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Automated sample preparation and purification of homogenized brain tissues

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Abstract

A robotic homogenized tissue sample transferring method has been developed by using a Packard MultiProbe II 8-tip system. It enables robotically transferring homogenized tissue samples from individual test tubes into a 96-well format plate for further sample purification and analysis. Extensive validation has been made to establish the accuracy and variability of this method. This automatic tissue sample transferring approach combined with automatic tissue homogenization, has significantly increased the throughput of tissue sample preparation in screening of drug candidates using liquid chromatography coupled with highly sensitive and selective tandem mass spectrometry (LC–MS/MS).

Keywords: Automation; Brain tissue

1. Introduction

Knowledge about pharmacodynamic and pharmacokinetic behavior of drug substances in the brain is very important in drug discovery and development, particularly when the therapeutic target is in the central nerve system (CNS) [1]. Quantitative determination of compound levels in tissue samples is often a challenge in bioanalysis in terms of sample processing, clean up and detection sensitivity. Even when tissue samples are homogenized by automated devices such as the Tomtec Autogizer, the homogenates need to be transferred from individual test tubes to a 96-well format plate for further sample purification when performed manually. This time-consuming and labor intensive transferring process considerably limits the throughput of tissue sample analysis [2]. In recent years, advances in modern analytical instrumentation and automation have enabled a paradigm change in drug discovery, in which the information from pharmacodynamic, pharmacokinetic and drug metabolism studies can be provided to medicinal chemistry researchers in a very timely fashion to guide molecule structure modification [3]. Clearly, it is of great interest to develop and implement a robust and high throughput approach for tissue sample analysis by utilizing automation technology.

Recently, automated 96-well sample preparations using robotics have been reported in numerous studies [4–8]. In these applications, robotic liquid handling, liquid sample preparation for plasma or urine analysis, as well as liquid–liquid extractions (LLE) and solid phase extraction (SPE) have facilitated high throughput quantitative analysis in drug discovery. However, there are few reports on using robotics in preparing and handling tissue samples, which can constitute a great portion of samples in pharmacodynamic and pharmacokinetic studies of drug compounds.

The objective of this work was to develop a robotic tissue homogenate transfer method using a Packard Multi Probe II 8-tip system. Samples were prepared in parallel using the robotic system and manual procedures, and subsequently analyzed by LC–MS/MS. For tissue samples, significant time savings can be achieved using the automated method and the resulting analytical accuracy is comparable to that of manual operation.

2. Experimental

2.1. Materials

All compounds used in this experiment were synthesized at Merck Research Laboratories, Rahway, NJ. HPLC grade acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (minimum, 95%) and ammonium

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formate (minimum, 98%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sprague Dawley rats were purchased from Charles River Lab (Germantown, NY, USA). C57/B6 Mice were obtained from Jackson Lab (Bar Harbor, ME, USA). Falcon brand 14-mL conical tubes were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The 96 square-well plates (2 mL/well) were obtained from Analytical Sales and Service, Inc. (Pompton Plains, NJ, USA).

2.2. LC/MS analysis

The LC–MS/MS system consisted of an Applied Biosystems Sciex (Thornhill, Ontario, Canada) API3000 mass spectrometer with a turbo ion spray interface and two Perkin-Elmer (Norwalk, CT, USA) Series 200 micro pumps. A Perkin-Elmer Series 200 autosampler was used. Multiple reaction monitoring (MRM) transitions in the positive ion mode and Analyst Software (Version 1.4) were used for data acquisition and processing. The analytical column was an Aquasil C18, 2.1 mm i.d. × 50 mm, with a 5-μm particle diameter from Thermo (San Jose, CA). The mobile phase was a mixture of 80% acetonitrile and water with 10 mM ammonium formate adjusted to pH 2.5 with formic acid. The typical run time for the experiment discussed in this paper was about 5 min, and typical analytes retention times were about 3–4 min at flow rate of 0.2 mL/min.

2.3. Packard MultiProbe II workstation platform

A Packard MultiProbe II workstation (Perkin-Elmer Co. Shelton, CT) equipped with a 8-tip robotic arm was used for tissue homogenate transferring. Programmable WinPrep Software controlled the workstation. Packard MultiProbe II 8-tip workstation platform was programmed to transfer homogenized tissue samples from individual test tubes (17 mm i.d. × 100 mm, 14 mL) in a custom-made 96-position rack to a designated 96-well plate. Fig. 1 shows the graphical user interface (GUI) of the software.

Both 1 mL regular and wide bore tips were evaluated in the study. The wide bore tips were chosen for this application because they are best suited for homogenized brain samples and can prevent sample from clogging. Robotic workstation parameters such as aspirate and dispense positions, height and speed were adjusted to ensure accurate data acquisition during the method development. Aspirating a small volume of air immediately after homogenate aspiration producing an air gap was applied to enhance the accuracy of tissue homogenate transfer. Two liquid touching (tip touch) height parameters were selected: one set below the liquid surface and one at a percent of well height above the liquid level. Below liquid surface touch was used for transferring blank tissue samples for establishing standard curves and quality controls (QCs). Above liquid touch was used for transferring brain tissue homogenate from drug-dosed subjects. Dispense height was chosen as percent above the well bottom.

2.4. Tissue homogenization

A Tomtec Autogizer (Hamden, CT, USA) was used for tissue homogenization. In this report, the tissue samples were rat and mouse brains. The brain tissue samples were weighted and placed in 14-mL conical tubes. A volume of water equivalent to twice the brain weight was added to each tube and homogenized by a Tomtec Autogizer (capacity of 95 sample positions) [9–10].

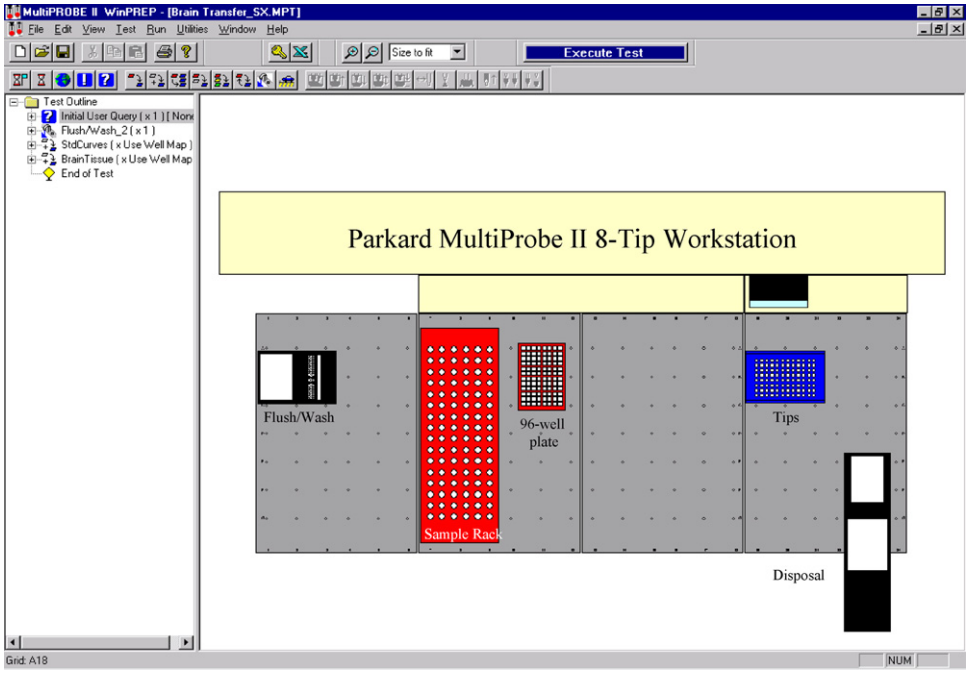

Fig. 1. Packard MultiProbe II workstation platform layout.

2.5. Tissue sample transferring and purification

The 96-well sample source plate was first prepared by using the Packard MultiProbe II to transfer 100 μL aliquots of homogenized blank brain tissue samples from the 14-mL conical tubes in the homogenizer rack to the wells designated for standard curve and QCs. Standard curve and QCs were prepared by adding aliquots of 25 μL working standard solutions, which were made from serial dilution of a 40 μg/mL stock solution at concentrations of 2, 4, 8, 20, 40, 80, 200, 400, 800, 2000, 4000 ng/mL. 100 μL aliquots of dosed brain tissue homogenates were transferred from their respective 14-mL sample tubes to the source plate. A 25 μL aliquot of acetonitrile:water 1:1 was added into each well containing dosed tissue samples to make the volumes of all samples, stds and QCs equal. Finally, a 25 μL aliquot of internal standard was spiked into each well. Protein precipitation was conducted in the 96-well source plate by adding 800 μL acetonitrile. The plate was then vortexed and centrifuged for 10 min at 3000 rpm. Aliquots of 600 μL of supernatant were transferred into a 96-well collection plate, dried under a stream of nitrogen and reconstituted with 200 μL mobile phase for concentration measurement by LC–MS/MS.

3. Results and discussion

3.1. Accuracy and precision

Accuracy and precision of the Packard MultiProbe II in transferring both aqueous and tissue homogenates were evaluated using manual transfer as a reference. The precision of eight-channel tips and the precision of one individual tip were first evaluated by transferring aqueous solutions of 2,6-dichloroindophenol at concentrations of 50, 25, 10 and 5 μg/mL and at volumes of 50, 100 and 200 μL. Manual pipetting was performed using a certified and calibrated Rainin 20–200 μL multiple channel pipette. UV/Vis readings of the solutions at 610 nm were obtained by a Spectramax Plus spectrophotometer (Molecular Devices Corporation). For the aqueous solutions, the deviation between robotic transferring and manual operation was in the range of 85–99%, and the precision of both eight-

Table 1
The accuracy and precision of aqueous solutions (robotic vs. manual)

	8-Tips				Individual tip ($n = 3$)			
	50[a]	25[a]	10[a]	5[a]	50[a]	25[a]	10[a]	5[a]
50 μL								
Robotic	0.387 ± 0.013	0.188 ± 0.010	0.069 ± 0.001	0.042 ± 0.001	0.303 ± 0.012	0.153 ± 0.007	0.060 ± 0.001	0.040 ± 0.002
Manual	0.335 ± 0.012	0.166 ± 0.005	0.066 ± 0.002	0.042 ± 0.001	0.280 ± 0.003	0.143 ± 0.002	0.058 ± 0.000	0.039 ± 0.001
%R.S.D.	3.3	5.1	1.5	1.3	4.0	4.6	1.7	5.0
%DV	84.5	86.7	95.5	100.0	91.6	92.5	96.8	98.5
100 μL								
Robotic	0.776 ± 0.020	0.408 ± 0.007	0.191 ± 0.003	0.089 ± 0.001	0.569 ± 0.024	0.251 ± 0.024	0.080 ± 0.000	0.046 ± 0.000
Manual	0.849 ± 0.007	0.455 ± 0.002	0.207 ± 0.001	0.093 ± 0.001	0.563 ± 0.001	0.271 ± 0.002	0.087 ± 0.000	0.045 ± 0.000
%R.S.D.	2.6	1.8	1.5	1.0	4.2	9.6	0.0	0.0
%DV	91.4	89.7	92.3	95.7	98.9	92.6	93.0	97.6
200 μL								
Robotic	1.700 ± 0.015	0.886 ± 0.006	0.391 ± 0.004	0.156 ± 0.022	1.177 ± 0.012	0.504 ± 0.044	0.129 ± 0.008	0.057 ± 0.000
Manual	1.683 ± 0.004	0.894 ± 0.002	0.383 ± 0.003	0.147 ± 0.001	1.142 ± 0.002	0.525 ± 0.002	0.140 ± 0.000	0.057 ± 0.000
%R.S.D.	0.9	0.6	0.9	14	1.0	8.7	6.2	0.0
%DV	99.0	99.1	97.9	93.9	96.9	96.0	92.1	98.9

%R.S.D. = (S.D./$AU_{robotic}$) × 100. %DV = 100 × [1−(|$AU_{robotic}$ − AU_{manual}|/AU_{manual})].
[a] Solutions (μg/mL).

Table 2
The accuracy and precision of transferring tissue homogenates

Nominal (ng/g)	Manual method			Robotic method		
	Measured (ng/g) ($n = 3$)	%R.S.D.	Accuracy	Measured (ng/g) ($n = 3$)	%R.S.D.	Accuracy
Compound A						
5	4.7 ± 0.4	9.0	95.0	4.3 ± 0.9	20.6	86.5
20	16.1 ± 1.2	7.3	80.4	15.5 ± 1.6	10.4	77.3
Compound B						
200	253.0 ± 10.6	4.2	73.4	229.0 ± 6.0	2.6	85.5
400	505.0 ± 24.8	4.9	73.6	445.0 ± 10.7	2.4	88.8
800	919.0 ± 17.7	1.9	85.1	822.0 ± 30.0	3.7	97.3
2000	1985.0 ± 47.6	2.4	99.3	1840.0 ± 15.2	0.8	92.0

%R.S.D. = (S.D./$Conc._{mean\ measured}$) × 100. Accuracy = 100 × [1 − ($Conc._{mean\ measured}$ − $Conc._{nominal}$)/$Conc._{nominal}$].

channel tips and individual tip was less than 10%. Table 1 is the summary of accuracy and precision of Packard MultiProbe II with aqueous solution.

Since the physical properties of tissue homogenates such as surface tension and viscosity may differ from those of aqueous samples, the accuracy and precision of robotic sample transfer were further evaluated using tissue samples. Two different classes of compounds were used in this study. The compound A solutions at concentrations of 5 and 20 ng/g, and compound B solutions at concentrations of 200, 400, 800 and 2000 ng/g, were spiked into blank brain tissue homogenates. Each 100 μL aliquot of tissue homogenate at a given concentration was transferred in parallel by both Packard MultiProbe II and manually. The concentration levels were measured by LC–MS/MS. Table 2 summarizes the measured concentrations of compound A and B, and the accuracy of robotic and manual tissue homogenate transferring method. Robotic tissue homogenate sample transferring method reached an accuracy ranging from 77% to 97% for a concentration range of 5–2000 ng/g. In the same table, the precision of both methods are also listed.

Table 3
Brain concentrations of compound X and Y in rat and mouse

	Compound X concentration (ng/g)	
	Manual (sample name)	Robotic (sample name)
Mouse		
0.25 h	729.0	753.0
	756.0	731.0
	853.0	664.0
Avg.	**779.3**	**716.0**
1.0 h	2610	2220
	2520	2180
	2560	2010
Avg.	**2563**	**2137**
2.0 h	2790	1880
	2810	2800
	2500	2740
Avg.	**2700**	**2473**
4.0 h	2890	2110
	2800	2570
	2270	2840
Avg.	**2653**	**2507**
	Compound Y concentration (ng/g)	
	Manual (sample name)	Robotic (sample name)
Rat		
0.25 h	26.4	27.0
	34.2	28.0
	28.0	22.1
Avg.	**29.5**	**25.7**
1.0 h	30.1	21.3
	32.9	31.6
	29.6	32.0
Avg.	**30.9**	**28.3**
2.0 h	31.3	30.9
	32.2	32.1
	34.3	34.3
Avg.	**32.6**	**32.4**
4.0 h	21.5	22.7
	24.7	29.0
	18.4	25.5
Avg.	**21.5**	**25.7**

3.2. Tissue concentration comparison between robotic and manual method

The robotic sample transferring method using Packard MultiProbe II was implemented in routine analysis of drug candidates, and its performance was compared to that of manual operation. For a fully loaded 96-well plate, the actual time needed for tissue homogenate transferring using a robotic method was less than 10 min. This improved sample preparation throughput significantly. Rat and mouse brain samples from two different studies following single intravenous dose [11] were analyzed using both robotic and manual transferring methods. The brain samples ($n = 3$) collected at 0.25, 1, 2 and 4 h post dose were homogenized, transferred and analyzed. The results of two methods are summarized in Table 3. *T*-test value showed that there is no significant difference between the pair of data (one obtained from robotic method and other obtained from manual method, confidence level is great than 95%, which is in the acceptable range for early stage of drug discovery environment.

4. Conclusions

A robotic tissue homogenate transferring method was developed and evaluated. The precision and accuracy of automatic sample transfer using a Packard MultiProbe II were comparable to those of manual operations. For brain tissue homogenates spiked with drug compounds, the robotic method demonstrated comparable accuracy and precision to those of manual transferring. The time required to transfer 96 tissue samples by the MultiProbe II 8-tip system is less than 10 min. Considerable time savings, work stress reduction and human error elimination are the most obvious benefits in using automated sample transferring. This helps to significantly increase the throughput for determining tissue distribution of drug candidates in the early drug discovery stage.

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