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Increase of the LC–MS/MS sensitivity and detection limits using on-line sample preparation with large volume plasma injection

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Abstract

Large volume injection (LVI) has systematically been studied to improve LC–MS/MS sensitivity (signal-to-noise ratio, or S/N) and detection limits. The method of LVI was combined with on-line solid phase extraction (on-line SPE) and LC–MS/MS detection for analysis of compounds directly in plasma. It was demonstrated that LVI of plasma with on-line SPE-LC–MS/MS allows for improvement of sensitivity and detection limits without compromising chromatographic peak shape and resolution and inducing significant matrix and signal suppression effects. Furthermore, sensitivity and detection limits improve linearly with the injection volume up to 100 μL. Quantification of the model compounds in plasma demonstrated comparable calibration curve statistics, precision and accuracy for 5, 50 and 100 μL plasma injections.

Keywords: Large volume injection; On-line SPE; Bioanalytical; Sensitivity improvement; LC–MS/MS

1. Introduction

The routine practice of bioanalytical sciences in the pharmaceutical industry involves broad utilization of liquid chromatography combined with mass spectrometry detection (LC–MS/MS) for qualitative and quantitative analysis. In particular, LC–MS/MS provides very high analytical quantitative detection sensitivity, selectivity and specificity for determination of drug candidates and their metabolites in samples prepared from in vivo and/or in vitro sources. Still, the perennial challenges facing bioanalytical scientists in the pharmaceutical industry require faster, more affordable, more sensitive detection of compounds of interests in samples.

Since the adoption of LC–MS/MS for quantitative analysis in the pharmaceutical industry, significant efforts have been invested to improve its sensitivity. Researchers have investigated the optimization of ionization mechanism [1] and evaluated different ion sources [2] on their effects on sensitivity, for example. A common cause of sensitivity loss in LC–MS/MS analysis of biological samples is the so-called matrix effect [3–5]. In addition to posing challenges to quantification, the matrix effect also results in the suppression of the analyte signal and lower sensitivity. Several approaches have been investigated to address matrix effects and improve sensitivity [6–14]. Some researchers have used post-column additions of methanoic acid [6] and trifluoroacetic acid in 2-propanol [7] into the LC flow to reduce or prevent analyte ionization suppression. Others investigated different off-line clean-up procedures [8] and on-line LC–LC [9–11] to clean up the samples and to address matrix effect and reduce signal suppression.

Another apparent way to improve LC–MS/MS sensitivity and limit of detection (LOD) is to inject a larger volume of samples. In fact, large volume injection (LVI) has long been widely implemented in GC [15]. LVI with HPLC and LC–MS have also been demonstrated for environmental analysis mostly in simple matrices [16]. However,

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direct LVI of complex matrix extracts into LC–MS/MS system may induce matrix signal suppression and also result in peak broadening and degrading chromatographic separation. Therefore a common practice for analysis in complex biological matrices is to utilize off-line sample preparation to clean up and concentrate the sample before injection. Indeed solid phase extraction (SPE) can be utilized for the large volume sample preparation approach. In SPE, the application of a larger volume of plasma or urine sample onto the SPE cartridge does not affect the SPE procedure in any significant way. However, conventional SPE method development is fairly time consuming. In addition, sample preparation using conventional SPE, even with the liquid handling robot with 96-well capability, still requires human intervention throughout the entire sample preparation process.

On-line sample clean up such as LC–LC–MS [17], LC–LC–MS/MS [18] and column switching [19,20] allows large volume injection and offers cleaner and faster sample preparation and analysis. Furthermore, most LC–LC methods require matrix dilution or off-line clean up before injection. On another front, recent advancement in on-line SPE instrumentation brings an integrated, robust, easier to set up, lower cost and efficient alternative to conventional SPE, LC–LC and column switching [21–24].

In this paper, we report a systematic study to improve bioanalytical sensitivity and detection limits (in terms of S/N) by using the LVI approach in combination with on-line SPE–LC–MS/MS. We studied and developed LVI methods for the analysis of drugs in plasma, which maintained the chromatographic separation and selectivity and, allowed for increased sensitivities and improved LOD without inducing matrix and signal suppression effects.

2. Experimental

2.1. Chemicals and materials

Propranolol and ketoconazole were purchased from Sigma (St. Louis, MO, USA). Fig. 1 shows the chemical structures of propranolol as well as the internal standard (IS), ketoconazole. HPLC grade methanol, acetonitrile and water were obtained from J.T. Baker (Philipsburg, NJ, USA). Ammonium acetate, ammonium formate, formic acid, acetic acid and ammonium hydroxide were purchased from J.T. Baker (Philipsburg, NJ, USA). C_{18} Luna column (2.1 mm × 50 mm, 5 μm) was purchased from Phenomenex (Torrance, CA, USA). The HySphere C_{18} HD cartridges (2 mm × 10 mm) were obtained from Spark Holland (Netherlands). Blank pooled rat plasma was purchased from Biochemed Pharmaceuticals Inc. (Winchester, VA, USA).

2.2. Chromatographic conditions

The new on-line SPE Symbiosis System (Spark Holland, Netherlands) is composed of two integrated units: autosampler (Reliance) with binary LC pumps and the on-line SPE unit with a pair of high-pressure solvent delivery pumps (HPDs). Up to two 96-well SPE cartridge plates can be used for a single batch. While one cartridge is eluting on the right clamp, the next one is being pre-conditioned in the left clamp. The ACN eluate, combined with LC gradient, is loaded onto HPLC column for LC–MS/MS analysis. The entire system is controlled by one software package (SparkLink), which interfaces with MS controlling software Analyst 1.3. LC and extraction methods as well as sample list run tables are created with the Spark-Link software and submitted to the Analyst 1.3. Flow rate was set at 0.8 mL/min. The LC pumps gradient profile is shown in Table 1. The mobile phase consisted of solvent A (5 mM ammonium acetate buffer, pH 7) and solvent B (acetonitrile).

2.3. Mass spectrometry conditions

Mass spectrometry detection was carried out in the positive electrospray (ESI) ion mode using API 4000 (Sciex, Concord, Ontario, Canada) triple quadruple system. Table 2 shows the MS parameters used for the detection of propranolol/ketoconazole (IS).

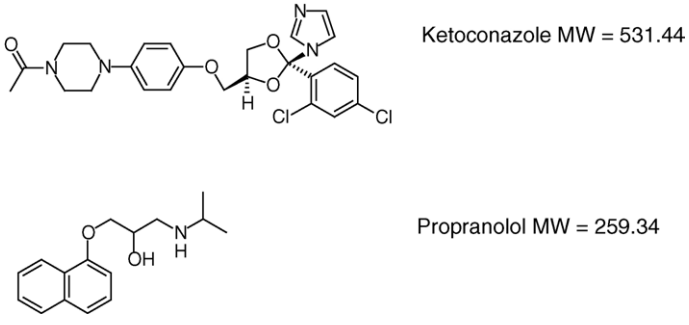

Fig. 1. Chemical structures of propranolol and ketoconazole.

Table 1
HPLC gradient table

Time (min)	B (%)
0:00	0
1:30	0
2:30	70
3:00	70
3:05	95
3:25	95
3:30	0
4:00	0

A: 5 mM ammonium acetate buffer, pH 7; B: acetonitrile.

Table 2
MS parameters for detection of propranolol and ketoconazole (IS)

	Propranolol	Ketoconazole
Ionization mode	Positive	Positive
Curtain gas (CUR) (psi)	10	10
Collision gas (CAD)	5	5
Ion spray voltage (IS) (V)	3000	3000
Temperature (TEM) (°C)	700	700
Ion source gas 1 (GS1) (psi)	85	85
Ion source gas 2 (GS2) (psi)	80	80
Declustering potential (DP) (V)	71	106
Entrance potential (EP)	10	10
Collision energy (CE) (eV)	27	43
Collision cell exit potential (CXP) (V)	8	10
MRM transition monitored (amu)	260/116	531/489

2.4. Sample extraction

The HySphere C_{18} HD (2 mm × 10 mm) was the cartridge of choice because it yielded the highest recovery, retention and satisfactory peak shape for both propranolol and ketoconazole. Table 3 lists the steps of the sample extraction process as performed by the Symbiosis system. On-line sample extraction procedure is discussed in details elsewhere [24].

2.5. Preparation of standards and calibration curves

Stock solutions of propranolol and ketoconazole were prepared directly from solids in acetonitrile (ACN) at 1 mg/mL concentrations and not corrected for salt and purity. Analyte (propranolol) working stock solutions of concentrations 10, 100 and 1000 ng/mL, as well as internal standard (ketoconazole) 1 μg/mL working stock solution were prepared from the stock solutions by dilution with distilled water. QC stock solutions were prepared from a different weighing of drugs than calibration standards and stored in −20 °C freezer along with the calibration standards. Blank rat plasma (850 μL) was spiked with 50 μL of internal standard stock solution and the proper volume of analyte stock solution and water to prepare the calibration curve and the quality control (QC) points. Final volume of the standard and QC solutions were 1 mL. Calibration curves were constructed of the following concentrations: 0.1, 0.2, 0.5, 1, 5, 10, 50 and 100 ng/mL, with three replicates at each point. Five QC replicates of each of the concentrations 0.2, 1 and 50 ng/mL were used to validate the calibration curves. The final ketoconazole (IS) concentration in plasma standards and QCs was 50 ng/mL. One double blank and one blank (blank + internal standard) were analyzed at the beginning of each batch.

2.6. Data analysis

Quantitative data processing was done using Analyst 1.3. Precision was calculated as percent relative standard deviation (% R.S.D.) of the analyte to internal standard peak area ratio obtained from replicates ($n = 5$) of each QC point. Accuracy was calculated as the percent bias of the calculated concentration relative to the nominal concentration of each QC point.

3. Results and discussions

Direct LVI of plasma samples using on-line SPE–LC–MS/MS was systematically investigated. Specific parameters studied included chromatographic characteristics, LOD, recovery and matrix effect, assay robustness and quantitative analysis parameters.

3.1. Chromatographic characteristics

To illustrate the advantages of LVI, 5 and 50 μL propranolol and ketoconazole neat samples (in water) were directly injected into the LC, bypassing the online SPE portion. Increasing the injection volume from 5 to 50 μL resulted in a chromatographic peak width increase of 50% (at FWHM). However, utilization of the on-line SPE to concentrate and clean up samples allowed retention of chromatographic sep-

Table 3
On-line SPE parameters

	Solvent	Flow rate (mL/min)	Volume (mL)	Duration (min)
Equilibration 1	Acetonitrile	5	1	0.2
Equilibration 2	5 mM ammonium acetate buffer (pH 7)	5	1	0.2
Loading	5 mM ammonium acetate buffer (pH 7)	2	0.5	0.25
Washing	5 mM ammonium acetate buffer (pH 7)	5	1	0.2
Elution	Acetonitrile	0.14	0.2	1.5

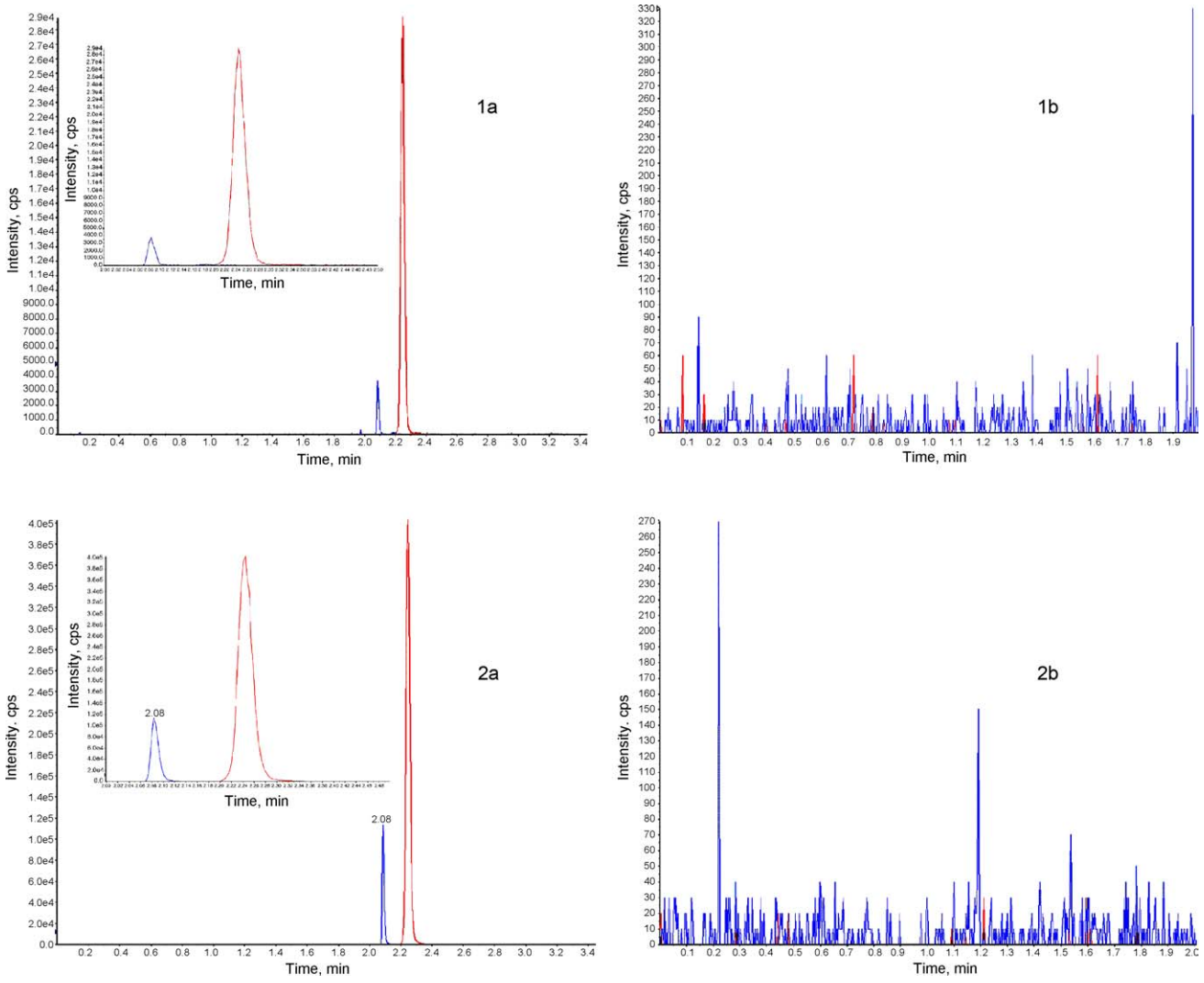

Fig. 2. Chromatograms of the 5 μL (1a) and 50 μL (2a) injections of propranolol (5 ng/mL) and ketoconazole (500 ng/mL) plasma samples and their background noise (1b and 2b, respectively) using on-line SPE–LC–MS/MS method.

aration and peaks widths. Fig. 2 shows representative chromatograms of the 5 μL (1a) and 50 μL (2a) injections of propranolol and ketoconazole obtained in plasma using on-line SPE–LC–MS/MS method. Note that the LC conditions in the on-line SPE–LC/MS/MS method were identical to the HPLC only method. Inspections of Fig. 2(1a and 2a) reveals significant increase of sensitivity for both propranolol and ketoconazole (i.e. peak height) while preserving chromatographic parameters and separation (i.e. retention time, peak width, peak shape and peak separation; see 1a and 2a insets). Therefore on-line SPE's near constant peak FWHMs with increased injection volumes lays one of the foundations for sensitivity improvement.

3.2. Increase of limit of detection (LOD)

Improvement of analyte S/N or LOD requires increase of peak height (area) while maintaining low noise and background levels. Fig. 2(1b and 2b) shows background noise for 5 and 100 μL injections of propranolol and ketoconazole plasma samples. Fig. 3 shows the Q1 scans of blank plasma at 5 μL (a) and 100 μL (b) injection volume, averaged between 2.0 and 2.4 min. Note that the maximum intensities of background noise peaks stay essentially the same. The 100 μL blank plasma injection has more background noise between 250 and 450 amu than the 5 μL injection, but the increase is far from proportional to injection volume, and is not reflected in MRM traces. The method developed for the analysis of propranolol and ketoconazole (see Table 3) allows for efficient clean up and washes out most of the matrix (plasma) components. SPE cartridge binding capacity allows for analyte retention despite increase of the matrix load with LVI. Therefore the matrix interference and background level stay low and essentially independent of the volumes of the injections studied. As a result, sensitivity, S/N and LOD increase with the sample injection volume. This is the other foundation for

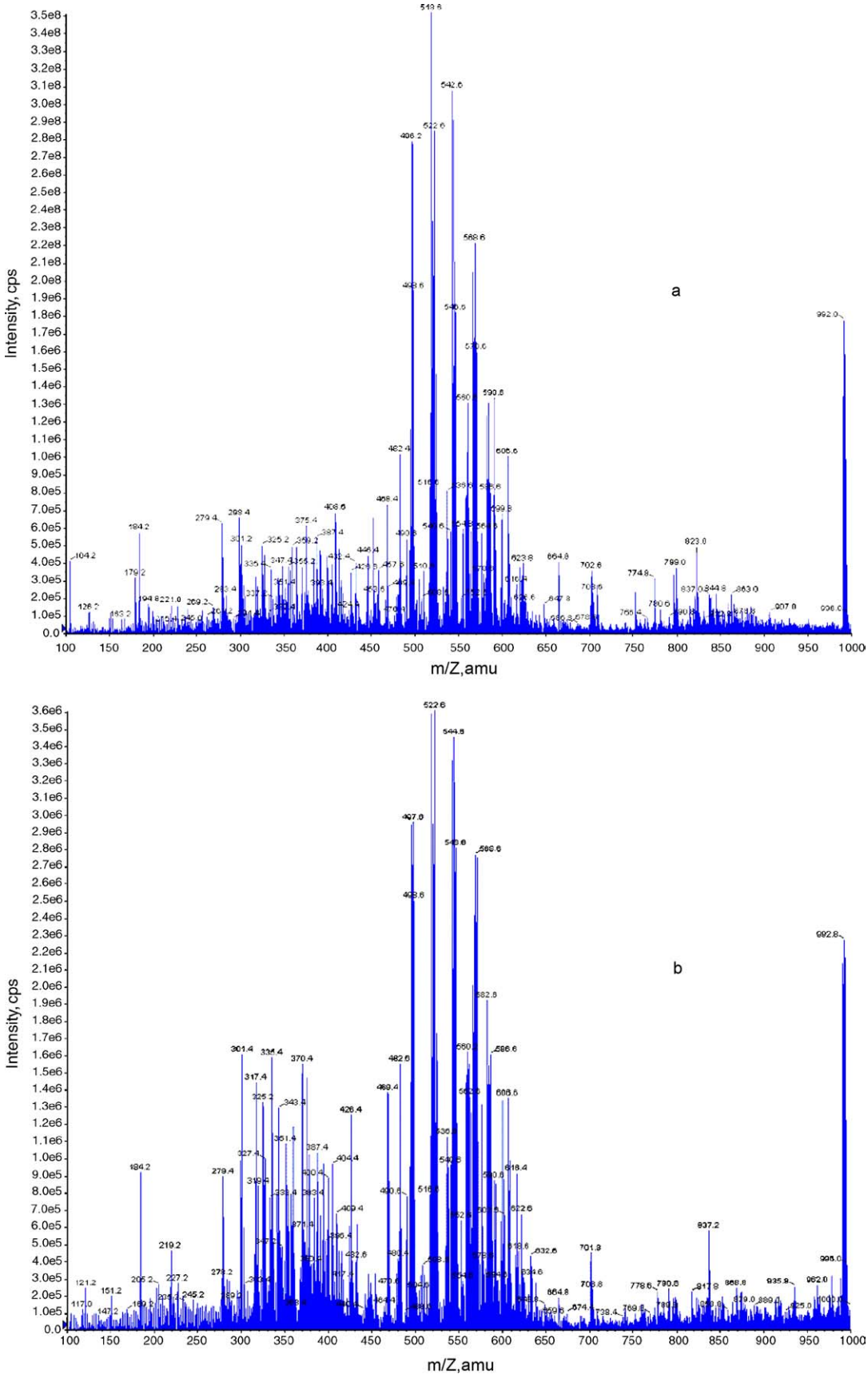

Fig. 3. Q1 scans for (a) 5 μL and (b) 100 μL blank plasma injections, averaged between 2.0 and 2.4 min.

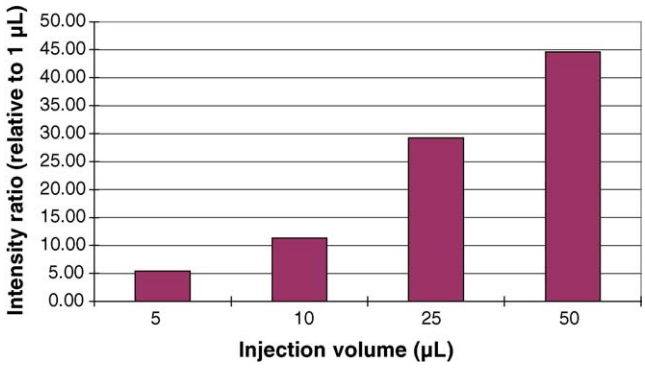

Fig. 4. Propranolol signal intensity vs. injection volume.

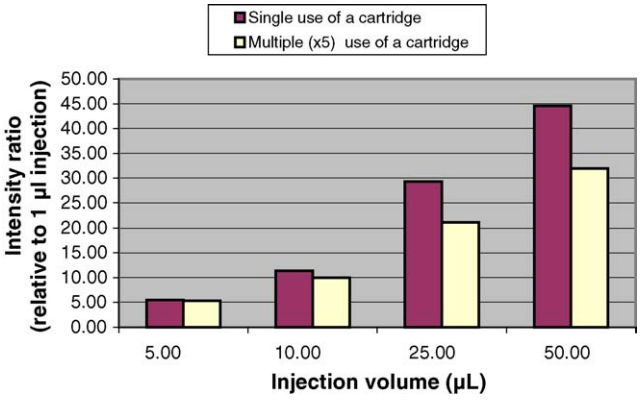

Fig. 5. Single vs. multiple use of a cartridge.

sensitivity and LOD improvement using LVI in combination with on-line SPE–LC–MS/MS.

Fig. 4 shows the signal intensity ratio (relative to 1 μL injection) obtained for propranolol in plasma versus injection volume. Increase of the injection volume by a factor of 10 (i.e. from 5 to 50 μL) resulted in increase of propranolol signal intensity by a factor of 9. The signal increase is not exactly proportional to the increase of the injection volume probably due to the complex interactions between analytes, SPE sorbent and plasma interferences. Nevertheless, Figs. 3 and 4 demonstrate that signal intensity, S/N and LOD can be improved using LVI. Furthermore this increase has near linear relationship to the increase of injection volume.

3.3. Recovery, matrix effect and assay robustness

Increase of sensitivity and LOD by increasing the injection volume in on-line SPE–LC–MS/MS is ultimately limited by the matrix effect and analyte recovery. Specifically, the volume of biological fluid that the extraction method and the SPE cartridge can handle without introducing matrix effect or failure to retain analyte is limited.

No significant signal suppression of the on-line SPE eluate was observed. Response of a single injection of plasma sample on the SPE cartridge was found to be above 90% of that of a neat sample, for up to 100 μL injection volumes. However, further increase of a single injection volume leads to a lower recovery and signal suppression effects. Fig. 5 shows the signal intensity increase vs. number of injections for single cartridge use and multiple uses (five injections on one cartridge). It demonstrates that utilization of a single cartridge for multiple injections results in smaller improvement of signal intensity compared to single injections. Significant change occurs after loading of 125 μL of plasma (25 μL × 5 injections) and further exacerbates for 250 μL (50 μL × 5 injections). This data demonstrated that a percentage of SPE sorbent capacity is taken with each injection. Eventually, sorbent capacity diminishes and will negatively affect the retention of analytes, causing low recovery and further signal decrease.

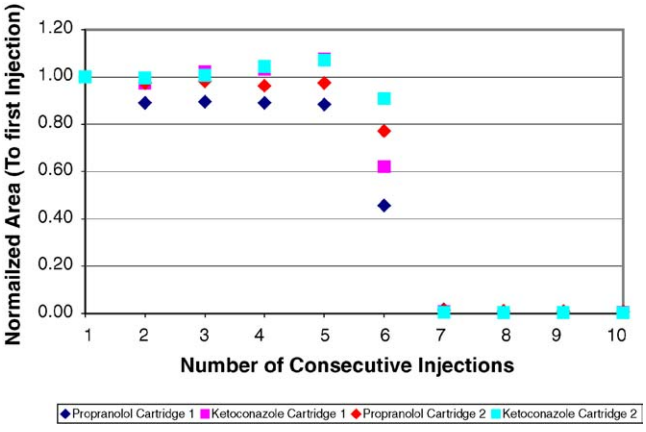

Fig. 6. Number of 50 μL plasma sample injections using the gradient LC method.

Fig. 6 demonstrates failure of the SPE sorbent after multiple injection of plasma. This experiment was repeated for different cartridges and different lots. In most cases each cartridge can handle up to 250 μL of plasma. Note that cartridge is reconditioned between injections. Therefore, in our case, individual injection of up to 100 μL plasma sample should be reliable as long as the total plasma volume passed is within 250 μL.

3.4. Quantitative analysis

To demonstrate the suitability of LVI of plasma samples using on-line SPE–LC–MS/MS, quantitative analysis was carried out with three injection volumes: 5, 50 and 100 μL. At each injection volume, the curve was repeated three times, with the QC samples bracketed by the standards. Three sets of SPE cartridges were used for the three 5 μL injections. The same sets were reused for 50 μL, and then 100 μL injections. Therefore, each individual SPE cartridge was loaded with no more than 200 μL of plasma samples. After a total of 235 injections the system pressure only increased slightly. Fig. 7 shows the calibration curves obtained for 5, 50 and 100 μL injections for 0.1–100 ng/mL range. Corre-

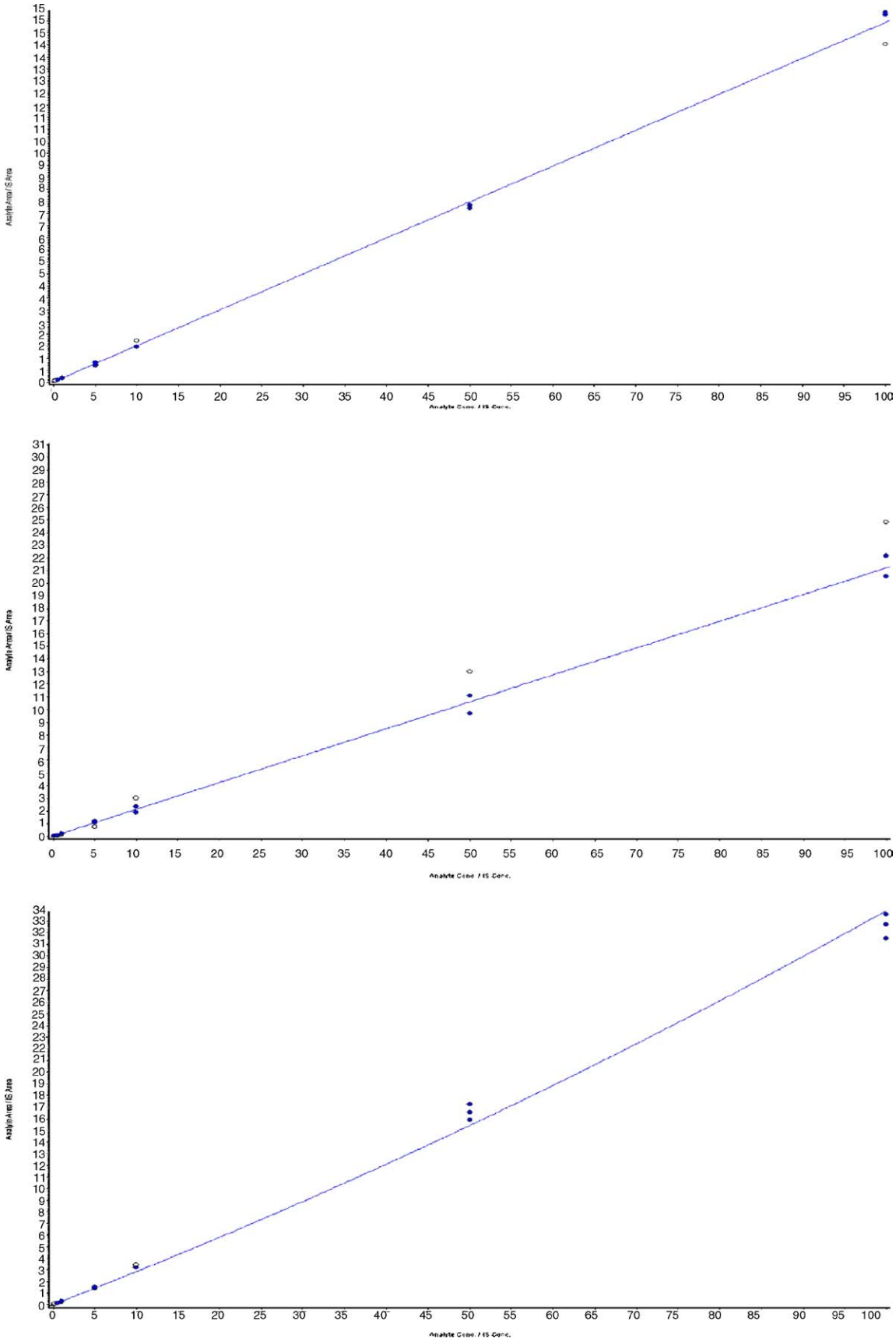

Fig. 7. Calibration curves of 5 μL (top), 50 μL (middle) and 100 μL (bottom) plasma sample injections.

Table 4
Quantitative analyses results

Injection volume (μL)	5	50	100
Regression	Linear	Quadratic	Quadratic
Weighting	$1/x$	$1/x^2$	$1/x^2$
Equation	$Y = 0.149x + 0.0319$	$Y = -9.89e{-}006x^2 + 0.213x - 0.00725$	$Y = 0.000617\,x^2 + 0.277x - 0.0028$
R^2	0.9990	0.9928	0.9924
Precision (% R.S.D. $n = 5$)			
QC 0.2	5.88	9.76	9.24
QC 1	6.99	6.58	3.52
QC 50	3.52	5.62	9.98
Average accuracy (%)			
QC 0.2	95.1	92.5	105.8
QC 1	93.3	94.8	102.4
QC 50	100.1	102.9	107.2

lation coefficients obtained for 5, 50 and 100 μL injection volume are greater than 0.99. Table 4 summarizes the precision, accuracy and calibration curve fitting results. Analysis accuracy and precision were comparable for all injection volumes. A close look at the chromatograms of the 100 μL injections of the 0.1 ng/mL plasma standard into the on-line SPE–LC–MS/MS system reveals that the S/N ratio for propranolol was about 40. Assuming that at LOD the S/N = 3, this on-line SPE system is capable of detecting a much lower level of propranolol in plasma. Based on results of multiple injections on one cartridge, injection volume may be increased up to 200 μL plasma with linear increase of sensitivity and detection limits. Further increase of sensitivity and detection limits would be limited by the cartridge binding capacity and can be addressed by increasing the SPE cartridge size. Another limiting factor is system carryover. Although carryover is insignificant for LVI of 0.1 ng/mL or more concentrated samples, at concentrations much lower than 0.1 ng/mL, system carryover becomes significant and hinders quantification. This issue is currently under investigation.

4. Conclusions

The method of LVI was combined with on-line solid phase extraction (SPE) for analysis of compounds directly in plasma using LC–MS/MS detection. It was demonstrated that LVI of plasma with on-line SPE–LC–MS/MS allows to improve sensitivity and detection limits without compromising chromatographic peak shape and resolution or inducing significant matrix and signal suppression effects. Furthermore sensitivity and detection limits improve linearly and proportionally to the injection volume up to 100 μL. Quantification of model compound in plasma demonstrated comparable calibration curve statistics, and measurement precision and accuracy for 5, 50 and 100 μL of plasma injections. Therefore, the methodology developed provides a reliable and simple alternative to improve sensitivity and detection limits for high throughput sample preparation and analysis of compounds in plasma and other complex matrices.

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