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High-throughput pharmacokinetics screen of VLA-4 antagonists by LC/MS/MS coupled with automated solid-phase extraction sample preparation

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Abstract

Automation of plasma sample preparation for pharmacokinetic studies on VLA-4 antagonists has been achieved by using 96-well format solid-phase extraction operated by Beckman Coulter Biomek 2000 liquid handling system. A Biomek 2000 robot is used to perform fully automated plasma sample preparation tasks that include serial dilution of standard solutions, pipetting plasma samples, addition of standard and internal standard solutions, performing solid-phase extraction (SPE) on Waters OASIS 96-well plates. This automated sample preparation process takes less than 2 h for a typical pharmacokinetic study, including 51 samples, 24 standards, 9 quality controls, and 3–6 dose checks with minimal manual intervention. Extensive validation has been made to ensure the accuracy and reliability of this method. A two-stage vacuum pressure controller has been incorporated in the program to improve SPE efficiency. This automated SPE sample preparation approach combined with liquid chromatography coupled with the high sensitivity and selectivity of tandem mass spectrometry (LC/MS)/MS has been successfully applied on both individual and cassette dosing for pharmacokinetic screening of a large number of VLA-4 antagonists with a limit of quantitation in the range of 1–5 ng/ml. Consequently, a significant throughput increase has been achieved along with an elimination of tedious labor and its consequential tendency to produce errors.

Keywords: High-throughput; Automation; SPE; LC/MS/MS; VLA-4

1. Introduction

VLA-4 is a member of the integrin family that is expressed on all leukocytes except platelets [1]. The inhibition of VLA-4 may produce a reduction in the migration and activation of cell types important to sustaining a prolonged inflammatory response. The potential therapeutic targets for such an agent might include asthma, rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease. A series of structurally diversified compounds functioning as VLA-4 antagonists has been discovered [2–5]. To build an appropriate in vivo profile of drugs at the early discovery stage, rapid pharmacokinetic screening of representative VLA-4 antagonists is essential. A reliable, sensitive and high-throughput bioanalysis approach is required to accommodate this strategy. Cassette

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dosing of mixtures of four to six compounds in rats has been widely used for rapidly acquiring preliminary pharmacokinetic information. Based on the PK results in cassette dosing, promising compounds can then be dosed individually to acquire more accurate PK parameters. The advantage of fast and efficient liquid chromatography coupled with the high sensitivity and selectivity of tandem mass spectrometry has revolutionized bioanalysis to ensure the feasibility of high-throughput cassette dosing [6–9]. However, biological sample preparation for liquid chromatography coupled with the high sensitivity and selectivity of tandem mass spectrometry (LC/MS)/MS analysis is usually labor intensive and time-consuming. Several approaches such as on-line separation, column switching, robotic automation of sample preparation have been applied [10–15]. Since fast turn-around time is important for the PK screening of the very often examined structurally diversified compounds in the discovery process, the use of 96-well format interfacing with robotic purification of biological matrices can meet our needs to increase overall throughput.

Fig. 1 shows representative structures of VLA-4 antagonists that have been used for analytical method development. Generally, these compounds had low plasma exposure in rats since most of the compounds were excreted into the bile as intact parent. Secondly, those highly protein bound dipeptide VLA-4 antagonists, in general, do not have good signal response in electrospray interface and are moreover thermally unstable in APCI. Thirdly, the physical properties of a set of VLA-4 antagonists used in cassette dosing can vary significantly, with log *D* value [16] ranging from 0 to 5. Therefore, developing sensitive LC/MS/MS and suitable sample preparation method for a mixture study to cover wide range of physical properties can be a challenge. After extensive comparison, a generic solid-phase extraction stands out to be the most effective purification method for plasma sample, a result which has been discussed in detail elsewhere [17]. This approach provides minimum signal interference related to dosing excipients and matrix components and still maintains reasonable extraction efficiency for VLA-4 antagonists to achieve sufficient limit of quantitation.

Automation of solid-phase extraction (SPE) has been reported by using either 3 M Empore or Waters OASIS 96-well format coupled with liquid handling stations such as Packard, Tomtec and Tecan [18–21]. The Biomek 2000, a liquid handling system from Beckman, has been reported for automation of a protein precipitation assay [22]. In this work, we report for the first time the use of a Beckman Biomek 2000 liquid handling system to perform automated plasma sample purification, which includes preparation of standards and quality control samples, transferring plasma samples, and performing solid-phase extraction of a wide variety of VLA-4 antagonists. This fully automated sample preparation method provides accuracy, extraction recovery, and sensitivity comparable to a manual SPE method.

2. Experimental

2.1. Materials

All investigated compounds were synthesized at Merck Research Laboratories, Rahway, NJ. Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ). Ammonium formate, formic acid, dimethyl sulfoxide (DMSO) were ordered from Aldrich (Miwaukee, WI) and poly (ethylene glycol) 400 (PEG400) was from Sigma (St. Louis, MO). OASIS TM HLB extraction cartridges (60 mg/3 cc) and extraction plates (30 mg per well, 96-wells per plate) were obtained from Waters (Milford, MA). All pipette tips (P200, P1000) and 96-square well plate (2 mg per well), 96-deep well plate (1 mg per well) were ordered from Beckman Coulter (Fullerton, CA). Tubes in 96-position format (0.75 ml per tube) which were used for collecting blood samples were obtained from Matrix Technologies Corp. (Cheshire, UK).

2.2. Liquid chromatography/mass spectrometry

All mass spectrometry was performed on a Sciex API 3000 triple quadruple instrument with Turbo Ionspray interface (ABI Sciex, Toronto, Canada). The HPLC system consisted two of PE 200 micro pumps and a PE200 autosampler (Perkin–Elmer, Norwalk, CT). The stationary phase used was BetaMax Base, with a 5 μM particle diameter (Keystone Scientific Inc., Bellefonte, PA). The column size was 2.0 mm (i.d.) × 100 mm. The mobile phase flow rate was

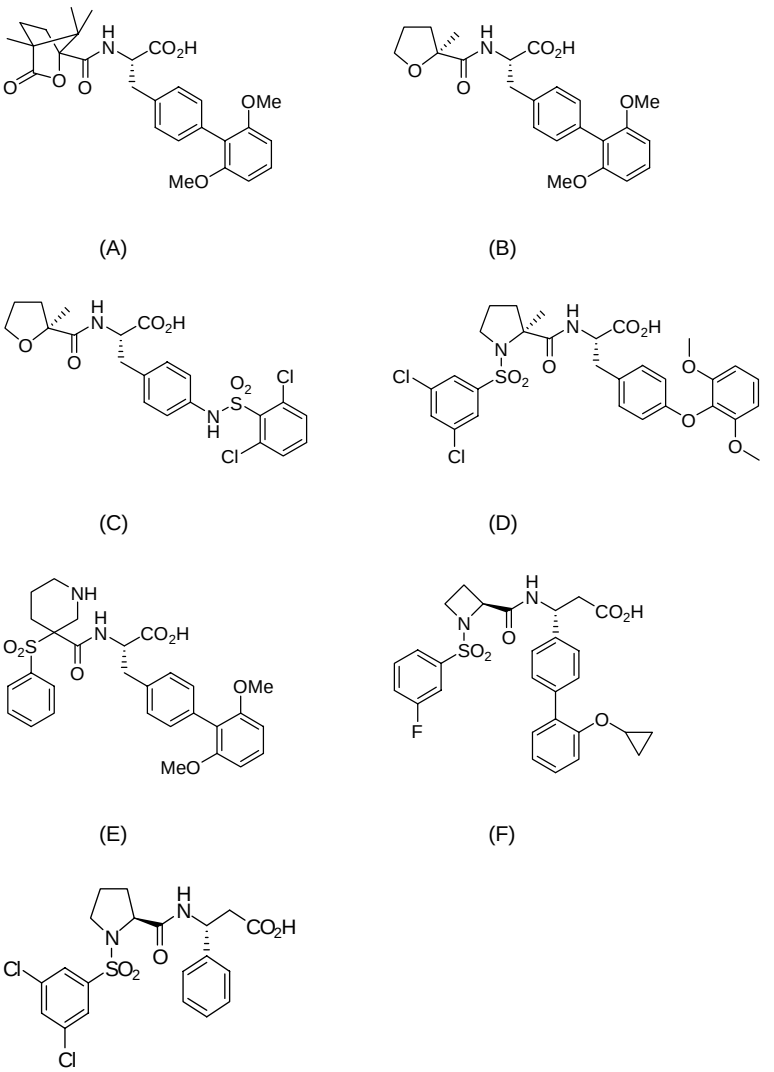


Fig. 1. Structures of compounds A–F, and internal standard.

0.2 ml/min and the mobile phase consisted of a mixture of acetonitrile and water with 10 mM ammonium formate adjusted to pH 2.5 with formic acid. The multiple reaction monitoring (MRM) transitions at positive mode for Compounds A–D (Table 1) were *m*/*z* 482.1/328.1, *m*/*z* 432.1/273.9, *m*/*z* 501.2/343/1, and *m*/*z* 654.2/591.2, respectively. One compound with similar structure was used for internal standard and monitored at *m*/*z* 427.1/278.1.

2.3. Biomek 2000 automated liquid handling station

The plasma sample preparation was performed on a Beckman Biomek 2000 (Beckman Coulter, CA) equipped with the following tools: pipette and wash tools, vacuum filtration manifold, vacuum valve unit, computer controlled six way multi-port valve and pump unit, and the gripper tool. Software was

Table 1
Summary of calibration and quality control data ($n = 6$)

Calibration data	Compound A		Compound B		Compound C		Compound D	
	Automated	Manual	Automated	Manual	Automated	Manual	Automated	Manual
Intercept	0.001	−0.002	0.018	0.002	0.004	0.003	0.011	−0.005
Slope	0.010	0.010	0.015	0.012	0.001	0.002	0.006	0.006
Correlation coefficient	0.995	1.000	0.996	1.000	0.992	0.999	0.994	1.000
5 (ng/ml)								
Mean	5.0	4.8	4.9	4.8	4.8	5.1	4.9	5.1
S.E.	0.4	0.7	0.3	0.1	0.3	0.2	0.3	0.3
Accuracy (%)	100.8	96.2	97.7	96.5	96.5	102.8	97.1	102.6
Precision (%)	8	14	7	3	6	3	6	6
50 (ng/ml)	49.5	51.9	50.0	53.4	51.1	50.5	51.5	50.3
Mean	49.5	51.9	50.0	53.4	51.1	50.5	51.5	50.3
S.E.	1.3	5.1	2.2	4.0	1.6	1.9	2.2	1.5
Accuracy (%)	98.9	103.7	100.1	106.8	102.0	100.9	103.0	100.5
Precision (%)	3	10	4	7	3	4	4	3
500 (ng/ml)								
Mean	501.0	478.8	485.0	509.3	479.2	506.8	499.8	494.3
S.E.	14	29.2	14.1	17.4	18.3	12.3	31.4	20.9
Accuracy (%)	100.1	95.7	97.0	102.0	95.8	101.3	100.0	98.8
Precision (%)	3	6	3	3	4	2	6	4

Bioworks 3.1c running on Windows NT 4.0. Pipette tools equipped with disposable tips were used for liquid transfer of varying volumes: a 200 μl single channel tool (P200) with liquid level sensing, and an eight-channel 200 μl tool (MP200), a 1000 μl tool (P1000) with liquid level sensing. The multichannel wash tool (Wash-8) was connected to three solvent reservoirs through a six-valve bulk dispense system. Water, MeOH, and ACN can be efficiently dispensed as bulk quantities via Wash-8 tool up to 10 ml. A Biomek 2000 96-filtration system that includes vacuum valve unit and vacuum filtration manifold was installed. A disposal unit was installed on the deck of Biomek 2000 for tip disposing.

2.4. Animal dosing

Individual rats (adult male, Sprague–Dawley) were dosed either at 1 mg/kg intravenously or at 2 mg/kg orally. Both cassette dosing and single compound dosing were performed based on the need of research projects. The dosing vehicle was DMSO:PEG400:water (15:40:45 v/v/v). Serial blood samples were collected from indwelling femoral artery catheters into Microtainer® tubes containing EDTA and placed on ice at the following time points: 2, 5, 15, 30 min, 1, 2, 4, 6, 8 h. Plasma was separated by centrifugation and stored frozen in 96-well format until assay. All animal procedures were reviewed and approved by Merck-Rahway Institutional Animal Care and Use Committee.

2.5. Manual plasma sample preparation method

2.5.1. Preparation of standard solutions and quality control standards by manual method

Standard stock solutions of test compounds were prepared as 40 μg/ml (free base) in 50/50 (v/v) acetonitrile:water. A set of standard solutions at concentration of 2, 4, 8, 20, 40, 80, 200, 400, 800, 2000, and 4000 ng/ml were prepared by serial dilution of the stock solution with 50/50 (v/v) acetonitrile:water manually in 1.5 ml Eppendorf tubes. Quality control solutions at three different levels (20, 200, 2000 ng/ml) were prepared separately.

2.5.2. Preparation of calibration standard curves, samples and QCs by manual method

All preparations were conducted in glass tubes. A series of 100 μl aliquots of blank control plasma was

pipetted into glass tubes. Aliquots of 25–75 μl plasma from subjects dosed with test compounds were pipetted into separate glass tubes and were adjusted to 100 μl total volume with blank control plasma. To each sample tube, 25 μl of acetonitrile:water (1:1 (v/v)) was added followed by 25 μl internal standard. To each control plasma tube, a 25 μl aliquot of the solution of working standards (for standard curve) or QCs was added followed by 25 μl internal standard. The contents of all tubes were further diluted with 1 ml water, and ready for transferring to conditioned OASIS HLB extraction cartridges.

2.5.3. Manual solid-phase extraction

The OASIS HLB extraction cartridges (60 mg/3 cc) were conditioned with 3 ml MeOH first and then 3 ml water. After loading the plasma samples to the conditioned cartridges, the cartridges were washed with 3 ml washing solvent and finally analytes were eluted off the cartridges into glass tubes with 2 × 1 ml methanol. The washing solvent was water except where specified. After manual SPE extraction, purified sample residues were evaporated to dryness under a stream of nitrogen, and reconstituted in mobile phase for further analysis.

2.6. Automated plasma sample preparation method

The configuration of initial layout of Biomek 2000 deck is shown in Fig. 2.

2.6.1. Preparation of standard solutions and quality control standards by automated method

Standard stock solutions of test compounds were prepared as 40 μg/ml (free base) in 50/50 (v/v) acetonitrile:water. A set of working standard solutions at concentration of 2, 4, 8, 20, 40, 80, 200, 400, 800, 2000, and 4000 ng/ml was prepared by serial dilution of the stock solution with 50/50 (v/v) acetonitrile:water in 1.5 ml Eppendorf tubes. These were placed on a 24-position rack sitting on the deck of Biomek 2000 (B2), and P1000 tool was used for serial dilution. The layout and dilution sequence of standard preparation are shown in Fig. 3. Separate quality control solutions at three different levels (20, 200, 2000 ng/ml) were prepared manually.

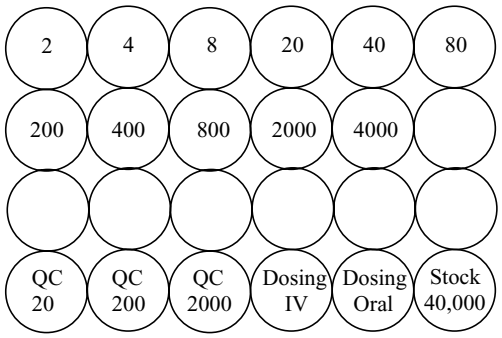


Fig. 3. The layout of standards, QC solutions, dosing solutions, and the dilution sequence of standard preparation.

2.6.2. Preparation of calibration standard curves, samples and QCs by automated method

All preparations were conducted in 2 ml 96-square well plates. A 100 μl aliquot of blank control plasma were pipetted into each well of the 96-well sample consolidation plate (B3) using the P200 tool (Fig. 2). A 25–75 μl aliquot of plasma samples from subjects dosed with test compounds were transferred from the 96-well plate in which they were stored to the consolidation plate (B3) by the MP200 and were adjusted to 100 μl total volume with blank control plasma. A 25 μl aliquot of acetonitrile:water (1:1 (v/v)) was added to

A1	A2	A3	A4	A5	A6
B1	B2	B3	B4	B5	B6

A1: P200, P1000, MP200 Tools	**A2:** Wash-8, Gripper Tools	**A3:** P200 Tips
A4: P1000 Tips	**A5:** Solvents Reservoir	**A6:** Vacuum Reservoir
B1: Plasma Plate in 96-Tube Format	**B2:** Standards, QCs and Dosing Solutions in 24-Tube Holder	**B3:** 96-Well Consolidation Plate
B4: Internal Standard in 96-Well Plate	**B5:** Waters OASIS 96-Well Extraction Plate	**B6:** 96-Well Collection Plate

Fig. 2. The configuration of initial layout of Biomek 2000 deck.

each sample well followed by the addition of 25 μl internal standard with the MP200 tool. The calibration curve and QCs were prepared by aliquoting 25 μl the working standards (for standard curve) or 25 μl QCs to the wells containing control plasma by the P200 tool followed by the addition of 25 μl internal standard with the MP200 tool. The contents of all wells were further diluted with 0.4 ml water by the Wash-8 tool before being transferred to conditioned SPE plate.

2.6.3. Automated solid-phase extraction by Biomek 2000

A OASIS HLB 96-well extraction plate (30 mg per well) was conditioned with 0.3 ml MeOH delivered by the Wash-8 tool of the Biomek 2000 and the wells were drained by vacuum pressure (5 in.Hg) for about 1 min. The SPE plate was further conditioned with 2 × 0.3 ml water in a similar fashion with vacuum applied for about 3 min to remove about 90% of the water. After transferring the plasma samples from consolidation plate (B3) to the wells of the conditioned SPE plate (A6) with the MP200 tool, the plate was loaded by applying very low vacuum pressure (<2.0 in.Hg). The loaded SPE plate was washed with 2 × 0.3 ml water delivered by the Wash-8 tool under vacuum pressure (5 in.Hg). This wash step took about 4 min. Using gripper tool to move a 96-well collection plate under the SPE plate, the analytes were eluted off the SPE plate by adding 2 × 0.5 ml MeOH with the MP200 tool. The elution was accomplished first by gravity first and then by applying low vacuum pressure (<2.0 in.Hg) for about 4 min total. After automated SPE extraction, the purified eluates were evaporated to dryness under a stream of nitrogen, and reconstituted in mobile phase for further analysis.

2.7. Physical and pharmacokinetic parameters

The log of the octanol–water partition coefficients (log *D*'s) for compounds were estimated from the HPLC chromatographic capacity factors (k') of the compounds at pH 7.3 according to a protocol described by Haky and Young [16]. Model-independent pharmacokinetic parameters were calculated using Watson software.

3. Results and discussion

In the drug discovery environment, the goal of automation of sample preparation is to establish a robust method that can be used for a wide variety of analytes with little or no modification. The whole process of automated SPE plasma sample can be divided into three distinct tasks. The first task is the preparation of standard solutions by serial dilution, the second task is the consolidation of standards, QCs, internal standard, and plasma samples into one 96-well plate, and the third task is to perform SPE to purify plasma samples by Biomek 2000.

The layout of the Biomek 2000 deck has been discussed in the Section 2 (Fig. 2). Our strategy here is to fully utilize 96-well format to eliminate the high degree of repetition in manually manipulating and transferring the standards, plasma samples, and labeling individual tubes or vials. As shown in Fig. 2, the plasma samples are supplied in a 96-tube plate located at position B1. Included in this plate are both dosed plasma samples and blank control plasma samples. The 8-channel MP200 is used to accurately and simultaneously transfer plasma samples from B1 to a 96-well consolidation plate at B3. Due to the relatively high viscosity of plasma, the aspiration and dispense rates are set at a very low speed to ensure accurate plasma volume transferring. The internal standard solution is supplied in the first column of a 96-well plate (1 ml per well) located on the deck at position B4 allowing the MP200 tool to transfer internal standard to all wells of the consolidation plate. The standard solutions, QC solutions, and dosing solutions are transferred to the consolidation plate individually using the M200 tool. A solvent reservoir located at position A5 that holds four different solvents (MeOH, ACN, water, and ACN:water (1:1 (v/v)) can be accessed by P200, P1000 and MP200 tools. The final layout of the consolidation plate is shown in Fig. 4. The MP200 tool is used extensively in this method because it can accurately aspirate and dispense up to 200 μl of liquid in eight-channels simultaneously which increases throughput. After consolidation, all samples are diluted with 0.4 ml water and ready to be transferred to the conditioned SPE plate for sample extraction (see Section 2 for details).

Time Points (hr)		0.033	0.083	0.25	0.5	1	2	4	5	8	24		
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
IV/Sub. #1	**A**	25∝l	25∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	x	QC	Dose-IV
IV/Sub. #2	*B*	25∝l	25∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	x	QC	Dose-IV
IV/Sub. #3	*C*	25∝l	25∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	x	QC	Dose-PO
PO/Sub. #4	*D*	x	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	x	QC	Dose-PO
PO/Sub. #5	*E*	x	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	x	QC	[a] Extr. Eff.
PO/Sub. #6	*F*	x	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	75∝l	x	QC	[a] Extr. Eff.
Standards	*G*	2	4	8	20	40	80	200	400	800	2000	4000	[b] DB
Standards	*H*	2	4	8	20	40	80	200	400	800	2000	4000	[c] B

a) SPE extraction efficiency, b) double blank, c) blank.

Fig. 4. The final layout of consolidation plate (B3).

3.1. Linearity, accuracy, and reproducibility of calibration curves

An additional goal of automation is to achieve high-throughput without compromising the accuracy, precision, and limit of quantitation inherent in the manual method. The transferring tools equipped on Biomek 2000 are the primary pipetting tools for preparing standards and transferring plasma samples. Thus, the accuracy and linearity of calibration curves generated by Biomek 2000 were evaluated extensively. First, we decided to use Eppendorf tubes for standard preparation instead of 96-well plates. Because Eppendorf tubes provide better sealing for the standards, and standards can be prepared in advance for method development and can be used repeatedly for multiple assays. The thorough mixing provided at each stage of dilution by repeated aspiration and dispensing ensures homogeneity of each dilution. The automated dilution by Biomek 2000 was compared head-to-head with manual dilution. Table 1 is a summary of calibration data for four compounds prepared either robotically using the Biomek 2000 or by manual preparation. All intercepts, slopes and correlation coefficients are in good agreement between two methods. The linear range of the calibration curves for these four compounds is from 0.5 to 1000 ng/ml. The accuracy measurements for all four compounds are within ±20% nominal which is the value acceptable for assay validation in a drug discovery environment [22].

Another strategy we consistently applied is that all QC samples were prepared manually to serve as true references avoiding any unnoticed Biomek 2000 pipetting failure which could happen during the preparation of standards. During method development, this helped us to detect and correct systematic errors caused by the robot.

3.2. Solid-phase extraction efficiency and limit of quantitation

A low limit of quantitation (LOQ) is essential for quantitation of interesting drug candidates in biological matricies and determination of accurate pharmacokinetic parameters. Thus, the automated procedure has to have sufficient extraction efficiency for compounds with different physical properties. Biomek 2000 is equipped with a filtration system that includes a vacuum manifold for use with 96-well format filters and collection plates. Flow rate through the SPE bed which is controlled by vacuum pressure is one of important parameters for high SPE efficiency. A series of compounds [1–5] with a variety of log *D* values (see Table 2) was chosen to evaluate the SPE extraction efficiency. The SPE efficiency was measured at three different concentrations (5, 50, 500 ng/ml) by comparing the area ratios of analyte to internal standard between samples spiked with analytes before the extraction and samples spiked post-extraction at the same level. Table 2 summarizes the SPE efficiency of two compounds evaluated individually. Also, since the cassette dosing is extensively used to increase high-throughput screening in drug discovery, a mixture of six compounds was evaluated and results are shown in Table 2. The SPE efficiency acquired

Table 2
Evaluation of solid-phase extraction efficiency

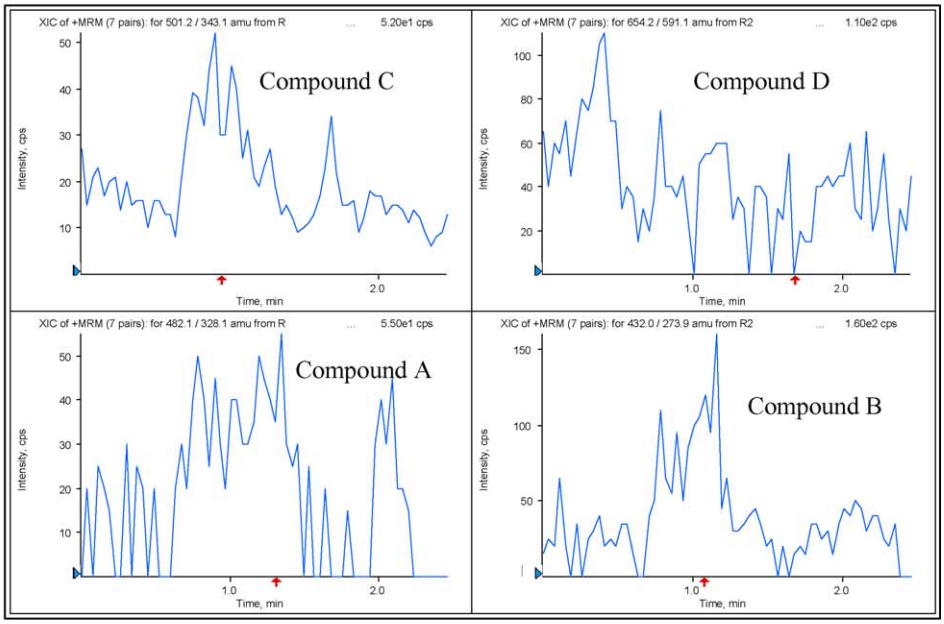
Compound	Concentration (ng/ml)	Automated (OASIS 96-well plate)		Manual (OASIS cartridge) (%)	log *D*
		High vacuum 10 in.Hg (%)	Low vacuum 2.5 in.Hg (%)		
Solid-phase extraction efficiency of individual compound					
E	5	74.0	93.0	85.4	2.7
	50	76.2	83.6	82.6	
	500	79.0	83.3	86.7	
F	5	46.4	80.9	85.7	3.4
	50	36.3	84.0	99.3	
	500	59.6	83.3	86.4	
Solid-phase extraction efficiency of a mixture of six compounds					
L	5	17.9	66.8	63.7	4.1
	50	30.1	48.8	42.7	
	500	27.5	51.8	52.4	
G	5	76.3	85.3	80.1	3.5
	50	71.6	94.1	83.5	
	500	74.0	88.5	79.9	
H	5	89.3	85.0	96.4	1.7
	50	72.4	94.3	72.3	
	500	101.6	96.9	86.0	
I	5	66.6	80.8	N/A	4.2
	50	70.6	101.8	80.8	
	500	48.9	89.6	67.0	
J	5	39.2	64.5	54.4	3.3
	50	23.6	80.8	68.3	
	500	32.0	70.7	57.0	
K	5	60.1	95.2	95.7	1.8
	50	58.9	98.5	N/A	
	500	54.6	92.8	74.5	

via OASIS cartridge with manual method has been served as a reference for evaluation of automated SPE method via Biomek 2000. Two vacuum pressures controlled by Boimek 2000 were compared to evaluate their effect on extraction efficiency. When high vacuum pressure (10 in.Hg) was applied in the all process, all tested compounds experienced low extraction efficiency in comparison to manual SPE efficiency due to insufficient interaction with OASIS polymer. Compounds with large log *D*, such as compounds F, G, I and J show less than 50% extraction efficiency. After switching to a low vacuum pressure (∼2.5 in.Hg), which provided enough time for the efficient interaction of compounds with the SPE absorbant, a significant improvement of SPE extraction efficiency was achieved for those compounds having relatively large log *D* value. With fine-tuning of the vacuum pressure, the SPE extraction efficiency on the OASIS SPE plate using the Biomek 2000 can reach more than 70% for all tested compounds and is comparable to the manual method.

In the SPE process, a relatively high vacuum pressure (5 in.Hg) is desirable during conditioning and washing steps to increase speed of the whole process, and to ensure that the final elution solvent is completely removed from the SPE plate at the end of the elution step. Therefore, a two-level vacuum switching valve was incorporated into the system with the software programmed to accommodate different needs during the SPE process.

Another key step in the SPE process is the volume of elution solvent. We assessed the effect of a variety of elution volumes on the SPE efficiency. One milliliter of MeOH in general provided sufficient recovery of

a) blank control



b) 2 ng/mL

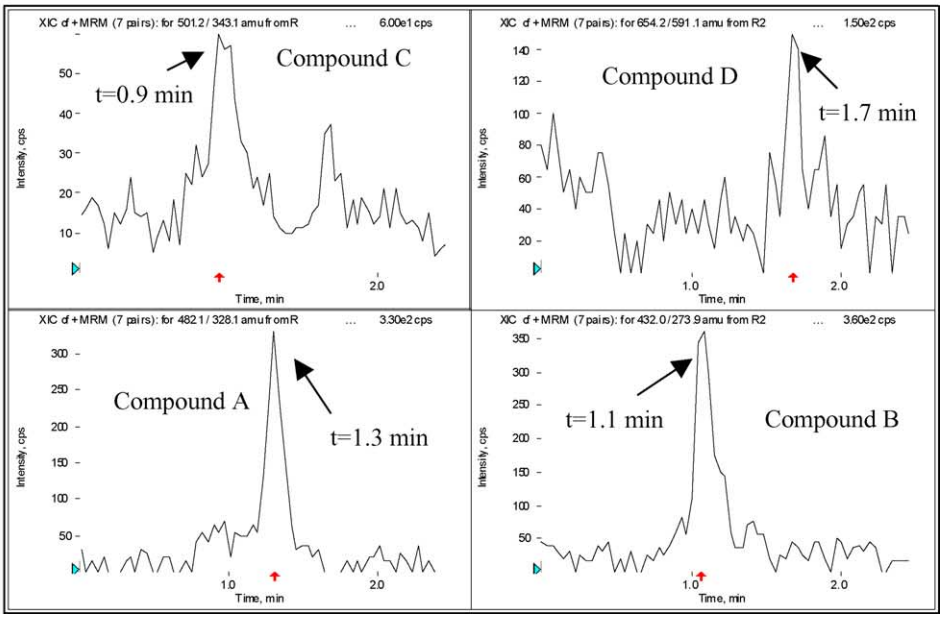


Fig. 5. LC/MS/MS chromatograms of compounds A–D in rat plasma after automated SPE purification: (a) blank plasma spiked with only IS and (b) blank plasma spiked with compounds A–D at 2 ng/ml.

Table 3
Pharmacokinetic parameters in rat

Compound	A		B		C		D	
	Automated	Manual	Automated	Manual	Automated	Manual	Automated	Manual
IV dose, 1 (mg/kg)								
$AUCN_{(0-\infty)}$ (μM h)	0.25	0.25	0.38	0.43	1.85	1.50	0.27	0.20
Cl_p (ml/(min kg)	141	139	101	91	18	22	99	134
Vd_{SS} (l/kg)	2.10	2.34	1.57	1.43	0.74	1.39	4.09	9.24
$t_{1/2}$ (h)	0.3	0.3	0.2	0.3	0.7	1.0	0.7	1.1
PO dose, 2 (mg/kg)								
$AUCN_{(0-\infty)}$ (μM h)	0.02	0.02	0.13	0.13	0.09	0.11	0.07	0.06
C_{max} (μM)	0.07	0.07	0.17	0.18	0.15	0.12	0.10	0.08
T_{max} (h)	0.14	0.1	0.28	0.1	0.14	0.1	0.25	0.5
F (%)	7.3	8.6	33.2	30.7	4.6	7.3	24.4	26.8

all test compounds from the biological matrix. Representative LC/MS/MS chromatograms of compounds A–D are shown in Fig. 5. For evaluating SPE purification of many compounds with different structures, the capacity of OASIS SPE bed has to be considered in the process of method development. For a single compound, we have found that the extraction efficiency is always comparable between SPE plate with 30 mg packing per well and individual cartridge with 60 mg packing. However, the capacity of the SPE plate can be an issue when a mixture of more than several compounds is involved. Reducing plasma volumes for the assay is an alternative way to avoid this capacity problem. Previously we examined in detail the choice of wash conditions for the purpose of reducing dosing excipients such as PEG400 which can produce signal interference in LC/MS [17]. Including 5% methanol in water as a wash solvent for SPE extraction is beneficial for the VLA-4 antagonists examined here.

3.3. Pharmacokinetic parameters

A mixture of four compounds was administered to rats via intravenous and oral routes. Serial blood samples were collected, assayed by LC/MS/MS and pharmacokinetic parameters were calculated. The dosing vehicle was DMSO:PEG400:water (15:40:45 v/v/v). Table 3 compares the pharmacokinetic parameters based on the plasma samples prepared using automated and manual SPE extraction methods. Very good agreement is seen between the results acquired by either fully automated robotic plasma sample preparation or manual preparation. Compounds A, B, and D showed very low plasma concentrations, high clearance, and short half-life in rats. Compound C demonstrated low clearance and long half-life in rats.

4. Conclusions

Overall, we have demonstrated that an LOQ of 1–5 ng/ml can be routinely achieved for many VLA-4 antagonists to acquire pharmacokinetic parameters in the high-throughput drug discovery mode. Reasonable solid-phase extraction efficiency has been achieved with 96-well format by careful optimization of the time and pressure of vacuum, the volume of solvents in the conditioning, loading and eluting steps. The linearity and accuracy of calibration curves, and limit of quantitation are equivocal by comparison samples prepared either with Biomek 2000 robot or manual method. The entire process of automated plasma sample preparation, from standard preparation to final SPE purification, takes less than two hours without manual intervention. The automation of sample preparation for in vivo pharmacokinetic studies significantly eliminates sample handling errors and the tedious labor involved in the nature of this work. Thus we are able to significantly increase the throughput for determining pharmacokinetic profiles of drug candidates in the early discovery stages.

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