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Automated sample preparation for drugs in plasma using a solid-phase extraction workstation

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Abstract

An automated solid-phase extraction workstation was used to develop, characterize and validate two separate HPLC methods for quantifying drugs in plasma. Method development was facilitated by workstation functions which allowed wash solvents of varying organic composition to be mixed and tested automatically. The precision estimates for the two methods were within 6.0 and 2.0% RSD across their respective calibration ranges. Accuracies for replicate determinations of quality controls were between -1.2 and $+4.8$% RE over ng ml^{-1} and μg ml^{-1} calibration ranges, respectively. Optimized recoveries were quantitative and were generally greater than 90% for the four analytes tested, and depended to a great extent, as expected, on the composition of the wash solvent. Sample throughput benchmarks for the two methods ranged from 3 to 10 min per sample, depending on the extent of air drying used. Because of parallel sample processing, 60 samples could be extracted in as little as 17 min. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Automated solid-phase extraction; Plasma; Parallel sample processing

1. Introduction

Several 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 the utility of this method for decreasing the time required to conduct a number of extractions is not well established, automated SPE will result in time savings by allowing the analyst 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 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.

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This work demonstrates the utility of such a workstation, a commercially available system dedicated to SPE, which uses a parallel processing algorithm to improve sample throughput relative to earlier serial processing versions. Extracted samples were then separated by analytical scale liquid chromatography systems prior to UV or fluorescence detection. The capability of such a system for making incremental changes in extraction variables during method development was examined. The precision, accuracy and recovery obtained from a system during extraction of drug substances from plasma have been assessed and are reported here.

Two compound classes were selected as analyte test systems. The first class is represented by Compounds I and associated internal standard (Compound II). Compound I is a cyclopentyl-fused 6-nitroquinoxalinedione, with the acidic tetrazole moiety at the eighth position used to increase aqueous solubility and provide additional binding interaction with neural receptors. An interesting pharmacological feature of this compound is that the drug probenecid inhibits its exportation from the brain via the organic anion transport system [5]. Compounds III and IV are amino acid analogs from a potent class of antagonists at the NMDA receptor. Compound III in particular is highly charged at physiological pH and has demonstrated anomalous characteristics of transport through biological membranes [6–8]. This behavior has prompted extensive transport studies in various biological systems to describe the ultimate delivery of Compound III to the central nervous system. The phosphonic acid moiety presents unique analytical challenges, particularly when coupled with progroups in an effort to mask charge [8]. This moiety is a common feature in many signal transduction targets.

2. Materials and methods

2.1. Analyte test systems

Two different analyte test systems were used to evaluate the workstation. The first of these involved extraction of PD 163223 (Compound I) and a related internal standard, PD 155011 (Compound II) from rat plasma by reversed-phase solid-phase extraction. An Octydecylsilica solid-phase sorbent was used exclusively for this work. The second test system involved extraction of PD 158473 (Compound III) and related internal standard PD 158474 (Compound IV) from rat plasma by exploiting the mixed mode ion exchange/partitioning characteristics of chemically modified silica in the form of C–18, C–8 and phenyl sorbents [9]. Structures for test compounds are given in Fig. 1. Compounds I through IV were synthesized and purified in-house (Parke-Davis Pharmaceutical Research, Ann Arbor, MI). Test compounds were selected on the basis of recent analytical interests in our laboratories.

2.2. Apparatus

Automated solid-phase extractions were performed on a Zymark RapidTrace workstation (Zymark, Hopkington, MA) equipped with six extraction modules, operating in parallel, and utilizing 1-ml 100 mg C–18, C–8 and phenyl solid-phase cartridges (Varian Sample Preparation

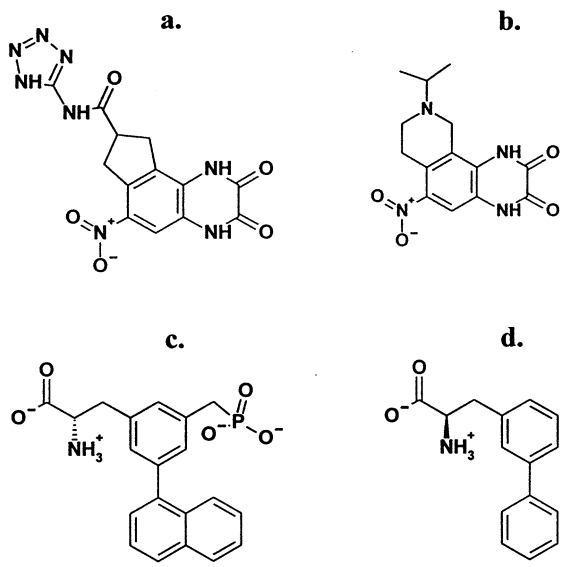

Fig. 1. Chemical structures for test analytes used in this work: (a) Compound I, (b) Compound II, (c) Compound III and (d) Compound IV.

Table 1
Workstation program sequences for solid-phase extraction of Compounds I and II from plasma

Step	Process	Reagent	Volume (ml)	Flowrate (ml min^{-1})
1	Wash cannula	H_2O	6.0	10
2	Cartridge Precondition	CH_3CN	1.0	10
3	Cartridge Precondition	CH_3OH	2.0	10
4	Cartridge Precondition	Phosphate buffer, pH 6.0	1.0	20
5	Load sample	sample	0.7	1
6	Wash cartridges	Phosphate buffer, pH 6.0	1.0	1
7	Wash cartridges	(9:1) Phosphate buffer:Methanol	0.5	1
8	Wash cartridges	H_2O	1.0	1
9	Dry cartridges	N_2 (g)	10.0	1
10	Elute	1% TFA in CH_3CN	1.0	1
11	Elute	1% TFA in CH_3CN	1.0	1
12	Purge elution line	N_2 (g)	3.0	10
13	Wash cannula	CH_3CN	6.0	10
14	Wash cannula	H_2O	6.0	10

Products, Harbor City, CA). The workstation was controlled by RapidTrace software operating under Windows for Workgroups (Microsoft, Bothell, WA) on a Laptop Computer (Xpi, Dell Computer, Round Rock, TX). Liquid chromatographic separations were performed on either a C–18 column (Compounds I and II; C18, Rx, 4.6 × 250 mm, Zorbax, MacMod, Chadds-Ford, PA) or a C–8 column (Compounds III and IV; C8, XDB, 4.6 × 150 mm, Zorbax, MacMod) using conventional HPLC pumps, autosamplers and detectors [2].

2.2.1. Chromatographic conditions

Compounds I and II were separated isocratically, using acetonitrile:water:trifluoroacetic acid (90:10:0.1, v/v) at 35°C and a flow of 1.0 ml min^{-1}. Test compounds were detected by absorbance at 340 nm. Compounds III and IV were separated isocratically, using pH 6.5 phosphate buffer, methanol, water and acetonitrile (51.3/30.1/12.9/5.7, v/v) at ambient room temperature and a flow rate of 1.0 ml min^{-1}. Test compounds were detected by fluorescence at 214 nm (excitation) and 360 nm (emission).

2.2.2. Reagents and standards

Acetonitrile, trifluoroacetic acid, methanol, phosphoric acid, ammonium hydroxide, sodium hydroxide and sodium phosphate monobasic 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). Rat plasma (heparinized) was prepared in-house from whole blood collected from Wistar rats.

2.2.3. Extraction procedure for Compounds I and II

To 100 μl of blank, pooled rat plasma or seeded quality controls in 13 × 100-mm borosilicate glass test tubes, 20 μl of calibration standard (0.5–100 μg ml^{-1} of I in 1 mM NaOH) or buffer (1:1, 1 mM NaOH:0.01 M H_3PO_4) and 20 μl of 10.4 μg ml^{-1} of internal standard solution (Compound II) were added. Final concentrations of Compound I ranged from 0.100 to 20.0 μg ml^{-1} in standards and 0.400, 2.40 and 16.0 μg ml^{-1} in quality controls. Final concentration of Compound II was 2.0 μg ml^{-1} in all samples. To each sample tube 500 μl of 0.1 M phosphate buffer, pH 6 was also added prior to vortexing (~ 3 s). Sample tubes were placed in the Zymark RapidTrace workstation which was programmed to processed the samples by solid-phase extraction on C18 sorbent according to the procedure shown in Table 1. Using two consecutive 1.0 ml aliquots of 1% trifluoroacetic acid in acetonitrile, analytes were eluted into 12 × 75 mm borosilicate glass test tubes. The eluent was evaporated to dryness at

Table 2
Workstation program sequences for solid-phase extraction of Compounds III and IV from plasma

Step	Process	Reagent	Volume (ml)	Flowrate (ml min^{-1})
1	Wash cannula	H_2O	3.0	30
2	Cartridge precondition	CH_3OH	2.0	20
3	Cartridge precondition	H_2O	2.0	20
4	Cartridge precondition	Buffer, pH 2.0	2.0	20
5	Load	Sample	1.5	5
6	Wash cartridges	H_2O	1.0	5
7	Add to mixer	Buffer, pH2.0	0.8	10
8	Add to mixer	CH_3OH	0.2	10
9	Mix reagents	CH_3OH:Buffer pH 2.0[a]	1.0	30
10	Wash cartridges	CH_3OH:Buffer pH 2.0[a]	1.0	5
11	Wash cartridges	H_2O	1.0	5
12	Elute	0.5% NH_4OH/CH_3OH[a]	1.0	5
13	Wash cannula	H_2O	3.0	30

[a] Indicates that the composition was a variable during method development.

50°C under a N_2 stream and residues were manually resuspended with 200 μl of chromatographic mobile phase: (1:9)/(1% TFA in H_2O:acetonitrile). Aliquots (150 μl) were injected into the HPLC system by autosampler. The extraction procedure was evaluated for analyte recovery, linearity, precision, selectivity and processing time.

2.2.4. Extraction procedures for Compounds III and IV

A procedure similar to that used for Compounds I and II was developed and used for preparation of samples containing Compounds III and IV. Aliquots (20 μl) of internal standard (Compound IV) at 2500 ng ml^{-1} were added to all samples, excluding some blanks, prior to extraction. The final concentration of Compound III in standards ranged from 10 to 1000 ng ml^{-1}, with quality controls positioned at 10, 100 and 1000 ng ml^{-1}. The extraction procedure outlined in Table 2 was executed on the workstation. Variables of the extraction sorbent (octadecyl, octyl, phenyl), wash solvent composition (20–60% methanol), and elution solvent composition (0.5% NH_4OH in methanol versus pH 6.5 phosphate buffer:acetonitrile (25:75 v:v)) were evaluated to optimize extraction recovery, selectivity, precision, accuracy and detection limit.

3. Results and discussion

3.1. Determination of Compounds I and II in rat plasma

Using the extraction procedure outlined in Table 1, implemented on a SPE workstation, Compound I was successfully determined in rat plasma over a concentration range from 0.100 to 20.0 μg ml^{-1}. Representative chromatograms for this procedure are displayed in Fig. 2a–c. Retention times were approximately 5.3 and 10.2 min for I and II, respectively. Excellent selectivities were obtained for Compounds I and II (capacity factors of 2.1 and 4.3, respectively) over the dynamic range of the assay. Chromatographic resolution (R_s) for Compound I was greater than 6, while that for Compound II was greater than 8, indicating more than adequate separation of analytes from potential interferences. Two unidentified metabolites of Compound I eluted at 14.7 and 15.7 min, and did not interfere with the peaks of interest.

3.1.1. Assay performance (Compounds I and II)

The extraction outlined in Table 1 apparently has significant ion-exchange character [9]. The use of 1% trifluoroacetic acid in the elution solvent was required to protonate any active silanol sites on the silica and enhance the recovery. Mean

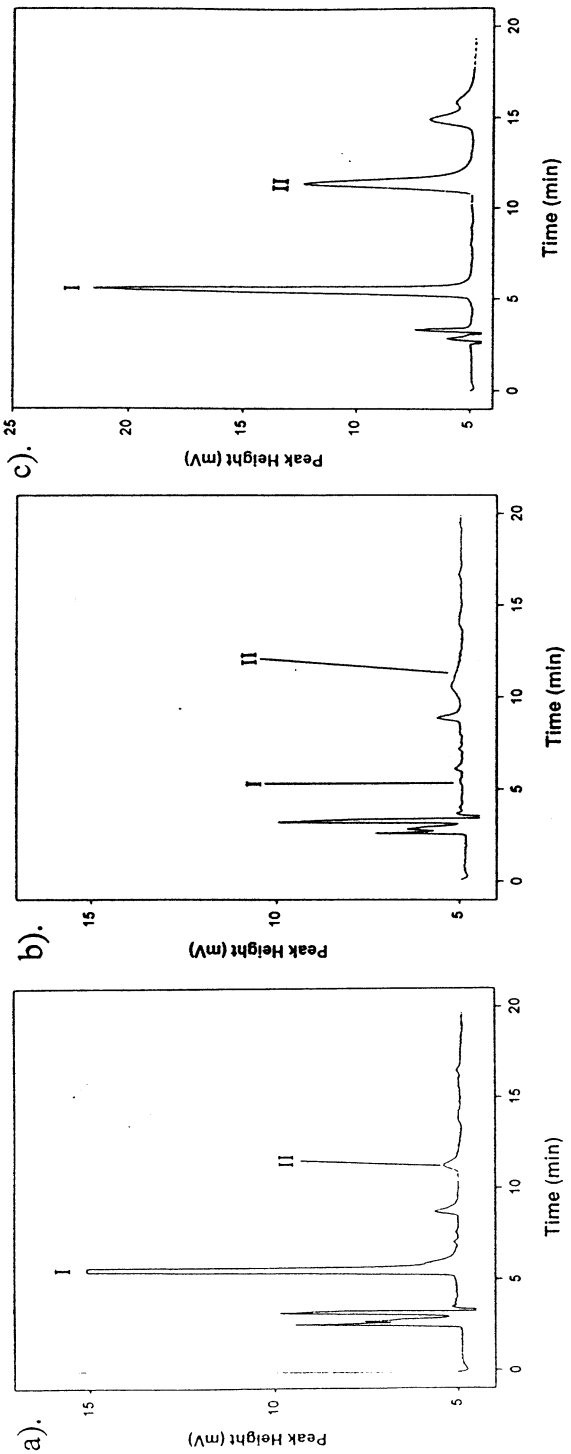

Fig. 2. Representative UV absorbance chromatograms for Compounds I and II from extracted rat plasma samples at concentrations of (a) 0.100 μg ml^{-1} (spiked), (b) blank and (c) 0.76 μg ml^{-1} (15 min after a 10-mg kg^{-1} h^{-1} dose by infusion).

Table 3
Concentrations and associated intra-day uncertainties for quality controls of Compound I in rat plasma

Nominal (μg ml^{-1})	Determined mean[a]	S.D.[b]	% RSD[c]	%RE[d]
0.400	0.400	0.024	6.0	0.0
2.40	2.41	0.022	0.9	0.4
16.0	16.4	0.406	2.5	2.5

[a] Determined mean for $n = 3$.
[b] Standard deviation in the mean.
[c] Percent relative deviation in the mean.
[d] Percent relative error in the determined mean as compared to the nominal concentration.

recoveries of 81.1 ± 9.2% were obtained for Compound I over the dynamic range of the assay, while those for Compound II were 89.6 ± 0.2% (2.0 μg ml^{-1}). Although some variability in the recovery was noted for Compound I, this apparently did not translate into higher variability for the assay overall. Table 3 summarizes the precision and accuracy estimates for Compound I, as assessed by replicate determinations of quality controls at three levels during a 1-day validation. The estimates for precision and accuracy, less than 6.0% RSD and less than 2.5% RE, respectively, demonstrated that acceptable assay performance was obtainable using a solid-phase extraction workstation. Similar results were obtained over four assay runs, containing approximately 200 rat plasma samples.

3.1.2. Linearity

A standard curve ranging from 0.100 to 20.0 μg ml^{-1} of Compound I yielded back-calculated standard concentrations which agreed with nominal values to within ± 8% (typical) and ± 15% (worst case outlier). The Pearson correlation (r) was 0.997 and a Y-intercept of − 0.0013 was statistically indistinguishable from zero. These results suggest acceptable assay linearity for a standard curve generated by extractions performed on the solid-phase extraction workstation.

3.2. Extraction development using the workstation (Compounds III and IV)

Using the program outlined in Table 2, a procedure for the extraction of Compounds III and IV in rat plasma was developed. Those variables which contributed most significantly to the selectivity and recovery of analytes, including sorbent selection, wash solvent and elution solvent pH and composition, were examined in some detail.

3.2.1. Recovery of analytes

Of the three sorbents evaluated, octadecyl gave clearly inferior selectivity and was not pursued. Octyl and phenyl sorbents gave acceptable recoveries and selectivities, with phenyl providing a slightly cleaner extract after a limited number of experiments. On this basis, phenyl was tentatively selected for further development.

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 zwetterionic compounds such as III and IV. Experiments conducted at higher pH or without pH control resulted in dramatically lower recoveries. Using the workstation to perform premixing, wash solvents with 30–60% methanol were automatically mixed and used to wash solid-phase extraction cartridges after application of sample spiked with 10, 100 or 1000 ng ml^{-1} of Compounds III and 2.5 μg ml^{-1} of Compound IV (internal standard). Recoveries were determined by comparison of analyte peaks to those of standards and the results are displayed in Fig. 3a and b, respectively. For either compound, an inverse curvilinear functionality between recovery percent and methanol percent composition was observed. Optimal recoveries for either compound were obtained at or below 30% methanol.

Acceptable selectivity was obtained for all recovery experiments when 0.5% methanolic ammonium hydroxide was used for elution. These results gave significantly better recovery than those obtained using pH 6.5 phosphate buffer/acetonitrile combinations. For this reason, and because rapid drydown was facilitated, 0.5% NH_4OH (methanolic) was selected as an elution solvent.

In a related experiment, the percent recoveries of Compounds III and IV as functions of concentration were determined and are summarized in Fig. 4a and b, respectively. For Compound III, although a higher recovery was obtained for the lowest concentrations studied (5 ng ml^{-1}), this level was not significantly different from the mean

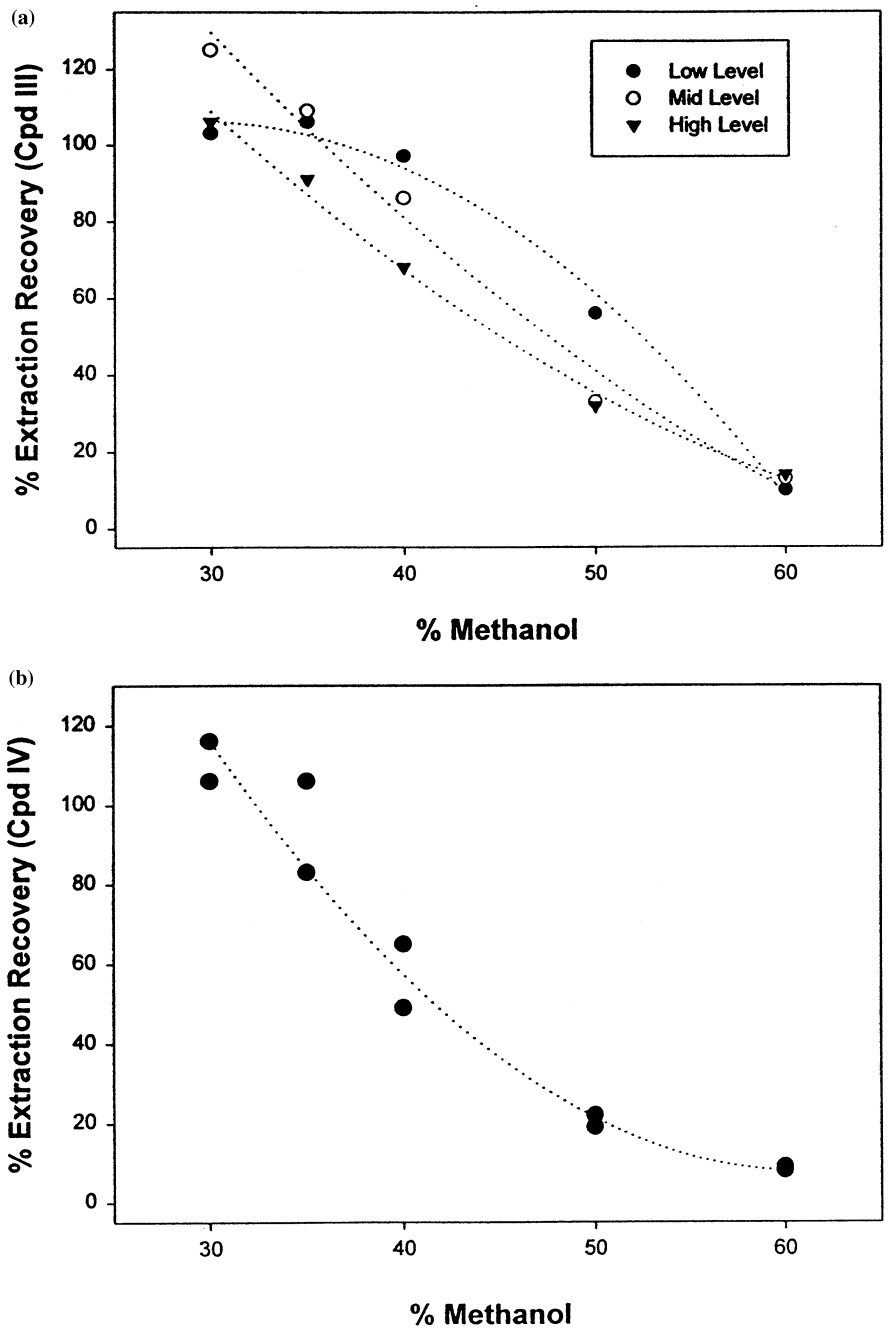

Fig. 3. Solid-phase extraction recovery of Compounds III (a) and IV (b) as functions of methanol percentage in wash solvent.

recovery ($P < 0.05$) over the calibration range. No trends in the recovery of Compound IV as a function of concentration were noted as well. The mean recoveries ($\pm$ S.D.) for Compounds III and IV were 90.8 ± 4.9 and $87.9 \pm 2.0\%$, respectively, over the calibration range.

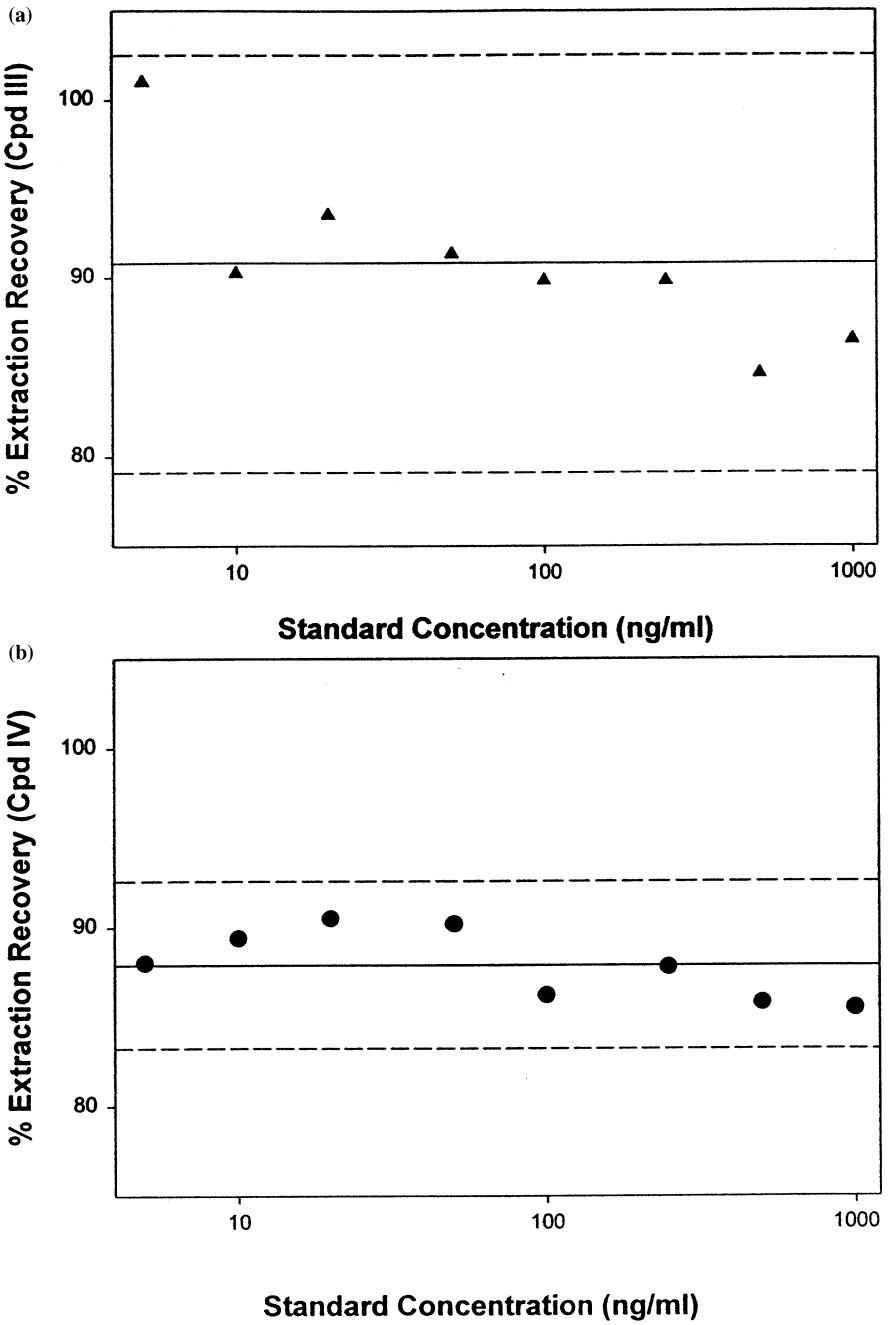

Fig. 4. Solid-phase extraction recovery of Compounds III (a) and IV (b) as functions of concentration. Dotted lines indicate 95% confidence intervals for mean recovery (solid line).

3.2.2. Chromatography

Representative chromatograms for the separation of Compounds III and IV in the post-extraction matrix are shown in Fig. 5A–C. Retention times for Compounds III and IV were 9 and 13 min, respectively, with associated capacity factors

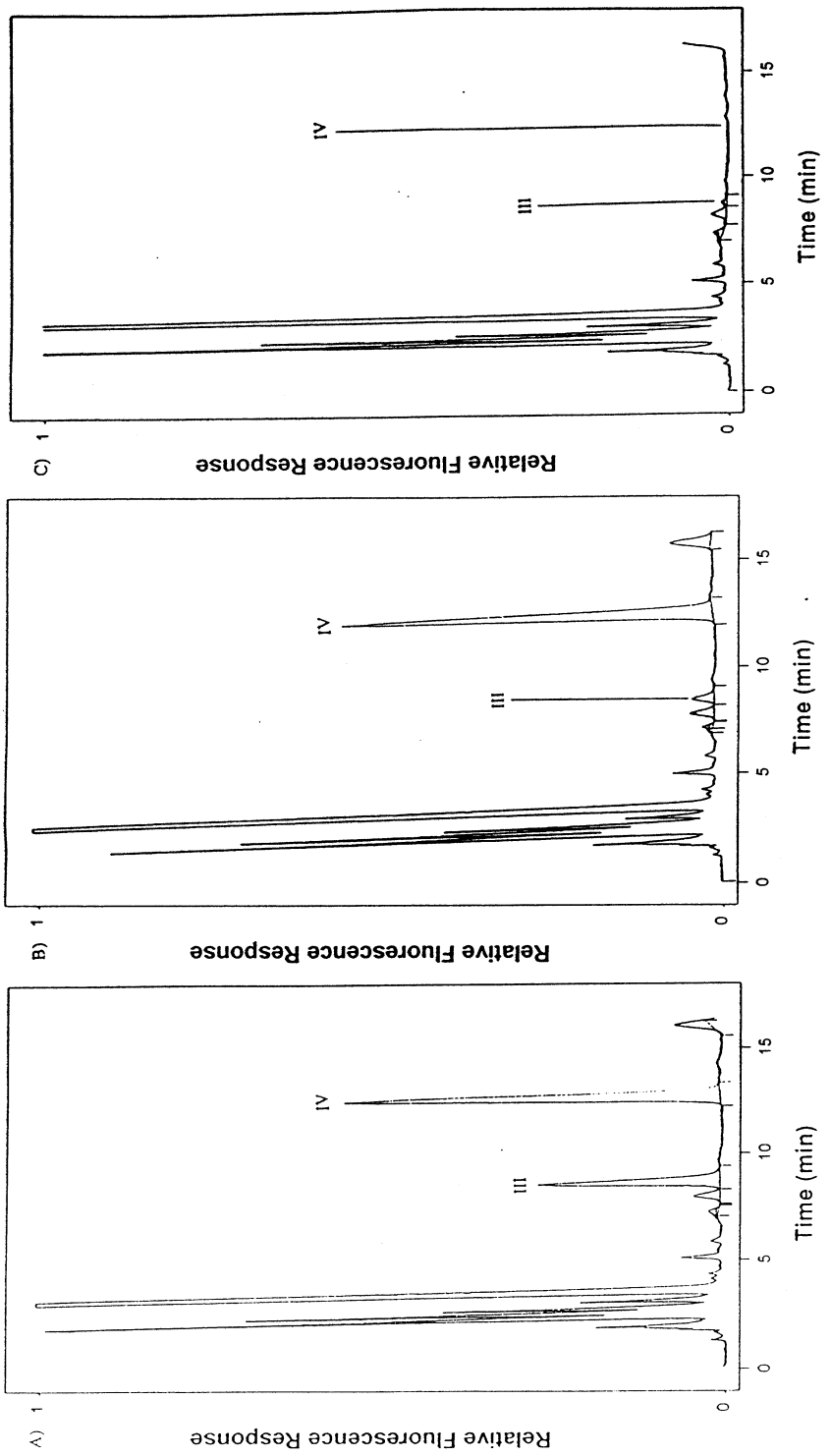

Fig. 5. Fluorescence chromatograms for Compounds III and IV from extracted rat plasma samples at spiked concentrations of (A) 100 ng ml^{-1}, (B) 10 ng ml^{-1} and (C) blank.

Table 4
Concentrations and associated uncertainties for quality controls of Compound III in rat plasma

Nominal (ng ml^{-1})	Determined mean[a]	S.D.[b]	%RSD[c]	%RE[d]
10.0	10.0	0.12	1.21	+0.1
100	105	2.10	2.00	+4.8
1000	988	18.9	1.91	-1.21

[a] Determined mean for $n = 3$.
[b] Standard deviation in the mean.
[c] Percent relative deviation in the mean.
[d] Percent relative error in the determined mean as compared to the nominal concentration.

of 6.5 and 9.8. The separation efficiency for these two analytes were 6000 and 9500 theoretical plates, respectively, and the resolution (R_s) between Compound III and the nearest matrix peak (8.3 min) was 2.7, indicating adequate chromatographic resolution. Interfering peaks were absent from the blank at the retention times for the two analytes. Chromatograms were consistent with reliable method performance.

3.2.3. Precision and accuracy of the method

The precision and accuracy of the method, as demonstrated by replicate quality controls at 10, 100 and 1000 ng ml^{-1} indicated a reliable analytical method (Table 4). Intra-day relative standard deviation did not exceed 2.0%, while intra-day relative errors were from −1.2 to 4.8%. As with the procedure for Compounds I and II, this workstation procedure demonstrated a high degree of precision and accuracy.

3.3. Workstation sample throughput

A single RapidTrace workstation module 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. As configured for this work, six modules were connected in parallel and were capable of processing up to 60 samples (10 × 6 modules) in a completely unattended manner.

For the extraction involving Compounds I and II (Table 1), 10 min were required to perform each complete extraction. Because samples were processed in parallel, up to six samples could be processed in this time and 60 samples (10 × 6 modules) could be processed in approximately 100 min.

Work involving Compounds III and IV (Table 2) required only 3 min for each complete extraction, with up to 60 samples being processed in approximately 30 min. If the workstation was expanded to a full set of ten modules, then up to 100 samples could be processed in this time. This latter throughput benchmark is much faster than could be performed by either manual processing (120 min for 100 samples), automated serial processing [1] or parallel processing in a 96-well format (90 min for 96 samples) [10].

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 and consistent recovery, as well as 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 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 should gain wide acceptance for its utility in rapid assay optimization using multiple variables, as well as high production throughput.

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