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Automated solid-phase extraction workstations combined with quantitative bioanalytical LC/MS

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Abstract

An automated solid-phase extraction workstation was used to develop, characterize and validate an LC/MS/MS method for quantifying a novel lipid-regulating drug in dog plasma. Method development was facilitated by workstation functions that allowed wash solvents of varying organic composition to be mixed and tested automatically. Precision estimates for this approach were within 9.8% relative standard deviation (RSD) across the calibration range. Accuracy for replicate determinations of quality controls was between -7.2 and $+6.2\%$ relative error (RE) over 5–1000 ng ml^{-1}. Recoveries were evaluated for a wide variety of wash solvents, elution solvents and sorbents. Optimized recoveries were generally $>95\%$. A sample throughput benchmark for the method was ≈ 8 min per sample. Because of parallel sample processing, 100 samples were extracted in less than 120 min. The approach has proven useful for use with LC/MS/MS, using a multiple reaction monitoring (MRM) approach.

Keywords: Automated solid-phase extraction; Plasma; Parallel-sample processing; Electrospray; LC/MS/MS

1. Introduction

Recent studies have shown solid-phase extraction (SPE) to be a practical and worthwhile application of laboratory automation [1–3]. Automated SPE can relieve the analyst from tedious sample processing tasks, assist in extraction method development and, in certain cases, improve the precision of an assay [2]. Although automated SPE will take roughly the same time interval as manual SPE, the analyst will now be free to redirect time to other tasks [3,4]. Until recently, major disadvantages of automated solid-phase extraction were the extensive time required to develop procedures and the lack of commercially available systems that could process samples in parallel [2–4]. These barriers have been largely overcome by the introduction of workstations that are dedicated to solid-phase extraction using parallel sample processing.

Previous work [5] demonstrated the utility of a commercially available system dedicated to SPE that used a parallel-processing algorithm to im-

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prove sample throughput. Extracted samples were separated by analytical scale HPLC systems prior to UV or fluorescence detection. The capability of such a system for making incremental changes in extraction variables was found useful for method development. It is now appropriate to evaluate this workstation with LC/MS. When an analytical method is changed from LC/UV to LC/MS, a new validation is required. Precision, accuracy and recovery of the extraction will not change, but the selectivity will change as a new detector is substituted. Also, the analyte ionization efficiency will be directly affected by the composition of the extract, and may not be consistent over the analytical range. Thus the precision and accuracy *of the method* will be affected [6]. Because LC/MS has become such an important technique for bioanalysis, we believe that these phenomena need to be investigated further.

This study demonstrates the utility of the same parallel sample processing workstation, for use with electrospray liquid chromatography/mass spectrometry (LC/MS). Extracted samples were separated by analytical scale liquid chromatography systems prior to injection. The capability of this system for optimizing a method through incremental changes in extraction variables during method development was examined. The precision and accuracy obtained from this system have again been assessed, this time with respect to electrospray–tandem mass spectrometric detection.

2. Materials and methods

2.1. Analyte test systems

To evaluate the workstation, PD 073953 (compound I, Fig. 1) and a related internal standard PD 105752 (compound II), were extracted from dog plasma by reversed-phase SPE. Compounds I and II were synthesized and purified in-house (Parke–Davis Pharmaceutical Research, Ann Arbor, MI).

2.2. Apparatus

Automated solid-phase extractions were performed on a Zymark RapidTrace workstation (Zymark, Hopkington, MA) equipped with six or ten extraction modules, operating in parallel, and utilizing 100 mg of sorbent contained in 1 ml solid-phase cartridges. Several solid-phase sorbents, including octadecyl-, cyclohexyl-, cyanopropyl- and ethyl-silica (International Sorbent Technology, Mid-glamorgan, UK or Empore, 3M, Minneapolis, MN) were evaluated for recovery in this work. RapidTrace software (version 2.1) operating under Windows for Workgroups (Microsoft, Bothell, WA) on a Laptop Computer (Xpi, Dell Computer, Round Rock, TX) controlled the workstation. Liquid chromatographic separations were performed on a C-8 column (5 μ, 2.0 × 50 mm XDB-C8, MacMod, Chadds-Ford, PA) using a quaternary solvent delivery system and autosampler (series 200, Perkin-Elmer, Norwalk CT). Injection volumes of 10 μl were used.

A Quattro II LC/MS system (Micromass, Beverly, MA) operating under MassLynx 2.3 was used for these experiments. The ion source for all experiments was a megaflow electrospray, operating with a high voltage counter electrode (500 V) under a negative ionization multiple reaction monitoring (MRM) mode. Typical source conditions for negative ion experiments were as follows: capillary 3750 v, skimmer 1.8 v, skimmer offset 5 v, RF lens 0.2 v, source temperature 140°C. Quadrupole 1 (Q1) parameters were: LM resolution 15.0, HM resolution 15.0, ion energy 1.0, ramp 0.0, and lens six 4 v. Quadrupole 2 (Q2)

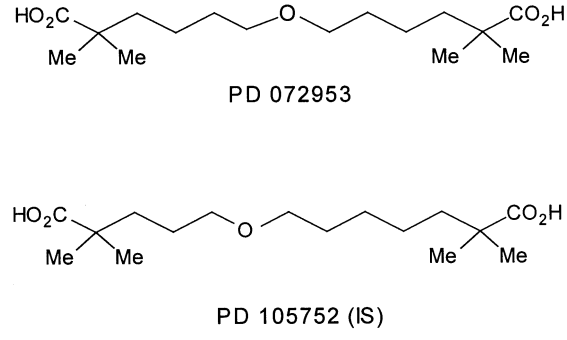

Fig. 1. Chemical structures for test analytes used in this work: Compound I (PD 072953), and Compound II (PD 105752).

Table 1
Generalized workstation program sequences for solid-phase extraction of compounds I and II from heparinized dog plasma

Step	Process	Reagent	Volume (ml)	Flow rate (ml min^{-1})
1	Wash cannula	H_2O	3.0	10
2	Cartridge precondition	CH_3CN	1.0	10
3	Cartridge precondition	H_2O	1.0	10
4	Cartridge precondition	Buffer, pH 2.8	1.0	10
5	Load	Sample	1.0	2
6	Wash cartridge	H_2O	1.0	2
7	Wash cartridge	*Wash solvent	1.0	2
8	Collect	*Elution solvent	0.2	2
9	Collect	Air	3.0	10
10	Wash cannula	H_2O	3.0	10

* Indicates that the composition was a variable during method development.

parameters were LM resolution 15.0, HM resolution15.0, ion energy 1.0, ramp 1.0, lens eight 40 v, lens nine 0 v, multiplier 650 v, respectively. MRM scan functions were: compound I transition 301 → 159 Da., dwell 0.1 s collision energy 30 v, cone energy 60 v. Compound II transition 301 → 173 Da., dwell 0.1 s collision energy 25 v, cone energy 50 v, with interchannel delay of 0.02 s, mass span of 0.0 Da., and scan time of 4.0 min.

2.2.1. Chromatographic conditions

Compounds I and II were separated isocratically, using methanol:water:acetic acid (70:30:0.1, v/v) at ambient room temperature (~ 23°C) and a flow of 0.25 ml min^{-1}. Typical retention times for either compound ranged from 2.8 to 3.1 min.

2.2.2. Reagents and standards

Acetonitrile, methanol, and acetic acid were obtained from EM Science (Gibbstown, NJ) and were used as received. Reagent grade water was prepared from in-house deionized water using a Milli-Q system (Millipore, Millford, MA). Heparinized plasma samples were prepared in-house from whole blood collected from Beagle dogs. A stock solution containing 100 μg ml^{-1} of compound I, was prepared in methanol:water (50:50). Working standards containing 1000, 500, 250, 100, 50, 25, 10, and 5 ng ml^{-1}, were prepared by volumetric dilution of the stock standard with dog plasma. Quality controls were prepared from a separate weighing of compound, using a similar dilution scheme. Concentrations of I in quality controls were 5000 (dilution quality control), 750, 75, 15 and 5 ng ml^{-1}. Quality controls were stored at −70°C until time of assay. A 10 mg ml^{-1} stock solution of compound II was prepared and volumetrically diluted to 100 ng ml^{-1} using methanol:water (50:50 v:v). This solution was used as internal standard.

2.2.3. Extraction procedure

To 200 μl of blank, pooled dog plasma or quality controls in 13 × 100 mm borosilicate glass test tubes, 50 μl of methanol water (or the appropriate standard solution containing compound I), 50 μl of internal standard solution (compound II) and 250 μl of 5% (v:v) acetic acid (aq) were added prior to vortexing (~ 3 s). Sample tubes were placed in the Zymark RapidTrace workstation, programmed to process the samples by solid-phase extraction on various sorbents (Table 1). The eluents from these procedures were collected into 12 × 75 mm borosilicate glass test tubes, evaporated to dryness at 40°C under a N_2 (g) stream and the residues manually re-suspended with 200 μl of methanol:0.1% acetic acid (v:v). Aliquots were injected into the HPLC system by an autosampler.

3. Results and discussion

The extraction procedure was evaluated for analyte recovery, linearity, precision, selectivity and processing time using various sorbents, wash solvents and elution solvents.

3.1. Extraction development using the workstation

A procedure for the extraction of I and II from dog plasma was developed, based on the Rapid-Trace program outlined in Table 1. Those variables that contributed most significantly to the selectivity for and recovery of analytes (sorbent selection, wash solvent and elution solvent composition) were examined in some detail. Sample loading was best accomplished when sample and SPE column were buffered at pH 2.0. This pH allowed for minimal ionization and maximal retention of acidic compounds such as I and II. Experiments conducted at higher pH or without pH control resulted in dramatically lower recoveries.

Throughout the course of this study, no appreciable endogenous plasma interferences were apparent in the chromatograms. Therefore, unlike previous work with optically based HPLC detection [2–5], where significant efforts were given to enhance the selectivity of the extraction methods little attention was given to chromatographic selectivity. The solvent and sorbent optimizations used here were driven primarily by recovery and precision needs.

3.1.1. Sorbent selection

Using generic wash (5/95 acetonitrile/0.1% acetic acid, (v/v)) and elution (100% acetonitrile) solvents, four packed bed sorbents (C18, C2, CH and CN) were evaluated for recovery and precision of extraction of compounds I and II (100 ng ml^{-1}) spiked into 200 μl of Beagle dog plasma. Octylsilica (C8) was expected to give results that were intermediate between C2 and C18, and was not evaluated. We reasoned that the selectivity of an aromatic sorbent, such as phenyl, would be inferior to that of straight chain hydrocarbons, and it also was omitted from the evaluation. The

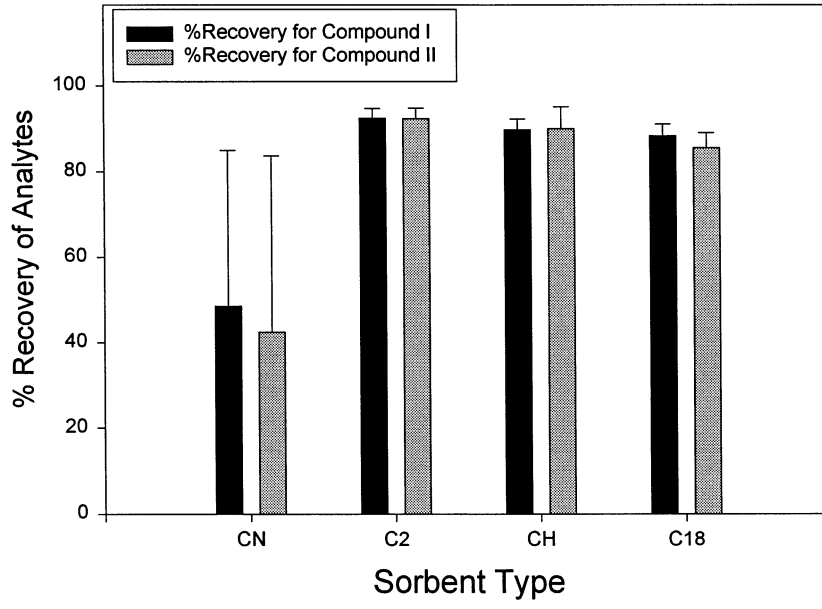

Fig. 2. Solid-phase extraction recovery of Compounds I and II from four different solid-phase sorbents using a wash solvent of 5% acetonitrile and an elution solvent of 100% acetonitrile. Solid lines indicate the coefficient of variation associated with each determination.

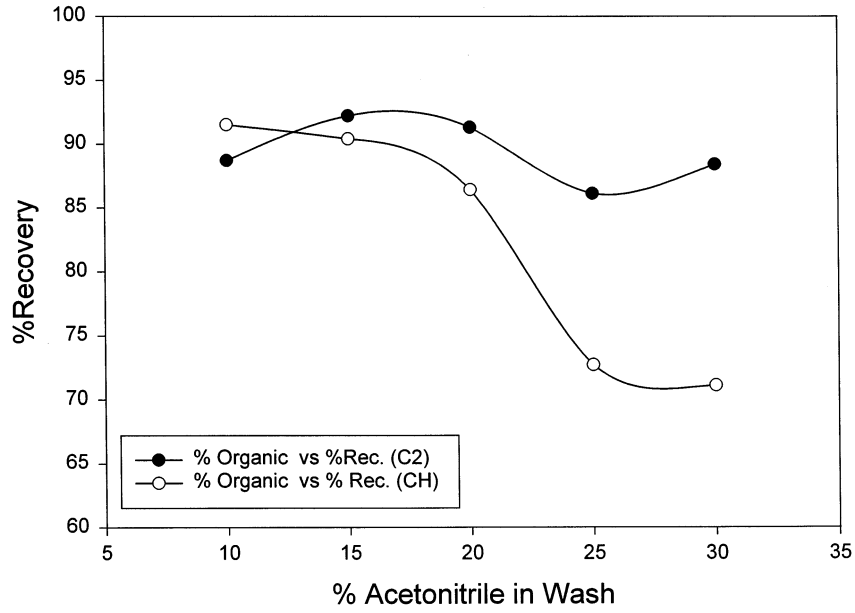

Fig. 3. Solid-phase extraction recovery of Compounds I as functions of wash solvent concentration and sorbent. Solid dots (●) indicate recovery for C2 sorbents, and hollow dots (○) indicate recovery for CH sorbent using 100% acetonitrile elution solvent.

results of this screen (Fig. 2) demonstrated that the ethyl silica (C2) gave both the highest recovery and the least variability, followed, in order, by cyclohexyl (CH), and octadecyl (C18). The cyanopropyl sorbent (CN) gave the poorest recovery with much higher variability. On this basis, a C2 sorbent was selected for further extraction optimization.

3.1.2. Evaluation of wash and elution solvents

Using the workstation to perform premixing of organic and aqueous components (1% acetic acid) in various compositions, solvents ranging in composition from 5 to 30% acetonitrile with 1% acetic acid were mixed and evaluated as wash solvents for the extraction of compounds I and II from dog plasma. The optimal recovery for the C2 sorbent was found to be nearly unchanged from 5 to 20% acetonitrile, then decreased slowly at higher organic strength (Figs. 3 and 4). On this basis, 15% acetonitrile was chosen to ensure a safe margin for day-to-day error, yet still deliver maximal recovery. Greater than expected variability (40–50%) in recovery was observed at any given wash solvent composition. This variability was found to arise from the organic/aqueous mixing process and was greatly reduced (to $\sim$10% RSD) when manually premixed solvents were used for the wash step.

Elution solvents were evaluated in a similar manner as wash solvents, and it was found that 70% acetonitrile/30% 1% acetic acid was the weakest solvent tested that gave maximal recovery of the analytes.

3.1.3. Physical difficulties associated with SPE

Because a significant pH adjustment (from 7.4 to 2.0) was required for analyte protonation and maximal retention during reversed-phase solid-phase extraction, a small amount of protein precipitation was observed in plasma samples prior to SPE loading. When solid-phase extraction disks were used in conjunction with the workstation, this protein precipitation resulted in a large increase ($\sim$2 to 3x) in back pressure and clogging for the SPE disks, leading to a high ($\sim$25%) extraction failure rate. The membrane disks were, therefore, a sub-optimum choice for this particular application.

Using the packed-bed sorbents described above, some increase in HPLC column backpressure typically was noted for the mid-bore (2.1 mm) chromatography columns used. This increase, generally on the order of 2 to 5 psi per injection, was minimal and could be managed by periodic replacement of a guard cartridge preceding the analytical column. We postulated that this backpressure increase was associated with collection of some fine sorbent particles by the column frit.

3.1.4. Recovery

After the selection of optimal sorbent, wash and elution solvents, recoveries (%RSD) were determined for replicate ($n = 6$) dog plasma quality controls at 15, 75 and 750 ng ml^{-1}. Results were 96.5 (10.7%), 92.1 (6.3%) and 106 (10.5%), respectively.

3.2. Determination of compounds I and II in dog plasma

Using the extraction procedure outlined in Table 1, implemented on a solid-phase extraction workstation, compound I was successfully determined in dog plasma over a concentration range from 5.0 to 1000 ng ml^{-1}. Representative chromatograms for this procedure are displayed in Fig. 2a–d. Retention times were $\approx$2.9–3.0 min for compounds I and II, respectively. Excellent selectivities were obtained for compounds I and II (capacity factors of 7–8) over the dynamic range of the assay.

3.2.1. Linearity

Standard curves ranging from 5 to 1000 ng ml^{-1} of compound I yielded back-calculated standard concentrations which agreed with nominal values to within $\pm$3% (typical) and $\pm$10% (worst case outlier). The Pearson correlation (r) was generally > 0.997 and a Y-intercept were typically statistically indistinguishable from zero. These results suggest acceptable standard curves generated by extractions performed on the solid-phase extraction workstation.

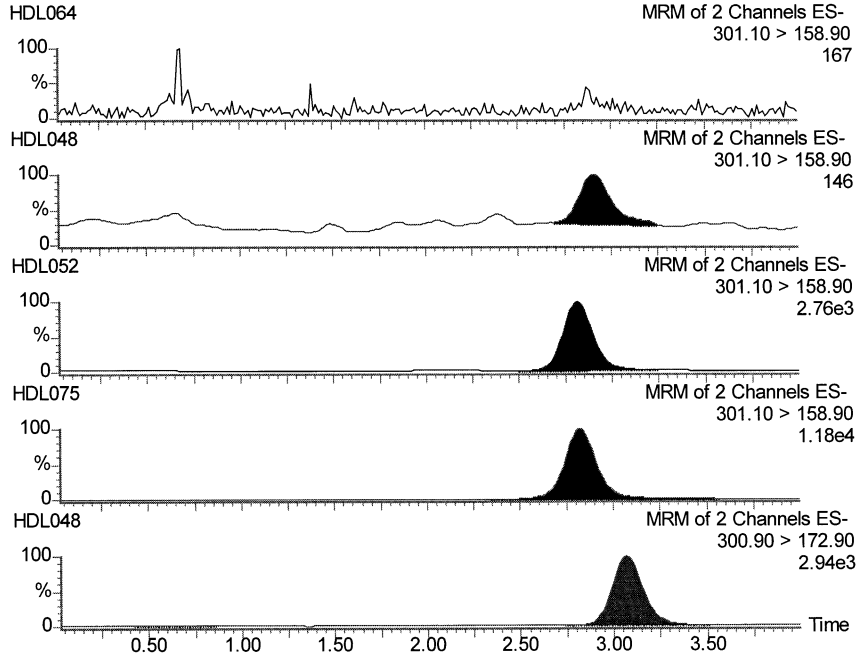

Fig. 4. Representative MRM chromatograms for extracted dog plasma samples (from top to bottom) at concentrations of (a) blank; (b) 5 ng ml^{-1} (spiked); (c) 100ng ml^{-1} (spiked); (d) 425 ng ml^{-1} (0.5 h after receiving an oral dose of drug; and (e) internal standard at an effective concentration of 100 ng ml^{-1}.

Table 2
Concentrations and associated intra- and inter-day uncertainties for quality controls of compound I in heparinized dog plasma

Nominal Concentration (ng ml^{-1})	Run No.	Intra-run % Rel. SD[a]	Inter-run % Rel. SD[b]	Intra-run % RE[c]	Inter-run % RE[d]
5[e]	1	14.4	13.4	−1.0	6.6
	2	5.8		2.1	
	3	13.2		18.5	
15	1	9.8	7.1	−2.4	2.3
	2	0.5		3.0	
	3	5.0		6.7	
75	1	2.4	4.9	2.8	−2.0
	2	2.6		−6.3	
	3	4.3		−2.4	
750	1	5.1	4.8	1.8	−1.3
	2	1.5		−6.2	
	3	2.0		0.6	
5000[f]	1	8.6	7.9	−3.0	−1.0
	2	2.9		−7.2	
	3	1.4		−7.0	

[a] Inter-run % relative for three replicates in each of three runs.
[b] Pooled intra-run % relative standard deviation for nine replicates over three runs.
[c] Inter-run % relative error for three replicates in each of three runs.
[d] Pooled inter-run % relative error for nine replicates over three runs.
[e] Quality control prepared at the limit of quantitation.
[f] Dilution quality control, reflecting 500× dilution factor.

3.2.2. Precision and accuracy of the method

The precision and accuracy of the method, as demonstrated by replicate quality controls at 5 (limit of quantitation), 15, 75, 750 and 5000 (dilution quality control) ng ml^{-1} indicated a reliable analytical method (Table 2). Intra-day relative standard deviation did not exceed 9.8%, except at the quantitation limit ($<14.4\%$), and typically ranged between 0.5 and 5%, while intra-day relative errors were from -7.2 to 6.7%. As in previously described work using HPLC-UV and HPLC-fluorescence, the extractions provided by this workstation demonstrated acceptable precision and accuracy.

3.3. Workstation sample throughput

A single RapidTrace workstation module as configured here, can process up to ten samples sequentially. Each workstation can have up to ten modules for a total sample capacity of 100 (10 × 10) samples in a completely unattended manner. The throughput advantages of parallel sample processing have been previously described [2–4]. For the extraction involving compounds I and II (Table 1), less than 8 min were required to perform each complete extraction. Because samples were processed in parallel, up to 10 samples could be processed in this time and 100 samples (10 × 10 modules) could be processed in less than 80 min (120 min, including extraction preparation). Work involving other compounds [5] required only 3 min for each complete extraction, with up to 100 samples being processed in ≈ 30 min. These throughput benchmarks are comparable to extractions performed by either manual processing (120 min for 100 samples), automated serial processing [1] or parallel processing in many types of 96-well format (90 min for 96 samples) [7].

4. Conclusions

Recently introduced solid-phase extraction

workstations, utilizing a parallel-processing algorithm are rapid, precise and accurate in performing bioanalytical extractions for either method development or production. High, consistent recovery, and good chromatographic selectivity were obtained for each of several test analytes. The time required to process samples was equal to or less than that required for several alternate forms of sample preparation. Combination of this system with an automated solvent delivery workstation could offer additional savings in time and effort. The approach has proven to be highly effective for rapid assay optimization using multiple variables, and in high production throughput in conjunction with electrospray LC/MS/MS. Fundamental studies of the effects of solid-phase extraction on ionization would be appropriate to improve the understanding of the electrospray ionization phenomena.

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