed by the preparation, extraction and injection of

blank plasma samples with and without internal standard, showed small analyte peaks equaling approximately 20–30% of the low standard. These small peaks were manageable and did not affect the quantitation in an appreciable way. The precision of determined drug concentrations at low (5 ng/ml), medium (100 ng/ml) and high (1000 ng/ml) levels were calculated based on replicates from four standard curves. Relative standard deviation (RSD %) did not exceed 9.2%, except for trimipramine at 5 ng/ml where the % RSD was 22.0. The accuracy, calculated by the mean of the four concentrations generally ranged from − 1.7 to 6.5% relative error (% RE), except for diphenhydramine at 5 ng/ml (12.0%). Limits of quantitation were 2.5 ng/ml for each of the four drugs in the assay method. Lower concentrations (to 1 ng/ml) could be detected (S/N > 3) but not reliably quantified. The correlation coefficients of each standard curve reached 0.999 or better in most cases. These numbers clearly indicated the reliability of the workstation and the analytical method.

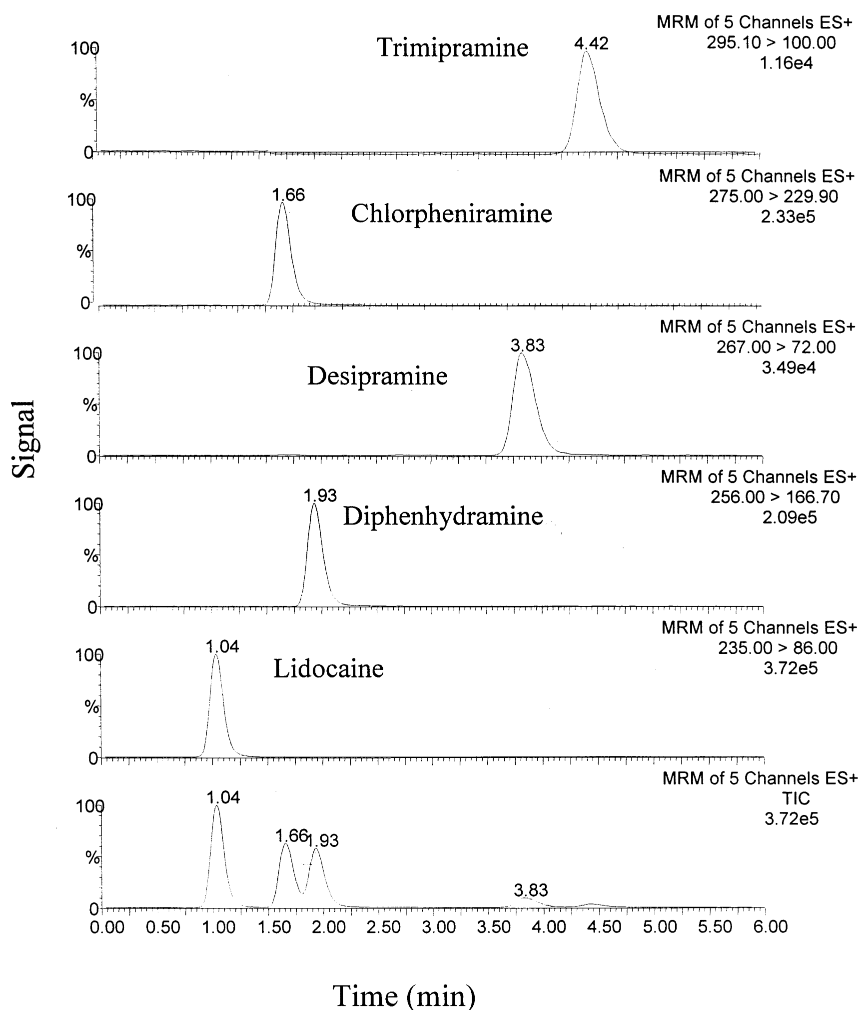

Fig. 3. Representative MRM chromatograms of drugs I–V from extracted rat plasma and internal standard drug V. The concentrations for drugs I–IV are 100 ng/ml (spiked).

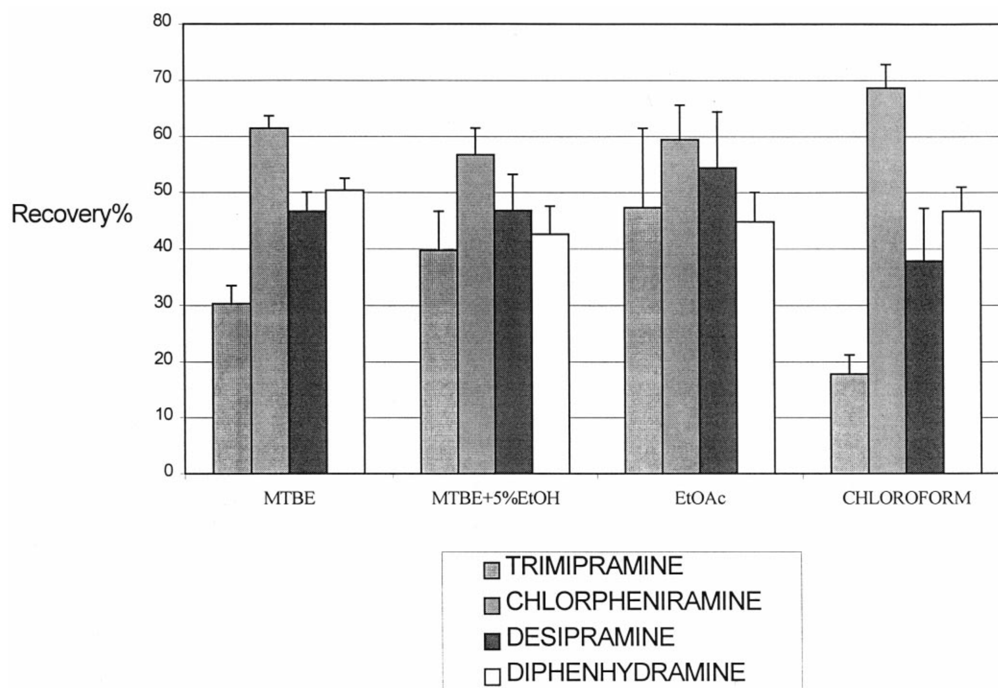

Fig. 4. Summary of recoveries for the semi-automated LLE as a function of analytes and organic solvents. Bar heights represent the average recoveries. Error bars represent the standard derivations ($n = 3$) in the means.

Table 2
Summary of method performance

	Conc. (ng/ml)	Precision (RSD %)	Accuracy (RE %)	Correlation[a] coefficient (r)	LOD[b] (ng/ml)	LOQ[c] (ng/ml)
Diphenhydramine	5	9.2	12.0			
	100	4.5	−0.7	0.9997	1	2.5
	1000	1.4	2.2			
Desipramine	5	8.6	0.4			
	100	3.5	−1.7	0.9977	1	2.5
	1000	0.76	1.0			
Chlorpheniramine	5	7.7	6.5			
	100	2.2	1.5	0.9994	1	2.5
	1000	0.57	0.5			
Trimipramine	5	22	0.9			
	100	6.7	0.8	0.9998	1	2.5
	1000	1.7	1.1			

[a] Curve type: second order; weighting: 1/x; origin: exclude.
[b] LOD: limit of detection (S/N > 3.0).
[c] LOQ: limit of quantitation is defined with RE % < 20.

3.4. Applicability of the approach

This semi-automated LLE method is a useful approach for processing large numbers of samples in parallel. The throughput of sample preparation has been improved because sequential manual operations on each sample have been replaced by 96 samples processed in parallel. Under circumstances of high sample load, automatic pipetting can reduce the human error introduced by tedious operations. The sample preparation time for one plate (96 samples) was approximately 1.5 h, which is approximately three-fold less than that of manual operation and compares favorably with previous reports for automated SPE [5,6]. In addition, the nature of LLE is such that minimum method development time is needed. The method introduced in this study is ideal for drug discovery work such as in vitro and in vivo screening, as well as ex vivo biological fluid samples from cassette-dosing studies [12]. Characteristic drug pharmacokinetic information such as bioavailability, $t_{1/2}$, T_{max}, C_{max}, and AUC can be obtained by processing one 96-well plate using the semi-automated liquid extraction. An application of this approach is described in the subsequent paper of this journal.

3.5. Future directions

Future directions for the automation of sample preparation could include fully automated liquid extraction and the further enhancement of recovery and throughput. Specifically, it could be feasible to combine other liquid handling functionality such as that demonstrated by reticulating probe liquid handlers (e.g. Packard Multiprobe) with liquid sensing and multifunctional facility to do more intelligent sample preparation. Also, 384 well plate processing could be possible, assuming that the volume ratio requirements of LLE can be met. The key component for the feasibility of these types of microextractions is instrumental sensitivity. As instrumental sensitivity is improved, the feasibility of micro-scale liquid extraction will become more apparent.

4. Conclusions

Through use of a parallel transfer 96-well format liquid handling workstation, LLE has been automated for biological fluid samples. The intensive labor and the possibilities of systematic error associated with manual volumetric transfer have been greatly reduced. Significant advantages over the manual process, in terms of throughput and efficiency have been achieved, while maintaining good precision and accuracy. The next step in the process, integration of front-end biological sample collection will be described in the following paper of this journal volume.

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