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Exploring Extra Sensitivity Using ionKey/MS with the Xevo G2-XS QTof HRMS for Small Molecule Pharmaceutical Analysis in Human Plasma

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APPLICATION BENEFITS

- High sensitive quantitation using high resolution mass spectrometry (HRMS)
- Fully integrated HRMS and microfluidics solution for bioanalysis
- Flexible HRMS platform with full scan, quan-qual (MS^E) and purely quantitative (Tof-MRM) modes of operation
- Trap and elute to enable high sample load with the ionKey/MS™ System
- Trap and elute to increase retention and peak shape of polar compound

WATERS SOLUTIONS

ACQUITY UPLC® M-Class System

ionKey/MS System

Xevo G2-XS QTof

iKey HSS T3 Separation Device

ACQUITY UPLC M-Class HSS T3 Trap Column

MassLynx® Software with TargetLynx™ Application Manager

KEY WORDS

Propranolol, verapamil, clopidogrel, buspirone, UPLC, HRMS, XS, QTof, Tof-MRM, LOQ, sensitivity, linearity, linear dynamic range, iKey Separation Device, ionKey/MS System

INTRODUCTION

The need for unambiguous data to support milestone transition and compound selection for drug discovery and development has fueled an unrelenting desire for instruments with higher sensitivity. Consequently sensitivity enhancement has been a critical attribute in each evolution of modern LC-MS instrumentation.

To address this need, Waters has focused on integrating and optimizing both the inlet and the MS detector. The ionKey/MS System integrates the separation into the source of the Xevo® G2-XS QTof Mass Spectrometer, yielding a single integrated platform. The iKey™ Separation Device is a microfluidic separation device that combines a traditional column, column oven, and electrospray emitter into one, and is integrated into the source of the mass spectrometer. Through enhanced ionization efficiency and reduced matrix interference, the ionKey/MS System produces signal enhancements for small molecule analysis, compared to analytical scale LC. The Xevo G2-XS QTof delivers enhanced mass resolution and sensitivity compared to its predecessor. This enhanced sensitivity is realized through a collision cell design that improves ion focusing and reduces losses in ion transfer through the cell. In addition to new hardware, innovative software, and acquisition methods were also introduced to enable simple Tof-MRM modes of operation targeted for routine bioanalytical work.

Here, a variety of drugs in human plasma were analyzed using several configurations of the ionKey/MS System with the Xevo G2-XS QTof. In the first series of experiments, the linearity and limits of quantitation (LOQ) were determined using direct injection and under Tof-MRM mode of operation. In the second set of experiments, a trap valve manager was configured to load samples onto the trap column for initial injection and wash, before being switched into the LC stream for compound elution, thus significantly increasing the injectable sample volume. Trapping using either single pump or dual pump configuration is described. The advantages of using trap-and-elute for optimum peak shape and higher sample volume is discussed.

EXPERIMENTAL

LC conditions

LC system:	ACQUITY UPLC M-Class System
Separation device:	iKey HSS T3 Separation Device, 100Å, 1.8 µm, 150 µm x 50 mm (p/n 186007260)
Trapping column:	ACQUITY UPLC M-Class HSS T3 Trap Column, 100Å, 5 µm, 300 µm x 50 mm (p/n 186008029)
Separation device temp.:	45 °C
Sample temp.:	10 °C
Injection vol.:	various
Flow rate:	3 µL/min
Mobile phase A:	water with 0.1 % formic acid
Mobile phase B:	90% acetonitrile/10% methanol with 0.1% formic acid
Gradient:	2–60 % B in 3.5 min, 60-95 % in 0.5 min, held at 95% B for 3 min before returning to the initial condition. The total run time was 10 min.

MS conditions

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI+, sensitivity mode (>30,000 FWHM)
Acquisition range:	50-1,200 *m/z*
Capillary voltage:	3.5 kV
Cone voltage:	30 V
Source temp.:	110 °C
Cone gas flow:	50 L/hr
Scan time:	0.2 s, continuum

Experiment

MS settings:	see results section
Tof-MRM settings:	product ions, see result section for details

Data management

MassLynx Software with TargetLynx Application Manager

Sample description

Human plasma was prepared by protein precipitation with the addition of acetonitrile (ACN) using a volume ratio of 3:1. The solution was centrifuged at 13,000 relative centrifugal force (RCF) and the supernatant was transferred to a new vial and further diluted 1:4 with water. The final %ACN in sample was approximately 19%. Test compounds (propranolol, verapamil, buspirone, and clopidogrel) were combined in a 100 ng/mL solution in diluted human plasma as previously described. The mixture was then serially diluted using diluted human plasma from a range of 100 ng/mL to <1 pg/mL.

Method conditions

The analytical LC-MS experiments were performed using the ionKey/MS System with the ACQUITY UPLC M-Class System and the Xevo G2-XS QTof Mass Spectrometer. MassLynx Software was used for data acquisition and TargetLynx Application Manager was used for data processing.

RESULTS AND DISCUSSION

Part I. Direct injection of sample using the ionKey/MS System

The instrument configuration for direct injection is shown in Figure 1, where the sample solution is introduced to the iKey Separation Device directly from the autosampler. Serially diluted samples in human plasma are analyzed using Tof-MRM acquisition mode. In this mode, the precursor ion is selected in the quadrupole (MS-MS) and passes through the collision cell, where collision energy is applied to produce a fragment ion. The Tof pusher at the detector entrance is then synchronized with *m/z* of the fragment ion for target enhancement. Figure 2 is a screen capture of the MS method editor, showing the MS function table and MRM target enhancement settings. Signal enhancement and advantages using Tof-MRM in relationship to MS and MS^E scans can be found in a recent application note.[1] For pure quantitation purposes, the Tof-MRM mode of data acquisition is generally recommended.

Figure 3 is a representative chromatogram of the sample with 98 fg on column using the Tof-MRM mode of data acquisition. The data was processed following bioanalysis validation criteria where the linear standard curve was fitted using 1/X2 weighing and 15% deviation used as the data exclusion criterion. Figure 4 is a sample screen capture of TargetLynx results for clopidogrel, where linearity, linear dynamic range, and LOQ were determined. Table 1 is a data summary of these three compounds.

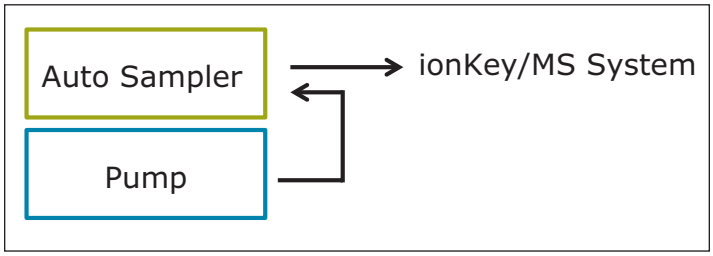

Figure 1. Instrument configuration for direct sample injection onto the ionKey/MS System.

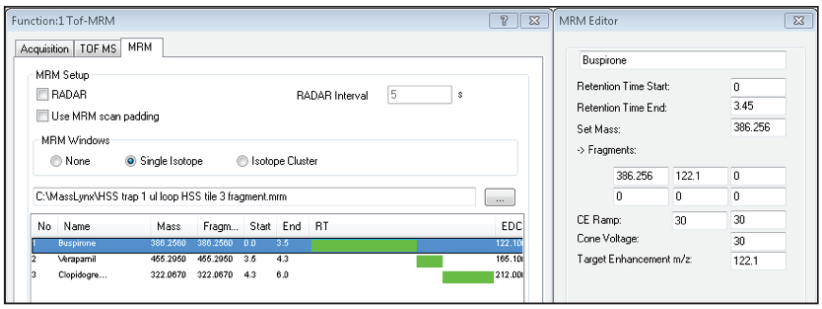

Figure 2. Screen captures of MS method editor in MassLynx Software. Function 1 table on the left shows three compounds monitored under Tof-MRM mode of data acquisition. Conditions for collision energy and target enhancement for each compound are defined in the MRM editor window. The buspirone example on the right shows collision energy of 30 kV and product ion m/z = 122 for the target enhancement.

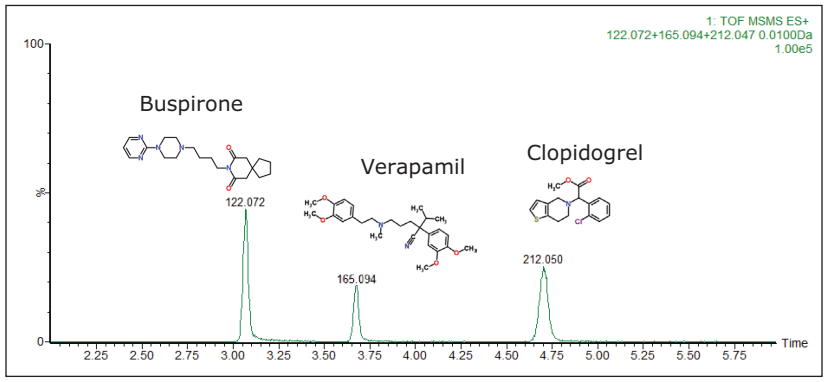

Figure 3. Structures of probe pharmaceuticals and extracted ion chromatograms (XIC) in human plasma using Tof-MRM mode of data acquisition.

	#	Name	Type	Std. Conc	RT	Area	IS Area	Response	Primar...	pg/mL	%Dev	S/N
3	3	Dec09_2014_SD037_1u_MRM_Frag_FL_P3	Standard	0.763	4.71	15.476		15.476	MM	0.8	-0.6	9
4	4	Dec09_2014_SD037_1u_MRM_Frag_FL_P4	Standard	1.526	4.70	26.792		26.792	bb	1.6	6.1	21
5	5	Dec09_2014_SD037_1u_MRM_Frag_FL_P5	Standard	3.052	4.70	49.339		49.339	bb	3.3	9.3	19
6	6	Dec09_2014_SD037_1u_MRM_Frag_FL_P6	Standard	6.104	4.70	93.076		93.076	bb	6.7	9.2	57
7	7	Dec09_2014_SD037_1u_MRM_Frag_FL_P7	Standard	12.207	4.71	171.784		171.784	bb	12.7	3.7	86
8	8	Dec09_2014_SD037_1u_MRM_Frag_FL_P8	Standard	24.414	4.70	294.832		294.832	bb	22.0	-9.8	163
9	9	Dec09_2014_SD037_1u_MRM_Frag_FL_P9	Standard	48.828	4.70	665.921		665.921	bb	50.3	2.9	298
10	10	Dec09_2014_SD037_1u_MRM_Frag_FL_P10	Standard	97.656	4.70	1217.343		1217.343	bb	92.2	-5.5	693
11	11	Dec09_2014_SD037_1u_MRM_Frag_FL_P11	Standard	195.313	4.70	2608.253		2608.253	bb	198.1	1.4	1699
12	12	Dec09_2014_SD037_1u_MRM_Frag_FL_P12	Standard	390.625	4.70	4404.746		4404.746	bb	334.9	-14.3	1956
13	13	Dec09_2014_SD037_1u_MRM_Frag_FL_P13	Standard	781.250	4.70	10025.908		10025.908	bb	762.7	-2.4	3839
14	14	Dec09_2014_SD037_1u_MRM_Frag_FL_P15	Standard	3125.000	4.70	40057.633		40057.633	bb	3048.6	-2.4	3427
15	15	Dec09_2014_SD037_1u_MRM_Frag_FL_P16	Standard	6250.000	4.70	82000.500		82000.500	bb	6241.2	-0.1	8642
16	16	Dec09_2014_SD037_1u_MRM_Frag_FL_P17	Standard	12500.000	4.70	170698.719		170698.719	bb	12992.6	3.9	14604

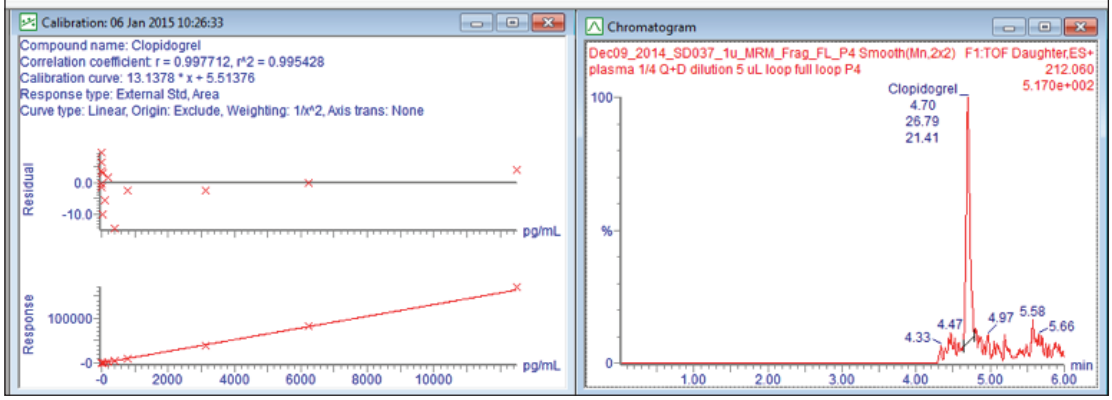

Figure 4. TargetLynx results for clopidogrel. Top is a summary table for the data set. Lower left is the calibration curve using linear fitting and 1/X2 fitting. Lower right is a sample chromatogram at 1.5 pg/mL or 1.5 fg amount on-column.

Compound	CE ramp	Transition	Linear range [Log] (pg/mL)	R^2	LOQ (pg/mL)	LOQ (fg) amount-on-column	S/N @LOQ
Buspirone	30	386 > 122	1.5–12,500 [3.9]	0.991	1.5	1.5	78
Clopidogrel	16	322 > 212	0.8–12,500 [4.2]	0.995	0.8	0.8	9
Verapamil	36	455 > 165	3.1–12,500 [3.6]	0.996	3.1	3.1	38

Table 1. Summary of MS method setting, linear range, linear dynamic range, and quantification limit for the three compounds analyzed. The data was acquired using a 1 µL sample loop.

Results indicate excellent linearity for all three compounds. The linear dynamic range ranges from Log = 3.6 for verapamil to Log = 4.2 for clopidogrel. The low quantitation limits for three compounds range from 0.8 to 3 pg/mL, which translates to 0.8 to 3 fg on column. The signal to noise ratio at the low quantitation limits ranges from 9 to 79. These attributes indicate that the ionKey/MS System with the Xevo G2-XS QTof is well suited to meet the needs for routine bioanalysis. The added advantages of the ionKey/MS System, such as user friendliness and greater than 90% reduction in solvent usage compared to analytical LC, make the ionKey/MS System with the Xevo GS-XS QTof an excellent choice for today's most demanding analytical needs.

Part II. Single pump trap-and-elute using the ionKey/MS System

It is well known in liquid chromatography that injection volume and sample solvent strength can affect peak width or resolution. The effect is more pronounced for early eluting peaks, as the sample solvent is typically stronger than the initial mobile phase strength in gradient elution. Thus, too large of an injection volume or a high organic concentration in the sample can cause peak distortion or broadening.[2] At the microfluidics scale of the ionKey/MS System, with smaller column I.D. and system volume, this effect is expected to be more pronounced.

The potential peak distortion is explained in Figure 5 in which 5 µL of the drug mixture in human plasma, containing ~19% ACN, was injected onto an iKey Separation Device. While the late eluting clopidogrel maintains excellent peak shape, the early eluting peaks for buspirone and propranolol are showing peak fronting. One approach to resolve this would be to dilute the sample further with weak solvent, however, this will also further dilute analyte concentration in the sample. The other approach is to inject the sample onto a trapping column before the iKey Separation Device. The trapping column has a larger inner diameter and runs at a higher flow rate making more mobile phase available for diluting out the strong sample solvent, resulting in effective focusing of the analytes onto the trapping column.

It should be noted that comparing to analytical scale column (2.1 mm I.D.), the volumes being injected on the iKey Separation Device (150 µm I.D.) are far higher relative to the column volume. To put this into perspective, injecting 5 µL onto an iKey Separation Device is approximately equivalent to injecting 1 mL onto a 2.1 mm column, which is typically beyond normal operating boundaries. At 5 µL injections, the iKey Separation Device can be used for many compounds, especially if the sample diluent is low organic, but trapping offers a mechanism to allow larger injection volumes in routine analysis.

The ionKey/MS System configured for single pump trapping is shown in Figure 6, using one pump and a trapping valve manager between the autosampler and the iKey Separation Device. The valve is set at trap position during sample injection, where weak solvent is pumped through the trapping column for dilution of the strong sample solvent, and concentrates the analyte at the head of the trapping column. After a set time, as defined by the user, the valve is switched to elute position, when the pump starts gradient elution to back flush the analyte onto the iKey Separation Device for separation and MS detection. Figure 7 shows the same 5 µL sample injection with trapping; the peak shape of both early eluting buspirone and propranolol, and late eluting clopidogrel are excellent.

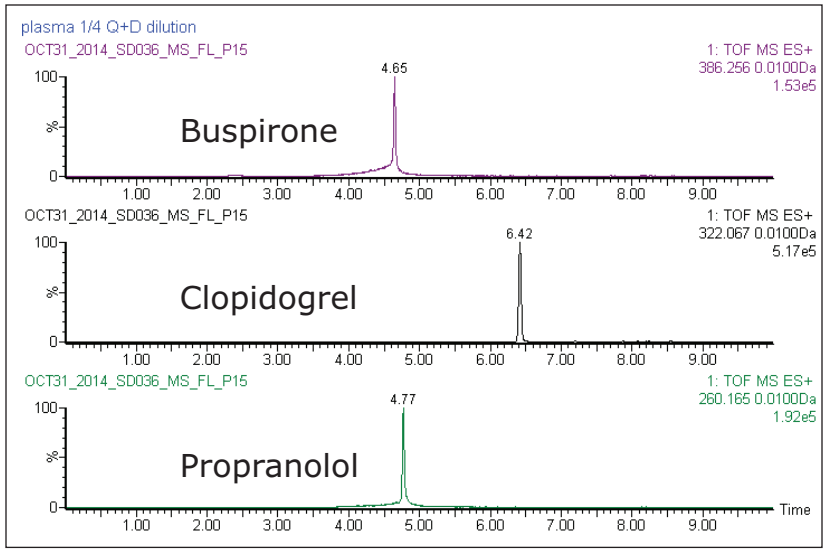

Figure 5. XIC of 5 µL injection of sample in human plasma containing ~19% ACN. Both early eluting buspirone and propranolol show peak fronting, while clopidogrel shows excellent peak shape (sample loop was 5 µL; sample concentration was 3 ng/mL.)

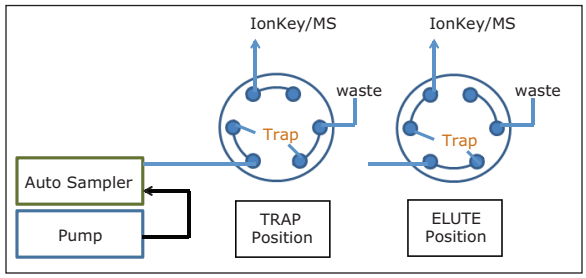

Figure 6. Instrument configuration using single pump trapping.

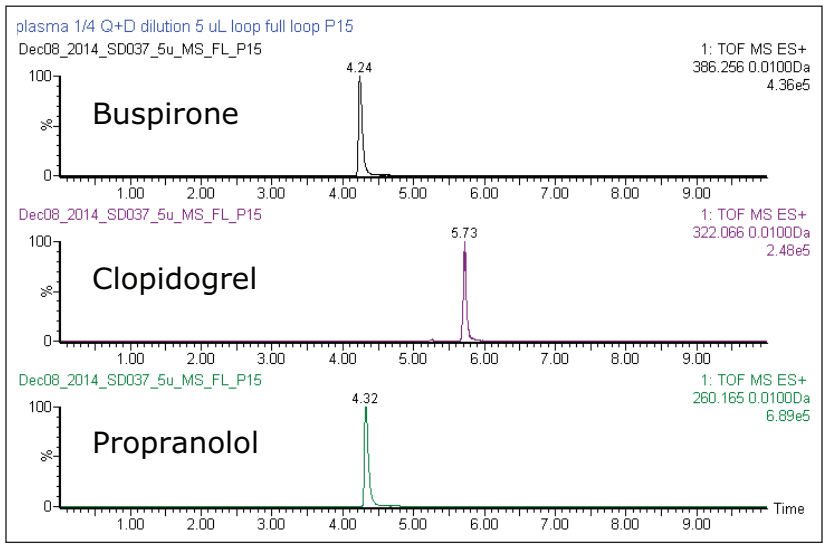

Figure 7. XIC of 5 µL injection of sample in human plasma containing 19% ACN using one pump trapping configuration. The sample concentration was 98 pg/mL or 98 femtogram on column. The trapping column used was an ACQUITY UPLC M-Class HSS T3, 5 µm, 300 µm x 50 mm Column (p/n 186008029). Trapping flow rate was isocratic at 15 µL/min 99.5% A/0.5% B. Trapping time was 2 minutes. Sample loop was 5 µL. MS scan mode of data acquisition was used.

Part III. Dual pump trap-and-elute for optimum iKey Seperation Device protection and enhanced sample loading

The trapping and subsequent sample elution can also be carried out using a two pump configuration as shown in Figure 8. The two pumps are identical ACQUITY UPLC M-Class Binary Solvent Managers. For the sake of differentiation, one pump is labeled "trap" and the other pump labeled "iKey". Under this configuration, both the iKey Separation Device and trapping column are under active flow at all times. Similar to the single pump trapping configuration, the valve is initially set at trap position during sample injection, where weak solvent is pumped through the trapping column for dilution of strong sample solvent, and concentration of analyte at the head of the trapping column. After a set time as defined by the "loading time" in the LC method, the valve is switched to elute position when the pump starts gradient elution to back flush the analyte onto the iKey Separation Device for separation and MS detection. After the analytes are eluted from the trap column, one has the option of switching the valve back to the trap position and washing the trap column with high organic mobile phase at high flow rate. This way, high lipophillic endogenous plasma components that are largely retained on the trapping column can be diverted to waste, rather than eluted onto the iKey Separation Device and MS. In this configuration, the trap column also acts as a pseudo guard column for the iKey Separation Device, and the wash cycle of the iKey Separation Device can be potentially shortened. More detailed description of inlet method parameters for the trap-and-elute can be found in the Appendix.

By installing a 20 µL sample loop into the autosampler of this dual pump trap-and-elute configuration, samples prepared in human plasma were injected with varying injection volume from 1 µL to 20 µL. Figure 9 shows an overlaid extracted mass chromatogram of samples at 1, 5, 10, and 20 µL injection. Data shows that peak resolution and peak shape were maintained for all volumes injected, and that the iKey Separation Device is capable of handling 20 µL injection of human plasma sample, with no adverse effect on peak shape or resolution.

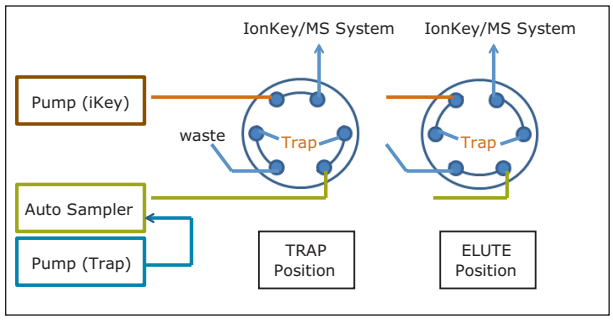

Figure 8. Instrument configuration dual pump trap-and-elute.

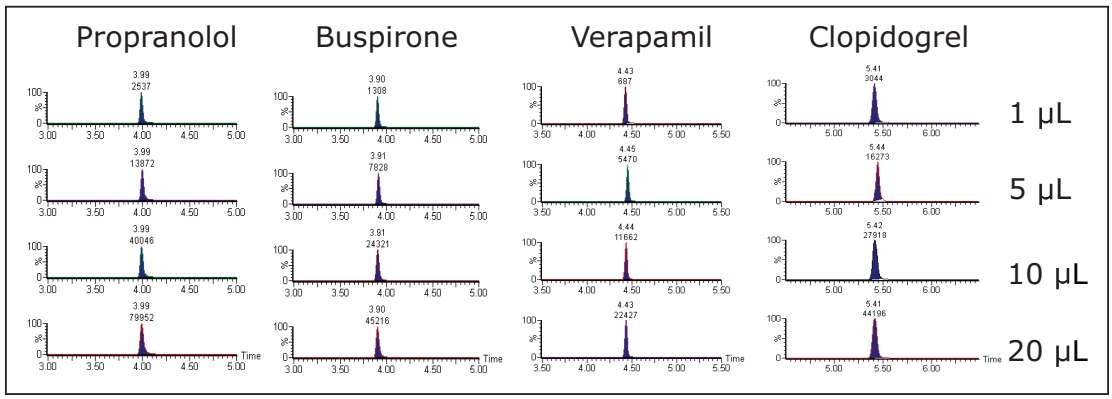

Figure 9. Overlaid XIC of sample with injection volume at 1, 5, 10, and 20 µL using a 20 µL sample loop. Peak retention and peak area are labeled in the graph. The solution concentration is 3 ng/mL for each compound in human plasma. MS full scan at scan rate of 0.2 s was used for the data acquisition. Trap and elute LC conditions are explained in the Appendix.

The peaks of propranolol and buspirone, that had fronting at 5 µL direct injection without trapping, showed no fronting at the high 20 µL injection. Figure 10 is the plot of injection volume versus peak area of propranolol, buspirone, and verapamil. A linear response with R^2 >0.995 were observed indicating complete sample recovery using the trapping column. For highly lipophilic, poorly soluble compounds, such as clopidogrel, a stronger mobile-phase solvent is needed to prevent sample precipitation from the sample loop. Figure 11 is the plot of injection volume versus peak area of clopidogrel at two trapping solvent compositions. At low 0.5% ACN in trapping mobile phase solvent, the plot shows a downward curve, indicating incomplete elution of sample post injection. Increasing %ACN in trapping mobile phase solvent to 5%, the response becomes linear indicating complete recovery.

It should be noted that even injecting 20 µL of human plasma sample to a 2.1 mm I.D. x 50 mm analytical column (an injection to column volume ratio of 0.11) is already challenging, and potentially results in poor peak shape or loss of resolution. At microfluidics scale of the iKey Separation Device, the 20 µL injected is 23 times that of an empty iKey Separation Device volume, a much higher injection to column volume compared to the analytical scale. This is equivalent to injecting 4 mL of sample into a 2.1 mm I.D. x 50 mm analytical column. Yet, excellent peak shape and resolution are maintained. This demonstrated how high volume injections using the ionKey/MS System would further broaden the usability and versatility of the system using traditional bioanalysis and DMPK lab sample volume workflows. To summarize, the trap-and-elute configurations allow analysts to extend the usable inject sample volume range in order to further enhance detection of weak signals.

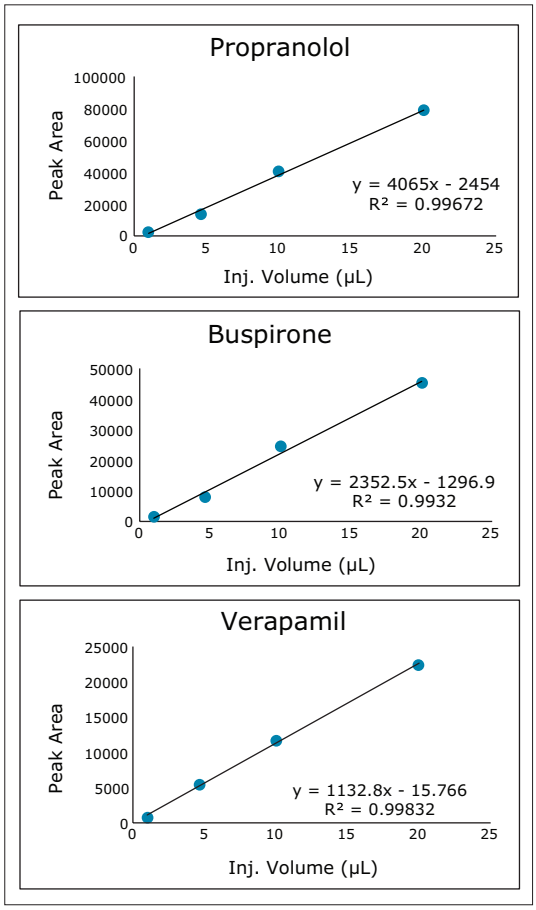

Figure 10. Plot of peak area versus injection volume. Trapping mobile phase consists of 0.5%ACN/99.5%H_2O.

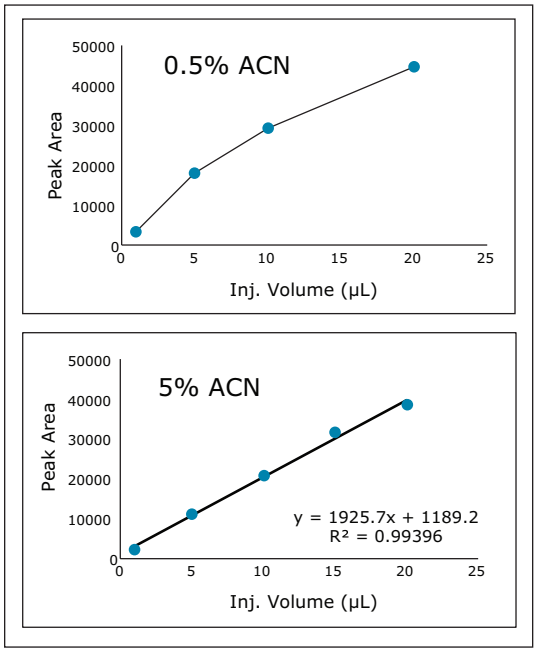

Figure 11. Plot of peak area versus injection volume of clopidogrel at two different trapping mobile phase composition: (left) 0.5%ACN/99.5%H_2O, and (right) 5%ACN/95%H_2O.

CONCLUSIONS

High sensitivity is demonstrated in the present study using the ionKey/MS System with the Xevo G2-XS HRMS. Using samples in human plasma, the system routinely reaches sub-femtogram on-column sensitivity using direct 1 µL injection and using Tof-MRM mode of data acquisition. The observed linear dynamic range and signal-to-noise ratio at quantitation limit fully support the HRMS system to be used in routine bioanalysis. Adding to this, the system can be extended using trap-and-elute configurations. The use of a trapping column can dilute strong sample solvent that might affect peak shape and peak resolution, especially those for early eluting compounds. The trap-and-elute configurations will also allow large volumes to be injected, which further enhances the sensitivity of the system. The option of using valve switching to divert late eluting endogenous plasma components to waste can help protect the iKey Separation Device and MS detector from contaminations by highly lipophilic components in the sample matrix. An injection volume as high as 20 µL is demonstrated to produce excellent peak shape and peak resolution for compounds with diverse polarity. All these observations coupled with user friendliness of the iKey Separation Device and tremendous solvent savings, suggest the ionKey/MS System with the Xevo-G2-XS HRMS is an ideal platform to meet modern challenges of both quantitative and qualitative analysis in pharmaceutical applications.

References

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APPENDIX

Programming inlet method conditions for the two pumps trap-and-elute

After the connection of two pumps and a trap valve manager in the UPLC, these modules are visible in the inlet panel as shown in Figure A-1. With inclusion of the trap valve manager in the system, a new tab called "trapping" is visible in each of the pump pages, where the LC mobile phase conditions are defined, when the trap valve is at trap stage. The "analytical" tab on the pump page is used to define LC mobile phase conditions, when the valve is switched to the analytical or elute position. The length of the valve spent at the trapping position is defined by "loading time", in this case, 2 minutes is used (the shaded box in Figure A-1). After trapping is completed, the system automatically switches to the analytical or elute position. The following screen captures are from the method used in the present two pump trap-and-elute method, more details are described in each figure caption.

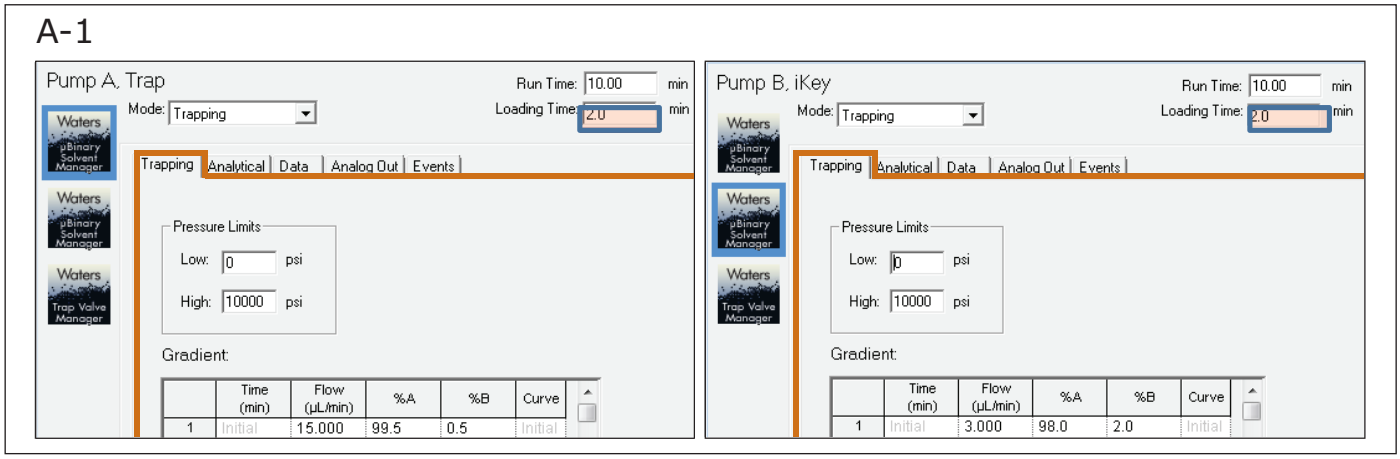

Figure A-1. LC pump mobile phase conditions when trap valve is set at trap position. The method shows that the trap pump is delivering 99.5% A/0.5% B isocratic to the trap column. The iKey pump is delivering 98% A/2%B isocratic, the initial gradient condition, to the iKey Separation Device. This is maintained for 2 minutes as shown in the "loading time" window.

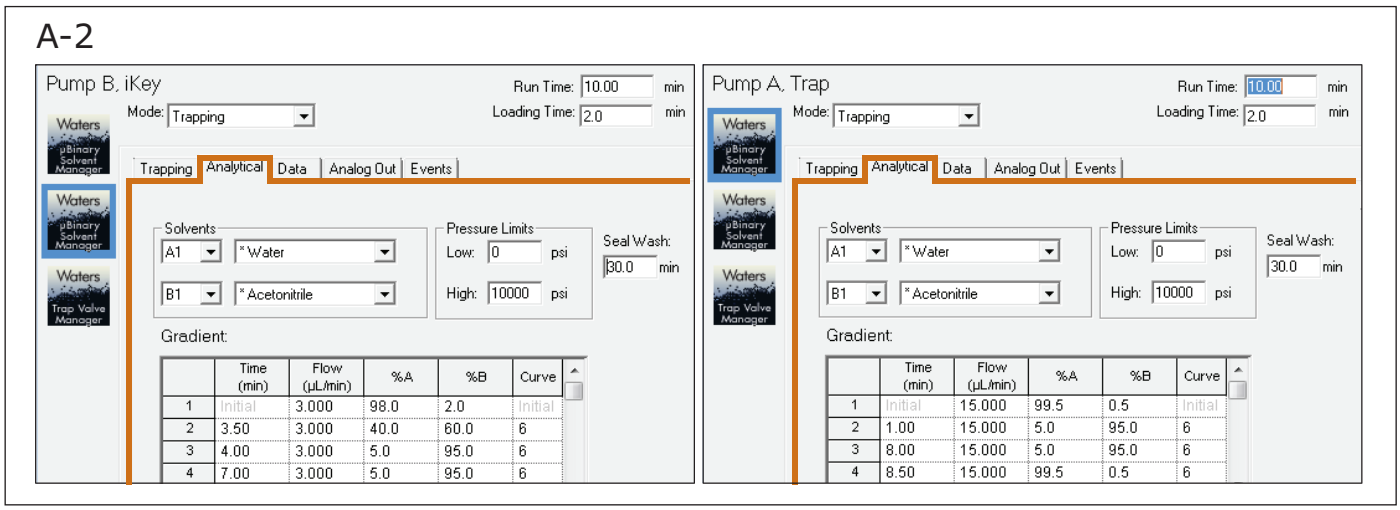

Figure A-2. LC pump gradient conditions for sample elution. After 2 minutes, the valve is switched to elute position. Mobile phase conditions for each of the pumps are defined by the analytical tab of the pump method panel. For the iKey pump, a full gradient cycle is applied to elude the sample from the trap column, to the iKey Separation Device, then into the MS. For the trap pump, a full gradient cycle is also written to wash the sample loop, the trap column (after it is switched back into loop) uses a strong organic solvent of 5% A/95% B, and then goes back to the initial condition.

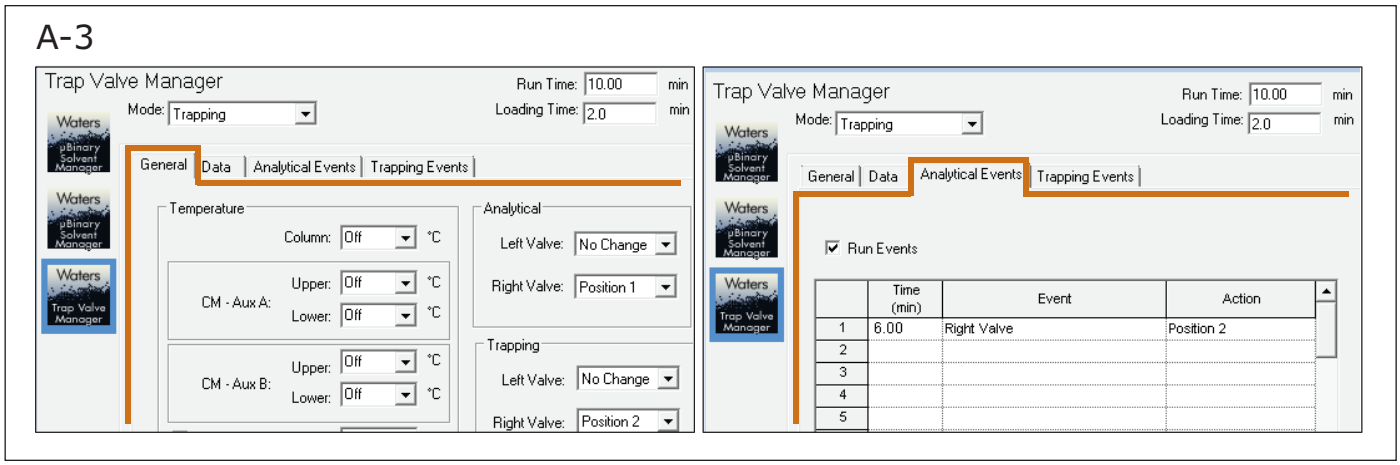

Figure A-3. Trap value parameter settings. The trap value method consists of two parts. Under the "general" tab, the exact valve position for trap-and-elute (analytical) is defined. In this case, position 1 is used for analytical and position 2 for trapping. A valve position diagram can be found on the inside panel of the trap valve manager. To wash late eluting components on the trap column into waste, the valve is switched back to trap position during the run by using the "analytical event" tap. In this case, the valve is switched after 6 minutes to the trap position, where the late eluting plasma components on the trap column are washed using the trap pump at 15 µL/min with 95% B/5%A.

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