Analyst

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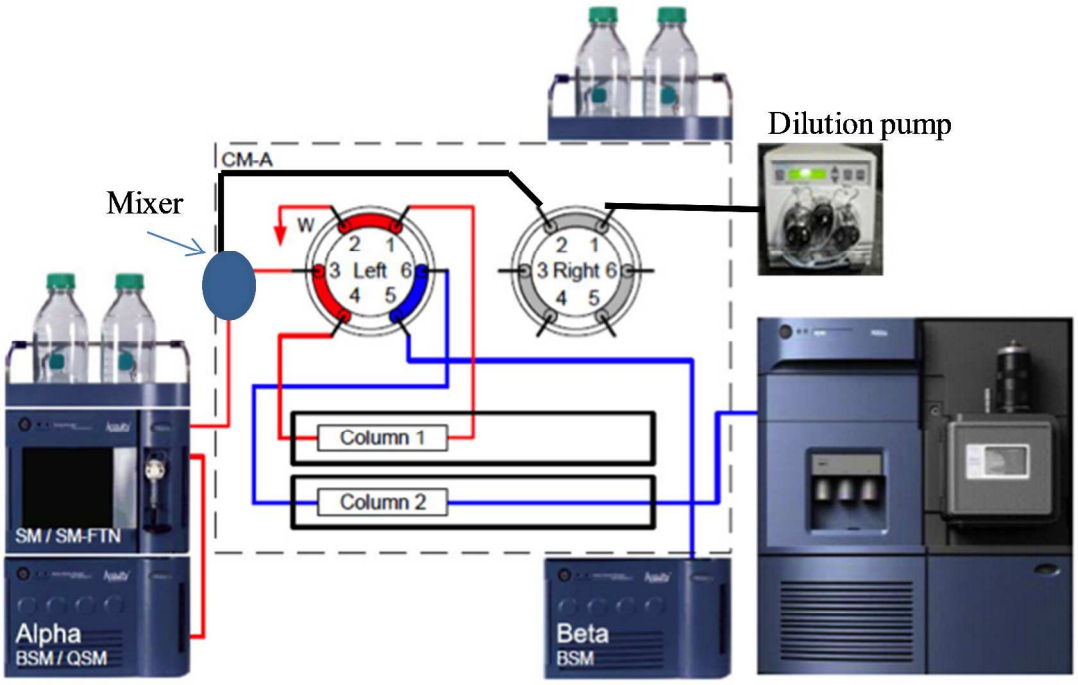
Journal:	*Analyst*
Manuscript ID:	AN-ART-11-2014-002090.R2
Article Type:	Paper
Date Submitted by the Author:	27-Jan-2015
Complete List of Authors:	Wilson, Ian D.; Imperial College, ; Imperial College, Rainville, Paul; Water Corporation, Simone, Jennifer; Waters Corporation, Root, Dan; Waters Corporation, Mallet, Claude; Waters Corporation, Plumb, Robert; Imperial College,

Colour graphic



Novelty of work:

This methodology provides increased assay sensitivity and facilitates small volume biofluid analysis in high percent organic samples.

A Method for the Direct Injection and Analysis of Small Volume Human Blood Spots and Plasma Extracts Containing High Concentrations of Organic Solvents Using Revered-Phase 2D UPLC/MS

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Abstract

The emergence of micro sampling techniques holds great potential to improve pharmacokinetic data quality, reduce animal usage, and save costs in safety assessment studies. The analysis of these samples presents new challenges for bioanalytical scientists, both in terms of sample processing and analytical sensitivity. The use of two dimensional LC/MS with, at-column-dilution for the direct analysis of highly organic extracts prepared from biological fluids such as dried blood spots and plasma is demonstrated. This technique negated the need to dry down and reconstitute, or dilute samples with water/aqueous buffer solutions, prior to injection onto a reversed-phase LC system. A mixture of model drugs, including bromhexine, triprolidine, enroflexacin, and

procaine were used to test the feasibility of the method. Finally an LC/MS assay for the probe pharmaceutical rosuvastatin was developed from dried blood spots and protein-precipitated plasma. The assays showed acceptable recovery, accuracy and precision according to US FDA guidelines. The resulting analytical method showed an increase in assay sensitivity of up to forty fold as compared to conventional methods by maximizing the amount loaded onto the system and the MS response for the probe pharmaceutical rosuvastatin from small volume samples.

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Keywords

At-column-dilution, dried blood spot, bioanalysis , LC/MS, 2D LC

Introduction

Over the last 30+ years the generation of pharmacokinetic data from pre-clinical studies and clinical trial samples has relied upon the analysis of blood-derived products in the liquid format, such as serum and plasma [1,2]. The use of a liquid format allows these samples to be easily and accurately sampled via pipetting, then the analytes isolated with techniques such as solid phase extraction, liquid-liquid extraction, or protein precipitation to remove the protein related-material [3,4]. A known quantity of the resulting liquid extract can then be quantitatively injected into the flowing stream of GC-MS or LC/MS systems. This allows the amount of sample introduced onto the system to be carefully controlled and assay sensitivity adjusted by the introduction of more or less volume [5].

Despite the benefits of liquid samples from an analytical chemistry point of view, there are several drawbacks including required sample volume, storage, handling and shipping. The collection of samples in tubes normally requires a minimum volume of 100-200 μL of blood. Although this size of sample is not a problem for large species, such as humans, some non-human primates or dogs, it severely limits the number of samples that can be taken from rodents such as rats, mice and guinea pigs etc. As a result there has, over the last few years, been significant interest in the use of alternative "microsampling" techniques, such as volumetric absorptive microsampling (VAMS) and dried blood spot (DBS) cards for the collection, storage and shipping of blood samples. The use of these formats can yield a dramatic reduction in the volume of sample required for analysis. This allows serial bleeding to be performed in rodents such that a whole pharmacokinetic

curve can be generated from one animal [6-13]. From a sample handling point of view, many researchers have reported that the use of DBS provides sufficient sample stability to allow the sample to be stored and shipped under ambient temperatures, thus significantly reducing storage and transportation costs. Several recent reports have estimated this saving in the region of $0.5 million per clinical trial study [14]. Along with the use of DBS, there has been interest in other forms of micro sampling approaches, such a capillary tube sampling and heat disintegrated blood sampling, and the aforementioned VAMS [15]. Whilst capillary sampling etc., does allow for the collection and reanalysis of the blood sample they do not, unlike DBS methods, allow for the shipping and storage of the sample under ambient conditions.

Notwithstanding the advantages that DBS sample acquisition delivers in terms of reduction in animal usage and reduced shipping costs, there are several analytical challenges to the routine application of the technique in bioanalysis. These include smaller sample volumes for analysis, additional sources of background matrix and the need to change sample format prior to analysis [16]. Processing of the DBS samples require the extraction of the sample from the cellulose card via punching out a fixed area at the center of the spot and then soaking the spot in organo-aqueous or entirely organic solvent. Most extraction solvents utilized for the extraction of compounds from DBS are of high organic composition [16-24]. The result of this is that only small volumes, generally less than 10 μL of the sample, can be directly injected onto a reversed-phase chromatography system without adversely affecting the chromatographic performance (especially if the composition of the extraction solvent is 100 % organic) [25].With orally

dosed compounds in the pre-clinical setting, a typical LC/MS system provides sufficient sensitivity to quantify the samples even at the 24 hour post dose time point. However, for compounds with low systemic exposure there is a need to load a greater portion of the sample onto the column to provide sufficient assay sensitivity. This requires further processing of the sample to reduce the proportion of organic solvent in the extracted sample or reduce the overall processed sample volume, using a process such as solid phase extraction or evaporation. These extra steps are not only time consuming but also risk introducing errors via e.g., losses of the analyte during the process or changing the sample via decomposition of thermally labile compounds etc.

To overcome some of the potential challenges with microsampling DBS formats, analytical methodologies have been developed to directly extract the DBS via the use of specialized devices such as thin layer chromatography (TLC) MS interfaces. Data from these studies showed that the direct extraction of probe pharmaceuticals from cards resulted in increased response for both chromatographic peak area counts and heights [26]. Further approaches utilizing chip based technologies (TriVersa NanoMate®) have been employed as well to create a liquid junction with the DBS paper and subsequent into the MS [27]. Direct MS analysis of DBS has further been illustrated via paper spray MS, desorption electropray ionization (DESI), and ambient sampling ionization [28-30]. Although these techniques do address the off line sampling issues of DBS many of these techniques do not offer opportunities for sample concentration or clean up thus failing to minimize matrix suppression or maximize the sensitivity of the technique.

Two dimensional (2D) chromatography has been used in both liquid and gas chromatography to provide increased resolution and selectivity in an analysis [31,32].

Over the last few years 2D chromatography has seen a resurgence in interest for both comprehensive chromatography of complex samples and targeted analysis of peptide therapeutics in a complex mixture [33,34]. In this LC format the analysis can be performed in several different modes including trap-elute, forward flush, backward-flush and heart cutting [35]. These approaches can deliver greater specificity to the analysis yielding more precise and accurate results. The trapping mode of analysis offers the analyst the opportunity to load a large volume of sample onto the LC system without degrading the chromatographic performance. This approach is used extensively in the field of nano-scale proteomics to increase the sample loading.

The application of small trapping columns to effect the isolation of drug related components from biological fluids prior to analysis by MS or LC/MS has been described previously by many authors [36-38]. The complexity / sophistication of the trapping process ranges from the use of a simple reversed-phase type columns, to the use of restricted access media and molecular imprinted polymers. Bower *et al* showed that by using a simple, single use, trapping cartridge plasma samples from clinical trial could be quickly and efficiently analysed without the need for prior processing [36]. However, all of these previous processes relied on the sample being in a solvent compatible with the extraction cartridge and, as previously mentioned, this is not the case with dried blood spot extracts. Thus in order to process these samples the elutropic strength of the solvent must be managed prior to the first column. In the majority of the published work the first dimension column was employed simply to trap the sample and remove protein related material rather than provide an “analytical quality” separation.

Here, we describe the use of 2D analytical scale chromatography, employing sub 2µm porous particles operated at greater than 6000 psi, with at-column-dilution (ACD) enabling the injection of large sample volumes of organic extracts of DBS spotted with as little as 15 µLs of blood and also plasma prepared by protein precipitation with acetonitrile as a means to both increase bioanalytical assay sensitivity and reduce endogenous contaminants.

Experimental

Chemicals

Methanol, acetonitrile, formic acid, and ammonium hydroxide were obtained from Sigma–AldrichChemicals (St. Louis,MO,USA). Probe pharmaceuticals procaine, brohexine, triprolidine and enrofloxacin were obtained from Sigma Aldrich (St. Louis, MO. USA) . Rosuvastatin (Figure 1) and the D6 deuterated internal standard were purchased from Toronto Research Chemicals (Ontario, Canada).

Human blood was purchased from Bioreclamation (Hicksville, NY) and stored refrigerated prior to use (blood was used within 7 days of purchase). Human plasma was also purchased from Bioreclamation and was stored at -80°C. All samples were collected according to ethical guidelines set by the Institutional Review Board (IRB).

Blood Spot Card Preparation

The samples, calibration curves and QCs, were prepared by spiking authentic standards in solution into fresh human blood over a concentration range of 0.1 to 100 ng/mL. Aliquots of 15 μL of blood were spotted onto Whatman DMPK type B cards. The centre of the resulting blood spots were sampled using a 3mm diameter punch from the center of the spot . These sample cores were suspended in 100 μL of methanol which was then shaken for one hour. The resulting extract was then centrifuged for 5 minutes at 13,000

relative centrifugal force (rcf). The extraction solvent was removed for injection onto the LC/MS system.

Plasma Preparation

Stock solutions for the pharmaceutical compounds used in this study were prepared in methanol and diluted 1 : 5 in water. The samples, calibration curves and QCs, were prepared by spiking the required concentrations of the authentic standards (normal and deuterated) in solution into human plasma over a concentration range from 0.1 - 50 ng/mL. The concentration of methanol, from the stock solutions in the samples, calibration curves and QCs were never greater than 5 percent organic. A 100 μL aliquot of plasma was mixed with 300 μL of acetonitrile and then vortex mixed with the resulting protein-precipitated sample centrifuged at 13,000 rcf for 5 minutes. The supernatant was then removed for injection onto the LC/MS system.

Chromatography

The chromatographic conditions described below were employed for procaine, brohexine, triprolidine, enrofloxacin and rosuvastatin. Analysis was performed using a 2.1 x 30 mm direct connect XBridge™ BEH C8, 10 μm as the trapping column and a 2.1 x 50 mm ACQUITY BEH C18, 1.7 μm column as the analytical column (Waters, MA, USA). The analysis was carried out on an ACQUITY Ultra Performance LC® system with 2D technology (Waters MA, USA) equipped with a sample manager, a binary solvent delivery manager for the analytical separation and a quaternary solvent delivery

manager to deliver the trapping column solvents. Aliquots of the samples (85 µl) were loaded onto the trapping column using the quaternary pump at a flow rate of 200 µL/min. The samples were diluted just prior to the trapping column via a mixer carrying 100 % water at a flow rate of 800 µL/min, thus producing a 1:5 dilution. The mixer utilized in this study is a standard Waters 50 µL mixer that contains zirconium beads. The mixer takes two fluidic pathways and combines them into a uniform single fluidic pathway. The trapping column (column 1) was maintained at 90 °C and the analytical column (column 2) at 35 °C. The elevated high column temperature of the trap column was chosen as a means to elute the analyte of interest in a minimum amount of organic mobile phase as a means to better re-focused the analytes on the analytical column. The analytes were eluted from the trapping column after the column had been washed with a mobile phase comprised of 20 % acetonitrile and 80% water (v/v). After washing, the analytes of interest were eluted from the trapping column and focused on the analytical column. The analytes were eluted from the analytical column using 95 : 5 – 45 : 55 % aqueous formic acid (0.1 % v/v): acetonitrile gradient over 5 minutes at 500 µL/min. Both the trapping and analytical columns were washed using 95 % acetonitrile 5% aqueous formic acid for 0.5 min after analyte elution. The columns were returned to the starting condition for 2 min prior to the next injection.

1D , 2D and 2D with ACD comparisons for aqueous and organic samples

Comparisons of four different LC configurations with aqueous and organic dissolved rosuvastatin samples were carried out on an an ACQUITY Ultra Performance LC®

system with 2D technology (Waters MA, USA) operating and configured in 1D, 2D, or 2D with ACD mode. The 2D method parameters that were utilized in these comparisions were identical to the parameters previously listed with the exception of having the ACD connected or not during the comparative experiment. The single dimension configuration comparison employed a solvent gradient from 95 : 5 to 45 : 55 % aqueous formic acid (0.1 % v/v): acetonitrile at 500 µL/min over 5 min. The column temperature was set at 35 °C. Mass spectrometry conditions were as described below. The rosuvastatin samples were either dissolved in 95 : 5 aqueous formic acid (0.1 % v/v) : acetonitrile or 100 % methanol.

Mass Spectrometry

The column effluent was monitored by positive ion electrospray MS operating in MRM mode on a Xevo™ TQ-S Mass Spectrometer (Waters MS Technologies, Manchester, UK). The collision energy was set to 32 eV, the capillary voltage to 1.0 kVand cone voltage to 60 V. For procaine, brohexine, triprolidine and enrofloxacin the following MS transitions were monitored: Procaine 237 ⇒ 164 , brohexine 377⇒ 114, triprolidine 279⇒ 208, enrofloxacin 360⇒ 316

Rosuvastatin was monitored using the transition 482⇒258 and the transition 488⇒264 was employed for the D6 internal standard.

Results and Discussion

The sensitivity of a bioanalytical assay is dependent upon the amount material which can be introduced onto the analytical measurement system. Thus with a reversed-phase chromatography system a careful balance that must be made between the solution which is employed to dissolve the sample, the mobile phase composition and the volume that can be injected onto the system. This can result in a compromise between having the optimal chromatography conditions and one that allows the maximum sample load on the column.

The 2D ACD LC/MS system was designed to allow the loading of samples containing a high percentage of organic solvent onto a reversed-phase LC system. To achieve this, the highly organic extract was diluted with water directly before the trapping column via the use of 50 µL LC mixer. Employing a flow rate of 100 % water (four-fold higher than that of the sample injection line flow) ensured that the 100 % organic injection composition was focused onto the trapping column with no breakthrough, even though it was maintained at 90 °C. The schematic in Figure 2 illustrates the set up of the chromatography system. The flow of the solvents was controlled by the left hand valve, which first directed the injection flow from the quaternary solvent manager onto the trapping column (column 1), while the analytical column (column 2) was washed with solvent from the binary solvent manager with the valve in position 1. When the left hand

valve switches to position 2 the analytical binary solvent flows through the trapping column eluting the analytes from the trapping column onto the analytical column. Once the analyte of interest was eluted from the trapping column onto the analytical column the valve was switched to the initial position of 1 whereby the analyte is eluted from the analytical column into the mass spectrometer.

The ability of the 2D ACD LC/MS system to inject large volumes of organic solvent on to the system is illustrated in Figure 3a-3d. In this Figure, we observe the comparison of a 10 μL injection of the probe pharmaceuticals procaine, brohexine, triprolidine and enrofloxacin dissolved in 100 % water or 100 % acetonitrile onto the 2D LC system with and without the incorporation of ACD. As can be observed in Figures 3a – 3d, when samples were injected in acetonitrile without the incorporation of ACD the chromatographic peak shape suffered greatly, indeed, the majority of the analyte was not retained on the trapping column and thus no prominent peak was detected. The lack of retention for the test analytes was further determined during the method development process by placing the first column (trapping column) in line with the mass spectrometer and monitoring for the MRM transaction of the compound under evaluation with and without the at column dilution option engaged. This result was also obtained for the cholesterol lowering statin rosuvastatin where the majority (based on peak response) of the drug was unretained without the use of ACD (Figure 4).

The maximum volume of the 100 μL organic extraction from the 15 μL DBS spot that was injected onto the 2D ACD method was determined to be 85 μLs due to the minimum

residual volume of the LC vials utilized in this study. This 85 μL injection volume on the 2D ACD method compares with the maximum volumes of organic solvent that could be directly injected onto the 1D reversed-phase LC system without prior aqueous dilution before chromatographic peak fronting and break through were detected of 2 μL or 4 μL when rosuvastatin was dissolved in acetontrile or methanol respectively. Thus, the use of the ACD method resulted in an increase in assay sensitivity for the DBS assay by 20-fold and 40-fold for the plasma prepared by protein precipitation with acetonitrile. With greater volumes than these peak fronting and lack of retention was observed. Therefore the application of 2D-LC significantly improved the mass of material that could be loaded onto the system and hence of the sensitivity of the assay.

A further advantage of the ACD configuration was that the extracted biological sample can be further cleaned up by employing an organo-aqueous solvent composition from the quaternary solvent delivery system that elutes the endogenous matrix components from the trapping column while leaving target analytes unaffected. The analyte can then be eluted from the trapping column with a mobile phase composition just sufficient to remove the analyte from the trapping column while leaving the more lipophilic matrix components such as phospholipids on the trapping column. Once the target analyte(s) had been eluted from the trapping column and focused on the analytical column, the valve was switched such that the solvent from the gradient was directed solely to the analytical column. This allows the trapping column to be washed and conditioned for subsequent injections while the analytical gradient is completed. The result of this is shown in Figure 5. Here we observe the unoptimized and optimized 2D LC conditions whereby the

residual phospholipid fraction contained in human plasma prepared by protein precipitation is diverted away from the analytical column (column 2) and the mass spectrometer. This provides for a means to keep the source of the mass spectrometer clean therefore leading to possibly more robust methods and also removing a major source of ion suppression. The use of a C_8 alkyl-bonded phase as the trapping column, maintained at 90°C, and a C_{18} alkyl-bonded phase as the analytical column maintained, at 35°C, aids the focusing of the analytes eluted from the trapping column onto the analytical column as the parameters set with the trapping column are less retentive to those of the analytical column under the conditions set.

Maximizing sensitivity is an integral method parameter and must be evaluated for any bioanalytical method. Therefore the area counts of the probe pharmaceutical rosuvastatin were evaluated during development of the 2D ACD LC /MS method. This evaluation included both 1D and 2D LC/MS configurations as well as aqueous and organic sample diluents. The data from this experiment are shown in Table 1. The same amount of analyte was injected onto each system.

The data displayed in Table 1 shows two important aspects. First, the rosuvastatin area counts between the 1D, 2D and 2D ACD methods showed excellent agreement. The reproducibility of each mode of operation was excellent with all %CVs being equal to or less than 3%. This indicates that the incorporation of additional fittings, tubing, valves, and mixer have not sequestered any of the analyte since comparison of the rosuvastatin area counts between each configuration remains fairly constant. Second, there was a

significantly higher MS response, approximately 20%, for rosuvastatin when a 100% methanol solution was injected. The explanation for this result may be due to either the increased solubility of rosuvastatin in methanol or perhaps a reduction in non specific binding that may be present due to interaction of the analyte in the injection vial or other components of the LC system. In either case, the overall sensitivity of the assay was influenced positively when the sample was injected in a 100% methanol environment.

This 2D ACD LC/MS approach was then evaluated for the analysis of rosuvastatin, in dried blood spots and plasma prepared by protein precipitation. Rosuvastatin is a competitive inhibitor of the enzyme HMG-CoA reductase, having a chemical structure and mechanism of action similar to that of other statins (Figure 1) [39]. Its approximate elimination half-life is 19 h and its time to peak plasma concentration is reached in 3–5 h following oral administration. The blood spot was extracted as described in the experimental section. Representative chromatograms for the lower limit of quantification and blank immediately following the injection of a high concentration standard is shown in Figure 6 for the blood spot analysis. From these data we can see that the blank chromatogram shows no interference in the region where the peaks elutes at 4.44 minutes. In addition, the chromatographic peak is very symmetrical and has a peak width in the order of 3 seconds at the base. The limit of detection for subsequent validation was set at 0.5ng/mL with a signal to noise value of 20:1 from a 15µL blood spot sample. This compares favorably with previously published work by Sangster et-al [40] where a limit of quantification of 0.1ng/mL was obtained from a much larger sample volume of 500 µL

of plasma processed by solid phase extraction and analysed by microbore reversed-phase LC/MS/MS.

The assay was validated using a 3 run protocol on 3 successive days according to the FDA validation guidelines May 2001 [41]. The method validation data is shown in Table 2 where the inter-day precision and accuracy showed bias of 14.8% and a CV of 4.0% at the 0.5 ng/mL level and bias of 1.4% and a CV of 1.2% at the 80 ng/mL level. An example calibration curve and residuals plot generated from this data is shown in Figure 7.

While these data illustrate the quantification of a pharmaceutical compound from a DBS extract using 2D ACD LC/MS, this approach could be applied to any analysis using an aqueous miscible organic solvent. To illustrate this point, the assay for rosuvastatin was repeated via analysis of rosuvastatin spiked into human plasma with protein precipitated with acetonitrile. A representative chromatogram for the LLOQ standard and the blank immediately following the 50ng/mL standard are shown in Figure 8. Here we can see that the 2D system provides excellent chromatographic performance and a very clean chromatogram. The plasma protein precipitation method was subjected to a one run validation using a 96 well sample plate. The method was demonstrated to be linear over a range of 0.1 – 50ng/mL with an r^2 value of 0.9995 obtained for the calibration line using a linear 1/x weighting. The results obtained are displayed in Table 3. At the 0.1 ng/mL level the bias was -6.2% with a CV of 7.1% and at the 35ng/mL level the bias was -1.0% with a CV of 1.8%. The difference in sensitivity obtained between the DBS and

protein precipitation method can be attributed to the difference in the volume of sample employed for each analysis. In the DBS method just 15μL of sample was sampled whereas for the protein precipitation assay, 100μL of plasma was extracted.

These results suggest that this approach of the 2D ACD LC/MS method can be employed for the analysis of organic eluents derived from either plasma protein precipitation or dried blood spots. This approach allows the direct injection of large volumes of organic solvent, removing the need for an evaporation step for organic based solutions. This not only saves time, it also eliminates the thermal energy cost involved in solvent evaporation, eliminates any potential loss involved in re-dissolving dried extracts and eliminates the potential for degradation of compounds during evaporation. Moreover this method improves the ability to obtain low limits of quantification for small sample volumes as it maximizes the amount that can be analyzed as well as reduces the potential for possible matrix suppression through the use of a second dimension of chromatographic separation.

Conclusion

The use of a 2D LC/MS approach with ACD shows great promise for the direct analysis of organic solutions resulting from the preparation of biological fluids such as dried blood spots and plasma. The implementation of ACD allowed for the highly organic composition sample to be directly loaded and focused onto a reversed-phase trapping column. Analytes of interest, such as rosuvastatin, can then be washed and selectively eluted from the trapping column onto the analytical column. This effectively removes a portion of sample matrix from being deposited onto the analytical column. With this approach, it was possible to inject 85μL of methanolic extracts of rosuvastatin onto the analytical system with no adverse effect on the chromatography providing between 20 and 40 fold increase in sensitivity compared to direct injection depending upon the solvent used to extract the samples. Using this approach for a DBS assay, precision and accuracy results for three separate batches was demonstrated to be well within the US FDA validation guidelines for LC/MS based assays with a limit of detection for rosuvastatin of 0.5 ng/ml. We further applied this approach using extracts of the same probe pharmaceutical, prepared by protein precipitation from plasma via addition of acetonitrile. Again the assay showed acceptable precision, and accuracy. Moreover the method showed an increase in the MS response, possibly related to increased solubility or

a reduction in non-specific binding due to injection of rosuvastatin in a 100 percent organic solution. This approach limits the need for evaporation and reconstitution in an aqueous solvent, which could result in analyte loss due to solubility, volatility, or non specific binding depending on the chemical nature of the analyte. Lastly this method maximizes the amount of organic extracted biofluid samples that can be loaded onto the chromatographic system making it useful for assays where sample volume may be limited.

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Tables and Figures

Table 1: Rosuvastatin area counts versus LC configuration and injection solvent.

Table 2: Three day inter-day accuracy/precision dried blood spot analysis of rosuvastatin.

Table 3: Day one intra-day accuracy/precision protein precipitation analysis of rosuvastatin.

Figure 1: Chemical structure of rosuvastatin.

Figure 2: Schematic of 2D ACD LC/MS System.

Figure 3: Comparison of probe pharmaceutical compounds injected dissolved in 100 % aqueous and 100 % acetonitrile with ACD and without ACD. Figure 3a) brohexine Figure 3b) triprolidine and Figure 3c) enrofloxacin and Figure 3d procaine.

Figure 4: Comparison of direct injection of rosuvastatin in 100 percent methanol with and without at-column-dilution.

Figure 5: Precursors of m/z 184 indicating the presence of the residual phospholipids from human plasma prepared by protein precipitation. Observed are the unoptimized (top

chromatogram) and optimized (bottom chromatogram) trapping conditions whereby the reduced signal of m/z 184 is observed.

Figure 6: Chromatogram of LLOQ and blank following analysis of rosuvastatin from dried blood spot at ULOQ.

Figure 7: Calibration line and residuals plot for the 2D ACD LC/MS analysis of rosuvastatin from dried blood spot.

Figure 8: Chromatogram of LLOQ and blank following ULOQ analysis of rosuvastatin in plasma following precipitation with acetonitrile.

Table 1

Area Counts

Injection	1D H_20	2D H_20	2D ACD H_2O	2D ACD MeOH
1	3405	3251	3374	4083
2	3503	3245	3350	3996
3	3562	3425	3591	4089
4	3596	3486	3603	4114
5	3535	3431	3507	4008
6	3683	3421	3494	4064
AVG	3547.3	3376.5	3486.5	4059.0
STDEV	93.1	102.3	106.1	47.1
%CV	2.6	3.0	3.0	1.2

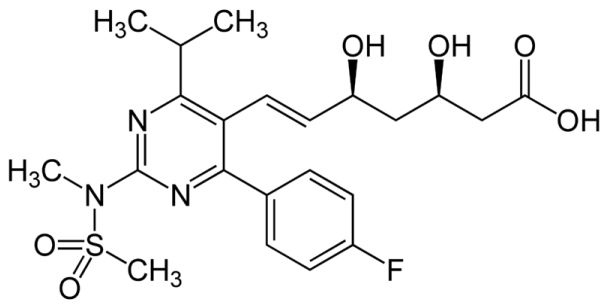
Table 2

	QC LLOQ 0.5ng/ml	QC Low 1.5ng/mL	QC Mid 30ng/mL	QC High 80ng/mL
Mean	0.574	1.55	29.9	81.1
St Dev	0.0230	0.0249	0.432	1.01
%CV	4.0	1.6	1.4	1.2
%Bias	14.8	3.2	-0.2	1.4
Replicates	18	18	18	18

Table 3

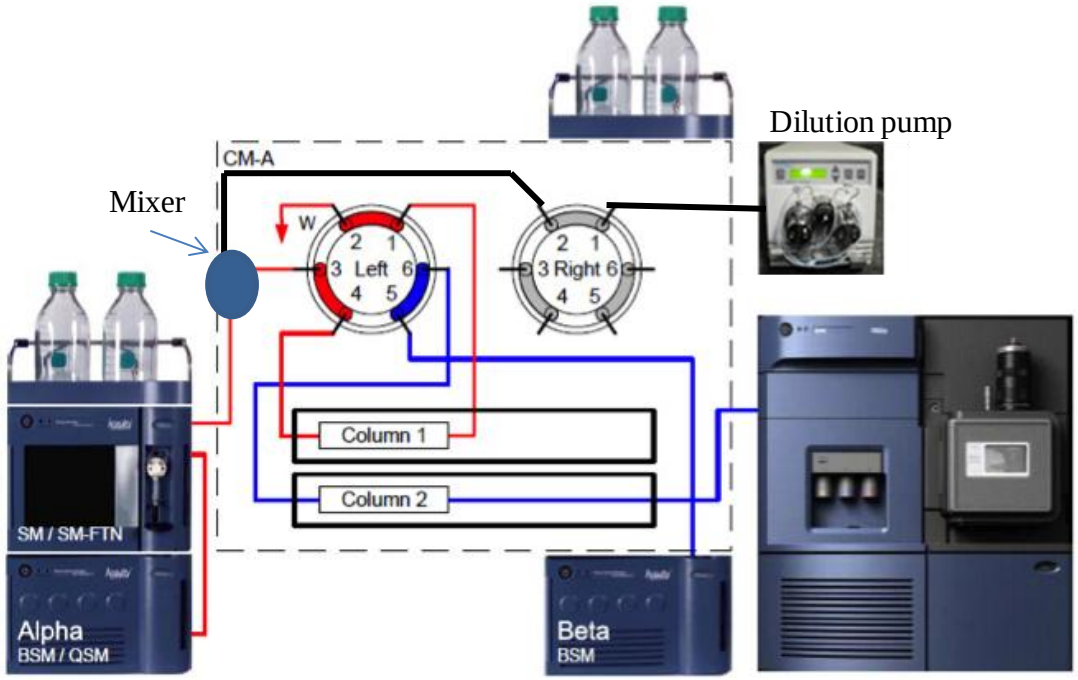
	QC LLOQ 0.100 ng/mL	QC LOW 0.300 ng/mL	QC MID 3.00 ng/mL	QC HIGH 35.0 ng/mL
Mean	0.0938	0.309	3.17	34.6
St Dev	0.00661	0.0173	0.116	0.613
% CV	7.1%	5.6%	3.6%	1.8%
% Bias	-6.2%	3.0%	5.8%	-1.0%
Replicates	6	6	6	6

Figure 1



683 Figure 2

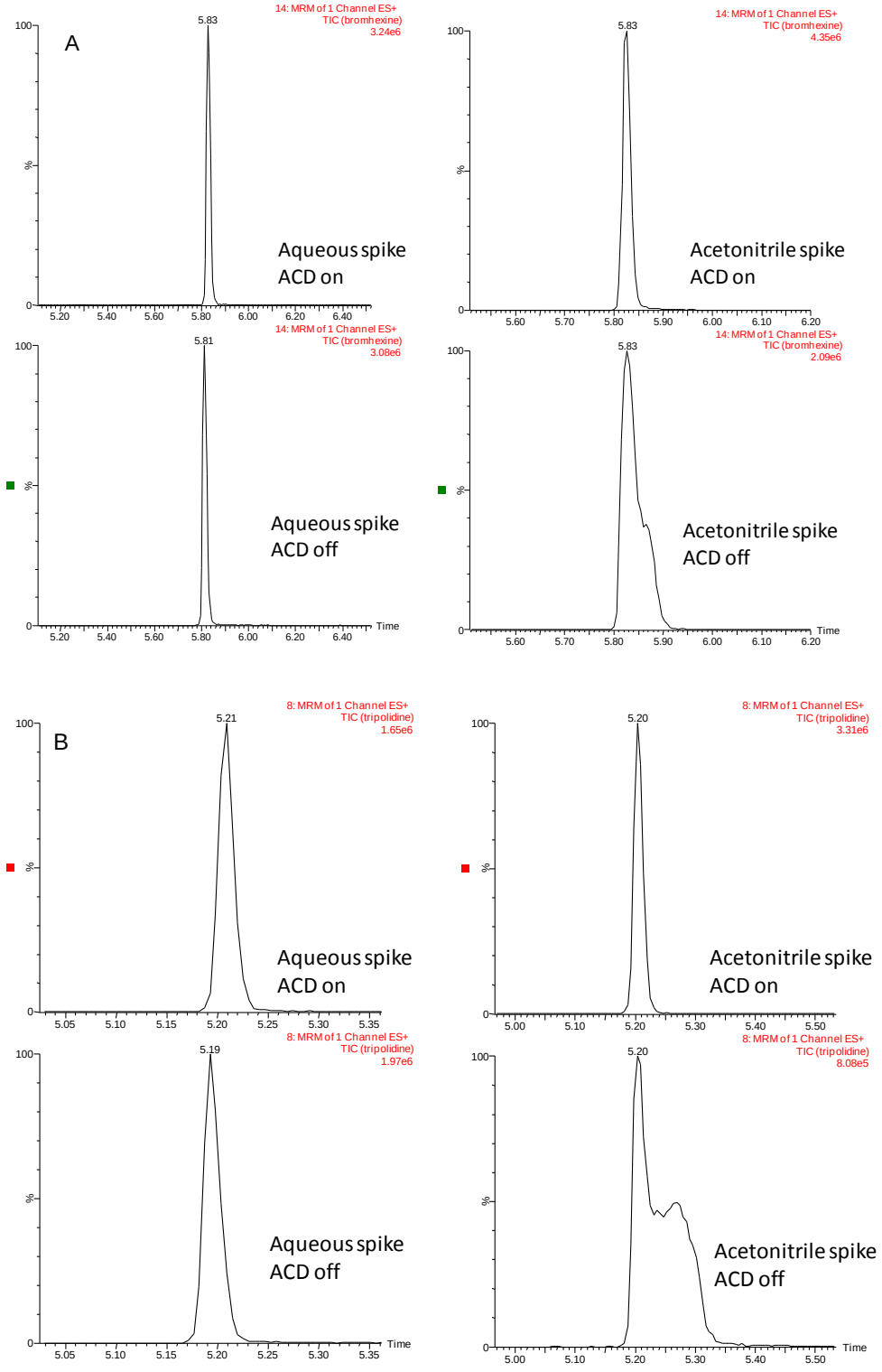
684

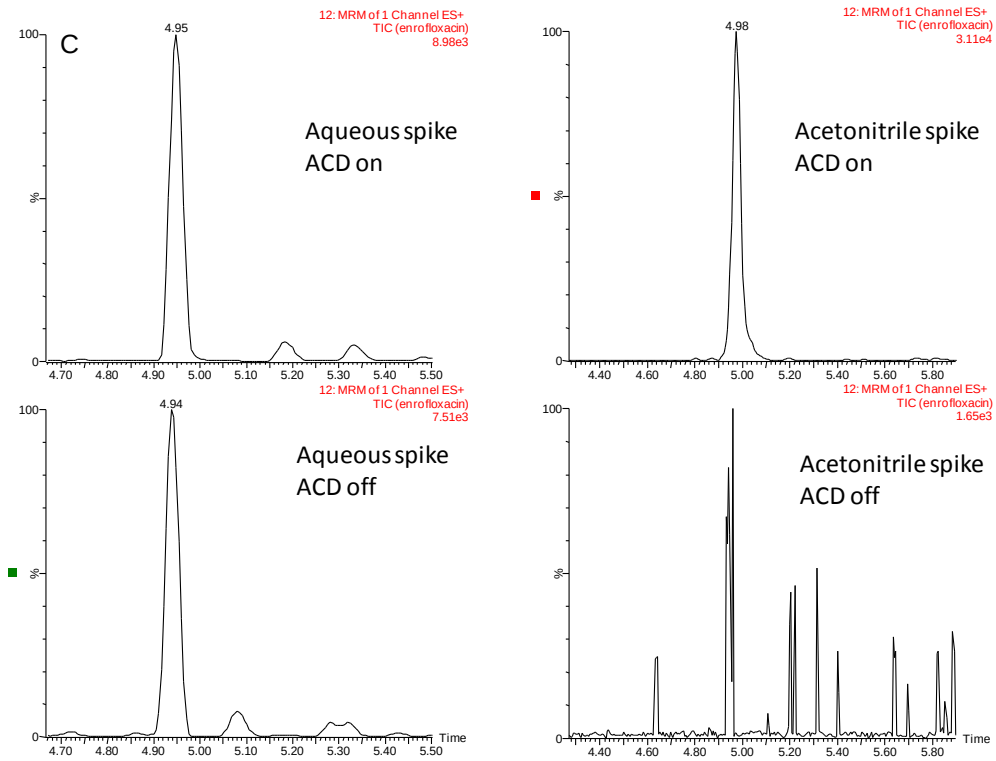


685

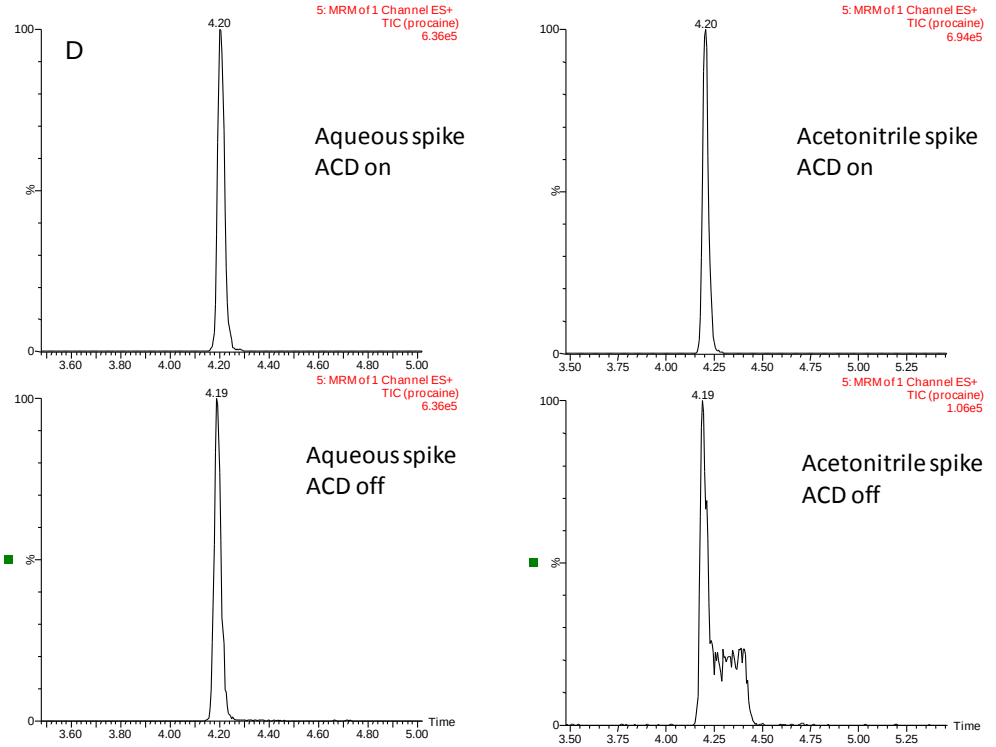
686

Figure 3a – 3d


A
14: MRM of 1 Channel ES+
TIC (bromhexine)
3.24e6
5.83
Aqueous spike
ACD on
14: MRM of 1 Channel ES+
TIC (bromhexine)
4.35e6
5.83
Acetonitrile spike
ACD on
14: MRM of 1 Channel ES+
TIC (bromhexine)
3.08e6
5.81
Aqueous spike
ACD off
14: MRM of 1 Channel ES+
TIC (bromhexine)
2.09e6
5.83
Acetonitrile spike
ACD off
B
8: MRM of 1 Channel ES+
TIC (tripolidine)
1.65e6
5.21
Aqueous spike
ACD on
8: MRM of 1 Channel ES+
TIC (tripolidine)
3.31e6
5.20
Acetonitrile spike
ACD on
8: MRM of 1 Channel ES+
TIC (tripolidine)
1.97e6
5.19
Aqueous spike
ACD off
8: MRM of 1 Channel ES+
TIC (tripolidine)
8.08e5
5.20
Acetonitrile spike
ACD off
Time



695
696
697



698
699
700
701

Figure 4

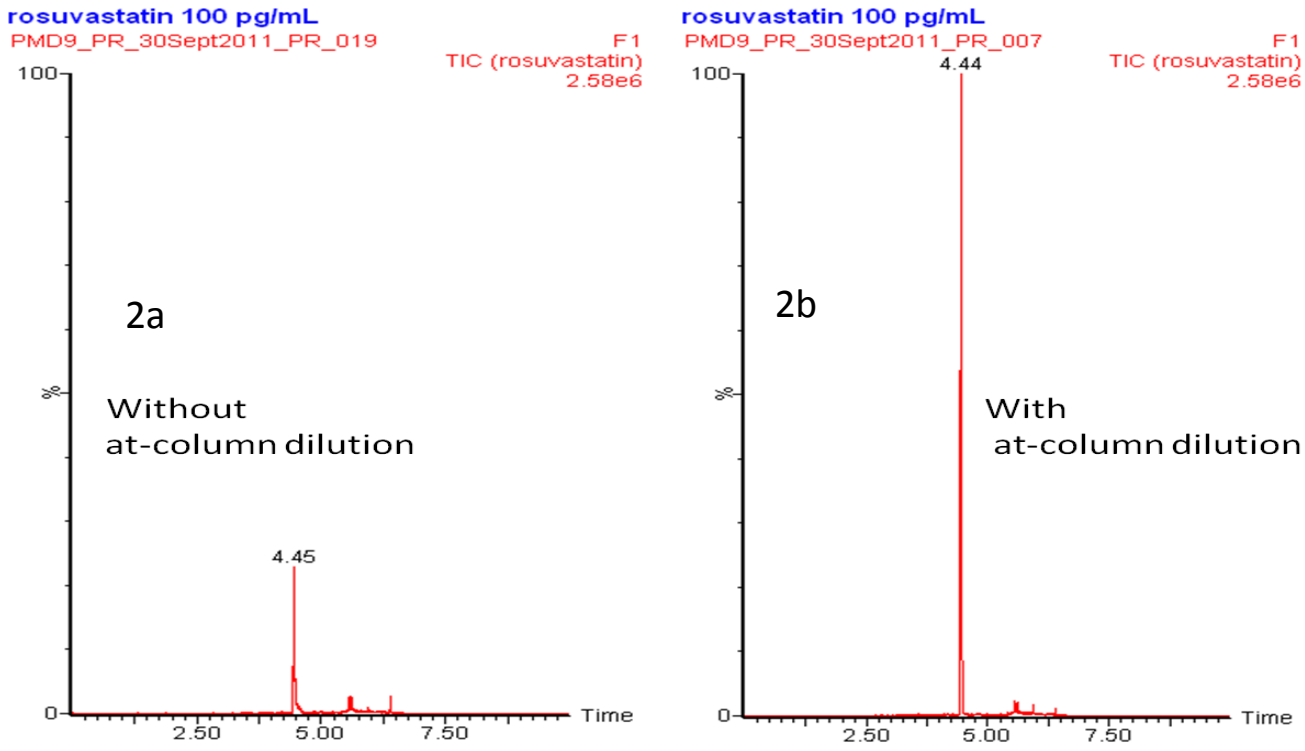

rosuvastatin 100 pg/mL
PMD9_PR_30Sept2011_PR_019
F1
TIC (rosuvastatin)
2.58e6
100
%
0
4.45
2.50
5.00
7.50
Time
2a
Without
at-column dilution
rosuvastatin 100 pg/mL
PMD9_PR_30Sept2011_PR_007
F1
TIC (rosuvastatin)
2.58e6
100
%
0
4.44
2.50
5.00
7.50
Time
2b
With
at-column dilution

Figure 5

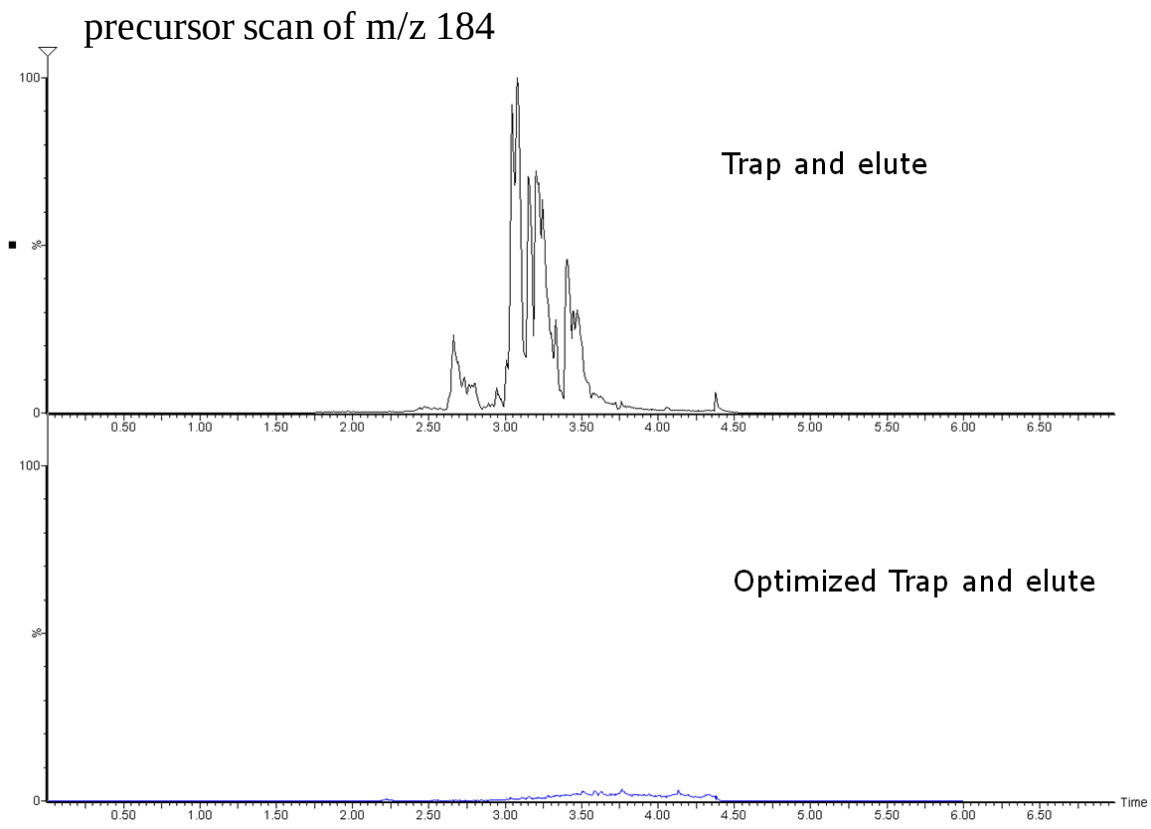

precursor scan of m/z 184
Trap and elute
Optimized Trap and elute
100
%
0
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50
Time

Figure 6

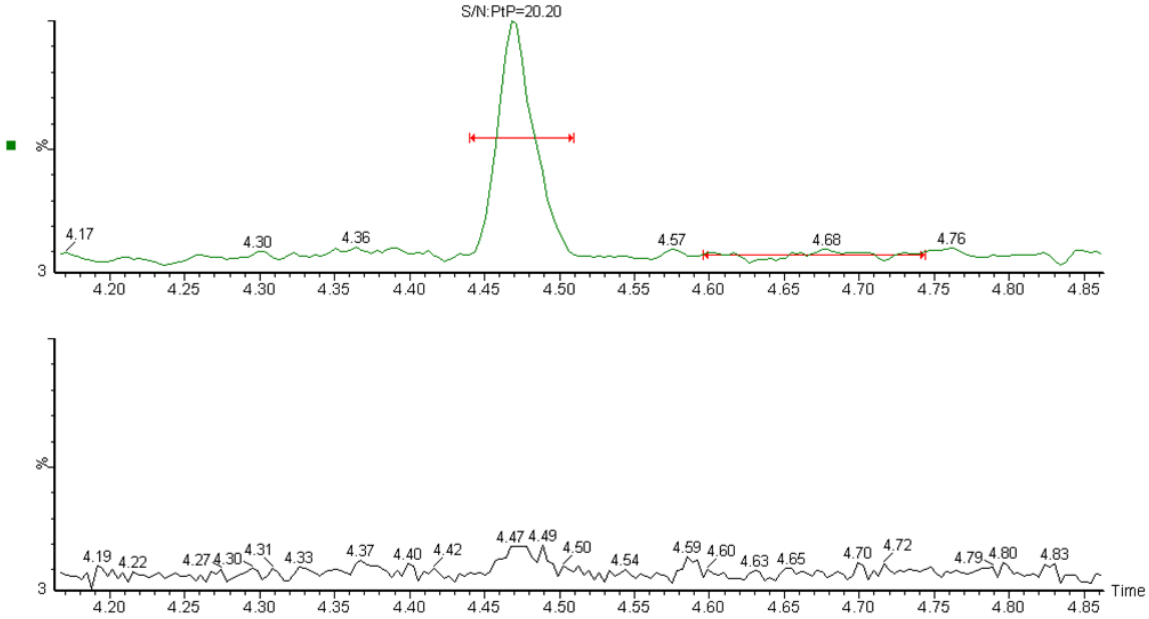

S/N:PtP=20.20
%
4.17
4.30
4.36
4.57
4.68
4.76
3
4.20 4.25 4.30 4.35 4.40 4.45 4.50 4.55 4.60 4.65 4.70 4.75 4.80 4.85
%
4.19 4.22 4.27 4.30 4.31 4.33 4.37 4.40 4.42 4.47 4.49 4.50 4.54 4.59 4.60 4.63 4.65 4.70 4.72 4.79 4.80 4.83
3
Time
4.20 4.25 4.30 4.35 4.40 4.45 4.50 4.55 4.60 4.65 4.70 4.75 4.80 4.85

Figure 7

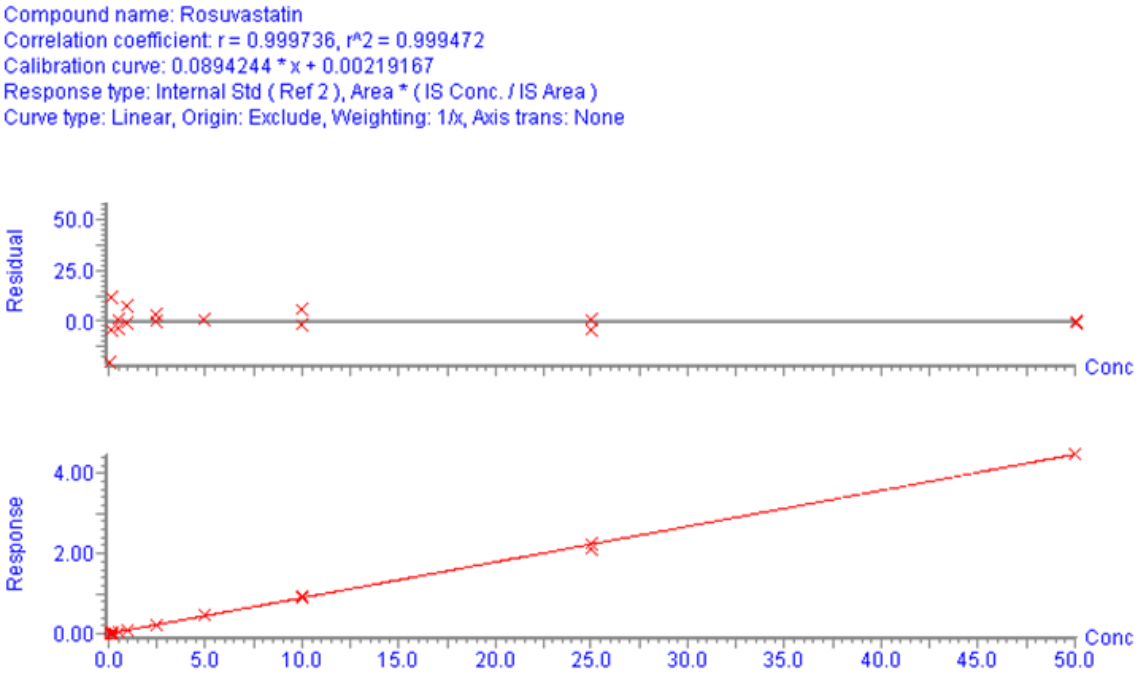
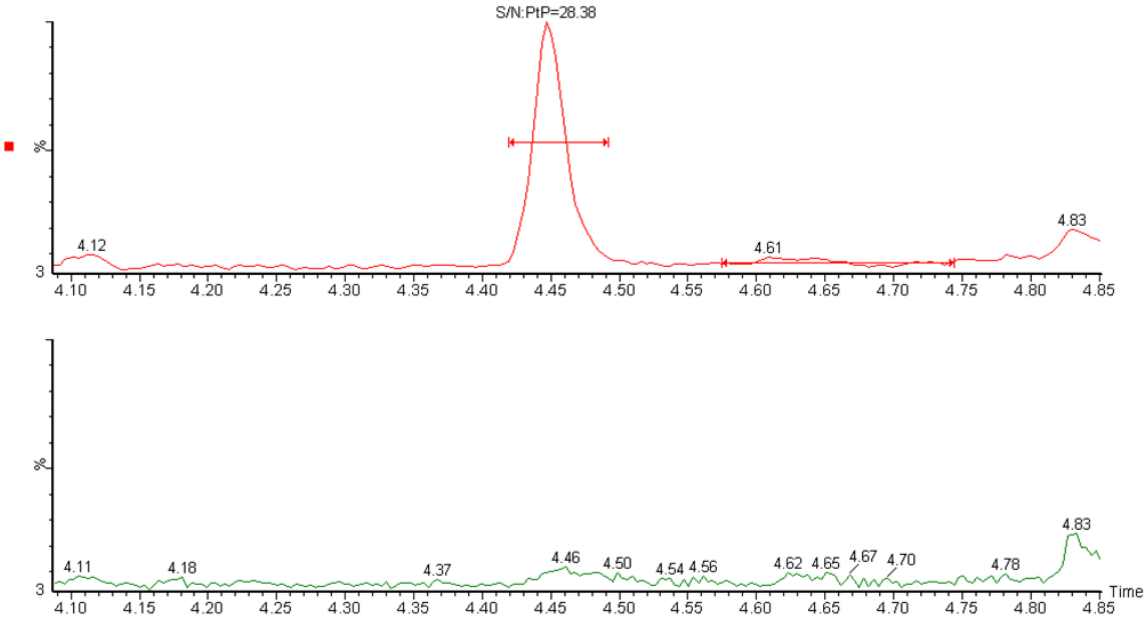

Compound name: Rosuvastatin
Correlation coefficient: r = 0.999736, r^2 = 0.999472
Calibration curve: 0.0894244 * x + 0.00219167
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None
Residual
50.0
25.0
0.0
Conc
Response
4.00
2.00
0.00
Conc
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0

Figure 8


S/N:PtP=28.38
%
4.12
4.61
4.83
3
4.10 4.15 4.20 4.25 4.30 4.35 4.40 4.45 4.50 4.55 4.60 4.65 4.70 4.75 4.80 4.85
%
4.11
4.18
4.37
4.46
4.50
4.54 4.56
4.62 4.65
4.67
4.70
4.78
4.83
3
Time
4.10 4.15 4.20 4.25 4.30 4.35 4.40 4.45 4.50 4.55 4.60 4.65 4.70 4.75 4.80 4.85