Comprehensive Forensic Toxicology Screening in Serum using On-Line SPE LC-MS/MS

SCIEX QTRAP® 4500 LC-MS/MS System and Spark Holland PICO

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Introduction

Comprehensive screening for the detection of drugs and toxic compounds in biological samples is an important function of forensic toxicological analysis. As the demand to monitor the ever increasing number of drugs continues to rise, so too does the need to detect and quantify these compounds in a simple, single and automated run, providing a fast turn-around time for the results.

This application note describes the rapid cleanup of serum samples and detection of over 100 drugs using On-line Solid Phase Extraction (SPE) LC-MS/MS screening and confirmation. The LC-MS/MS was operated in Multiple Reaction Mode (MRM) for detection. Dependent MS/MS spectra were acquired in the Enhanced Product Ion (EPI) mode after being triggered from a *Scheduled* MRM™ Pro Algorithm Information Dependent Acquisition (IDA) survey scan. Combining MRM and product ion spectral acquisition allows for compound identification with highest confidence based on mass spectral library matching. The automated workflow monitors large panels of analytes, detecting and quantifying these compounds in a single run.

The *Scheduled* MRM™ Pro algorithm allows for excellent data quality covering the broadest range of drugs possible by only spending time collecting useful data and eliminating triggering on secondary MRMs and product ion spectra if the compound of interest is not present.

Figure 1. Performing On-Line SPE LC-MS/MS using the Spark Holland Pico and SCIEX QTRAP® 4500 LC-MS/MS System for automated drug screenings.

Materials and Methods

Materials

130 neat standard solutions of different drug classes were purchased from Cerilliant. An analyte stock solution was prepared by combining all the drugs with methanol, at appropriate concentrations to evaluate the automated serum cleanup method for all the compounds. A detailed list of the drugs used for this study is available upon request. A selected panel of deuterated analogues were purchased from Cerilliant and used for quantification.

Blank serum was spiked with drug mixture stock solutions making concentrations ranging from 0.5 to 2000 ng/mL to prepare the calibrators; 10, 1000 ng/mL for QCs.

Sample pretreatment

Sample Clean-up and LC separation was performed using a Spark Holland Pico System. Figure 2 shows the sample preparation procedure. On-line sample clean-up was achieved using HySphere Resin GP (general purpose) 10; polydivinyl-benzene cartridges.

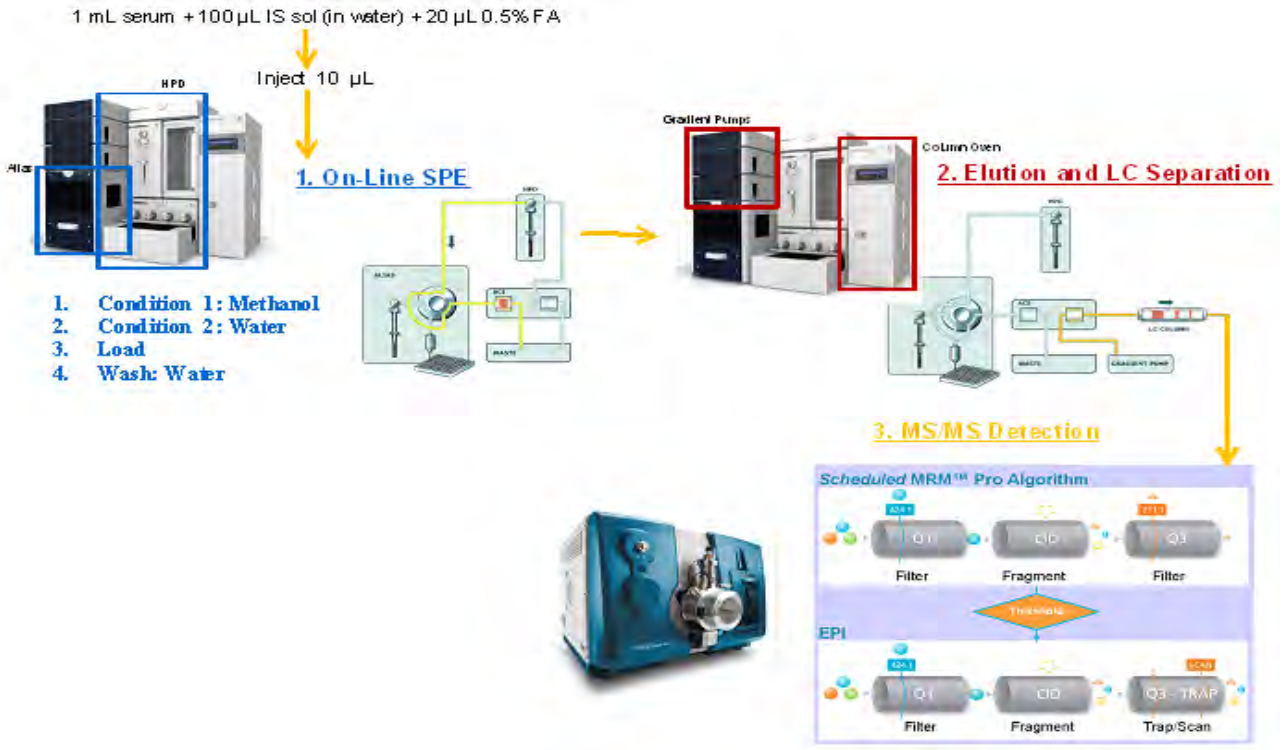


Figure 2. Graphical representation of the automated On-Line SPE LC-MS/MS workflow with Spark Holland PICO and SCIEX QTRAP® 4500 LC-MS/MS System

HPLC Conditions

- Phenomenex Kinetex column (C18, 3 x 50 mm, 2.6 µm, 100 Å); 30°C
- Mobile phase A: Water + 10 mM ammonium formate
- Mobile Phase B: Acetonitrile + methanol (1:1)
- Flow rate: 400 µL/min flow rate.

Time (min)	Flow (mL/min)	%B
0.00	0.4	2
1.00	0.4	2
10.00	0.4	100
13.00	0.4	100
13.10	0.4	2
15.50	0.4	2

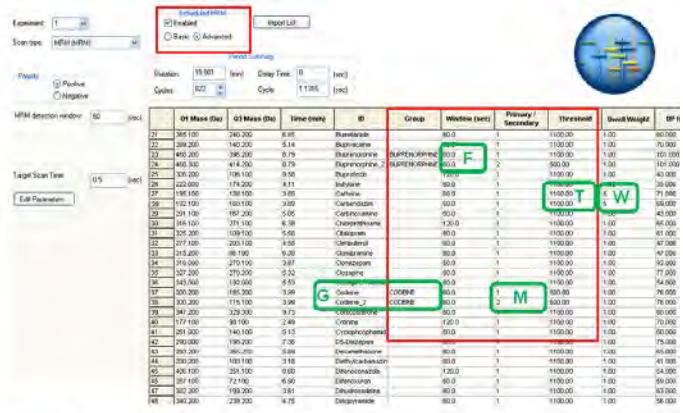
MS/MS Conditions

A SCIEX QTRAP® 4500 LC-MS/MS System operating in *Scheduled* MRM™ Pro Algorithm mode was used for detection, in positive TurboIonSpray® probe mode. EPI spectra at a scan speed of 10000 Da/s were triggered from the MRM survey scan and acquired using a dynamic fill time and dynamic background subtraction for optimal MS/MS quality. EPI spectra were generated using standardized Collision Energy (CE) of 35 V with Collision Energy Spread (CES) of 15 V to ensure a characteristic MS/MS pattern independently on the compound's fragmentation efficiency. MS/MS spectra were searched against the iMethod™ Application Meta Library.

Analyst® Software Version 1.6.2 and *Scheduled* MRM™ Pro Algorithm

SCIEX introduced the *Scheduled* MRM™ Algorithm in 2008 in Analyst® Software version 1.5. The algorithm adjusts detection windows automatically depending on the retention time of the compound. This means no compromise has to be made between dwell times for each compound or the cycle time. It allows for addition of detecting many more MRM transitions from a single method and gives better signal to noise, accuracy and reproducibility.

The new *Scheduled* MRM™ Pro algorithm retains all the benefits of *Scheduled* MRM™ algorithm but also has extended functionality with Grouped MRM-triggered MS/MS, MRM triggered MRM, Dwell Time Weighting, Flexible Window Width and Dynamic Window Extension. All these features allow the mass spectrometer to only spend time collecting useful data.



Flexible Window Width (F), Dynamic Window Extension (T), MRM-triggered MRM (M, T), Group MRM-triggered MS/MS (G, T), Dwell Time Weighting (W)

Figure 3. Analyst® Software version 1.6.2 with *Scheduled* MRM™ Pro Algorithm

Group MRM-triggered MS/MS

Full scan product ion MS/MS in combination with library searching is the best evidence for confirmation of a compound. Triggering a full scan MS/MS for any single transition in a complex matrix can lead to many false IDA triggers and acquiring this data wastes precious cycle time. Using a group of MRM transitions as a trigger focuses the acquisition of MS/MS to the best time to confirm the compound. A threshold is set over which all primary transitions must rise before MS/MS is triggered Product ion spectra are therefore triggered less often but more often on the compound of interest.

MRM-Triggered MRM

One or more primary transitions are monitored during the whole acquisition window and only trigger secondary MRMs when the intensity of all primaries exceeds the defined threshold. Secondary MRMs are monitored until primaries drop back below the threshold, thus are monitored across the LC peak. Therefore accurate and quantifiable areas are obtained for all transitions.

Dwell Time Weighting

Higher dwell times improve the signal to noise of MRM transitions. Spending the same dwell time on an internal standard and a difficult to ionize compound is wasteful. However, calculating optimal dwell times for each compound without impacting cycle time during a scheduled run would be difficult. Dwell time weighting allows a desired dwell time to be expressed relatively. Internal standards can be assigned a low weight (<1) while difficult compounds can be assigned a high weight (>1). During the run, the available dwell time will be assigned based on this weighting.

Flexible Window Width

This allows setting acquisition time windows individually for each compound, based on the retention time reproducibility or peak width.

Dynamic Window Extension

MRM detection windows are automatically extended for transitions where the peak has not completely eluted (when the signal is still above the user defined threshold). This functionality allows increasing the robustness to shifts in retention time and ensures the collection of the entire signal of heavily contaminated samples.

Results and Discussion

Despite the high selectivity that MRM provides, there is always a risk of false positive findings due to endogenous compounds that have the same mass. Typically a second MRM is monitored per analyte and the ratio of quantifier to qualifier transition is calculated for each unknown sample and compared to the MRM ratio of standards for identification. However, targeted compounds with low fragmentation efficiencies (i.e. Low Intensity Ions) have been reported to produce false positive results for compound identification (4-6).

For improved accuracy, compound identification was performed using full scan MS/MS experiments in Enhanced Product Ion (EPI) mode with automated library searching capabilities to compare the unknown with a standard spectrum. The dependent

MS/MS spectra were acquired using the EPI mode of the QTRAP® system after being triggered from a *Scheduled* MRM™ Pro Algorithm IDA survey scan and searched against mass spectral libraries for compound identification. The information of the complete molecular fingerprint saved into EPI spectra significantly reduces the risk of false positive and negative results (7-10). In this method, a large library of MS/MS spectra (SCIEX iMethod™ Meta MS/MS Spectral Library) containing more than 2400 compounds including pesticides, mycotoxins, veterinary drugs, pharmaceuticals and drugs of abuse compounds was used to search against in order to confirm compound identifications. Quantitative analysis was performed in the same run allowing for both quantification and qualitative data to be collected simultaneously.

Figure 4 shows representative *Scheduled* MRM™ chromatograms for over 100 different drugs. Figure 5 shows the signal-to-noise levels for selected compounds obtained from 1 ng/mL spiked serum. Figure 6 shows example calibration curves (0.5-1000 ng/mL) created for compounds identified from the library matching from the same run.

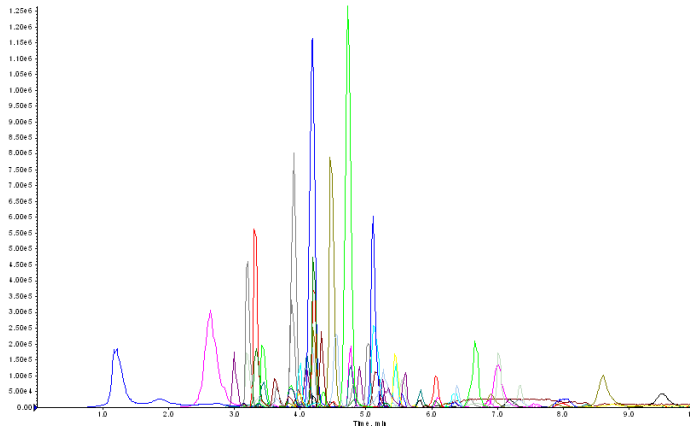


Figure 4. Extracted ion chromatograms for 130 drugs from a 10 ng/mL extracted serum calibrator

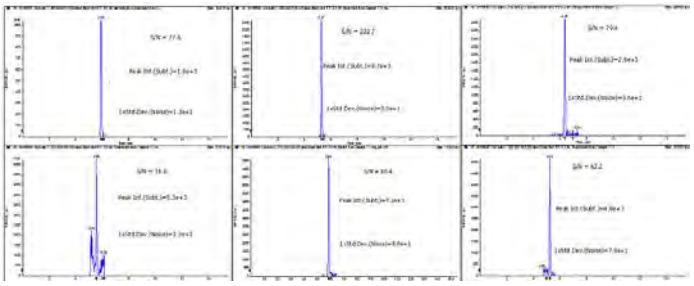


Figure 5. Chromatogram of select analytes in spiked serum at 1 ng/mL. The signal to noise was calculated by dividing the average background signal intensity from the peak by a 1 times the standard deviation of the noise region

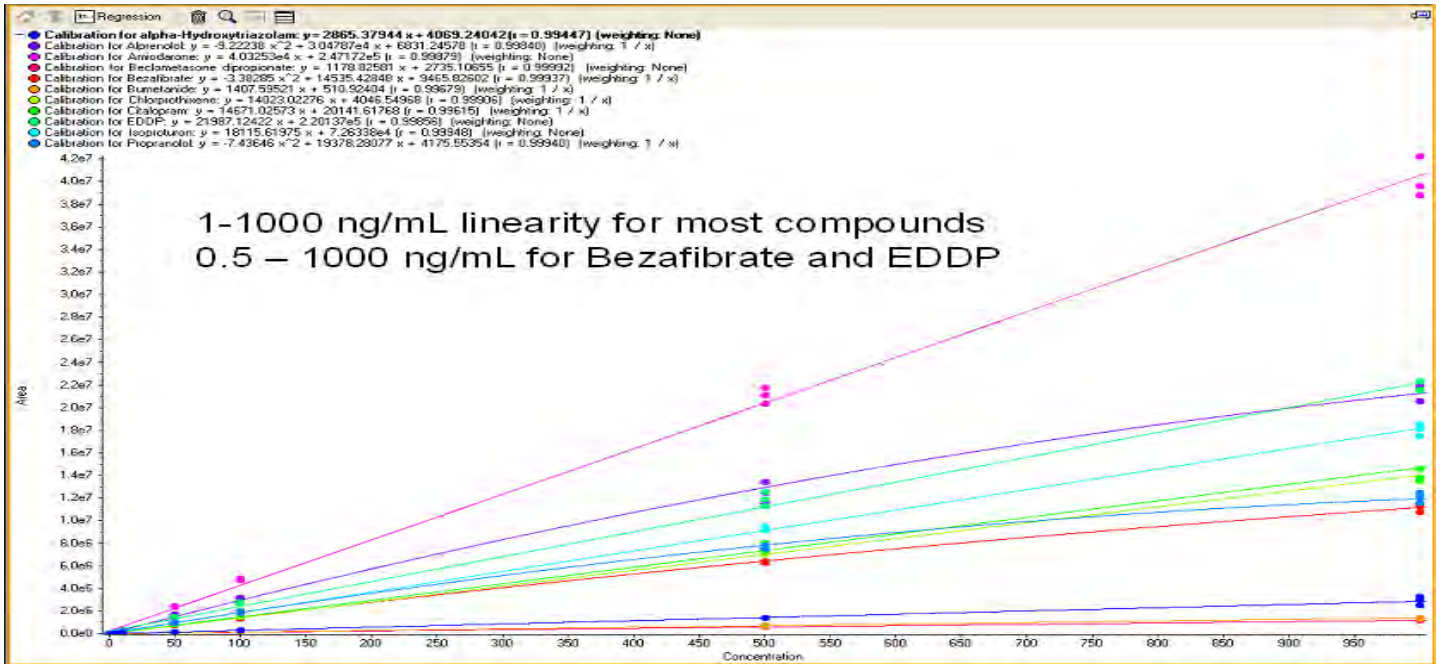


Figure 6. Selected example calibration curves for spiked serum calibrators, generated from the same run in which qualitative information was obtained for confident identification through library searching.

Regression analysis for the analyzed samples within this method resulted in R^2 values of 0.99 or greater. Typical recoveries were greater than 80% (Table 1). Matrix effects were evaluated at 10 ng/mL concentrations, using one lot of serum, and % accuracy differences were typically less than 20%. Figures 7 and 8 show specific examples with % CV and % Accuracy values across the calibration curve concentrations.

	% CV 1ng/mL LOQ (n=3)	% Accuracy	Matrix Effect (% accuracy difference)	Recovery (%)
Acetominophen	0.9	102	*15*	*98*
Beclometasone	8.5	*109*	*6*	*61*
Bezafibrate	*20*	*80*	*23*	*55*
Clonazepam	*20*	*112*	*4*	*112*
Nalorphine	*9*	*87*	*1*	*81*
Norfentanyl	*5*	*99*	*3*	*14*
Oxycodone	*3*	*97*	*11*	*86*
Propanolol	*6*	*90*	*49*	*61*
Sotalol	*2*	*103*	*15*	*97*
Terfenadine	*5*	*87*	*16*	*76*

Table 1. Typical recoveries and matrix contribution values as represented by these selected examples

Extracted spectra and library search Purity Score values using an MS/MS library search algorithm are shown in Figure 9 for extracted serum samples with low analyte concentrations.

The developed method was quickly imported into the Cliquid® Software as a pre-configured method. The Cliquid® Software allows the simple selection to run the method and after sample batch set up the software performs automated acquisition, data processing and reporting of the results. Figure 10 shows an example report that is generated by the Cliquid® Software.

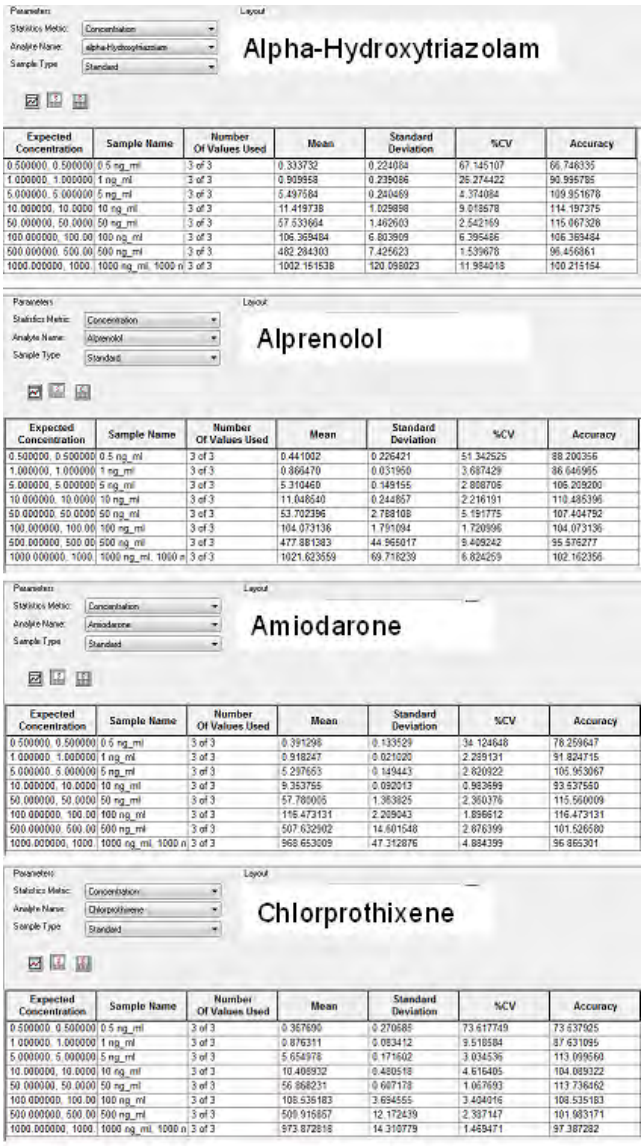
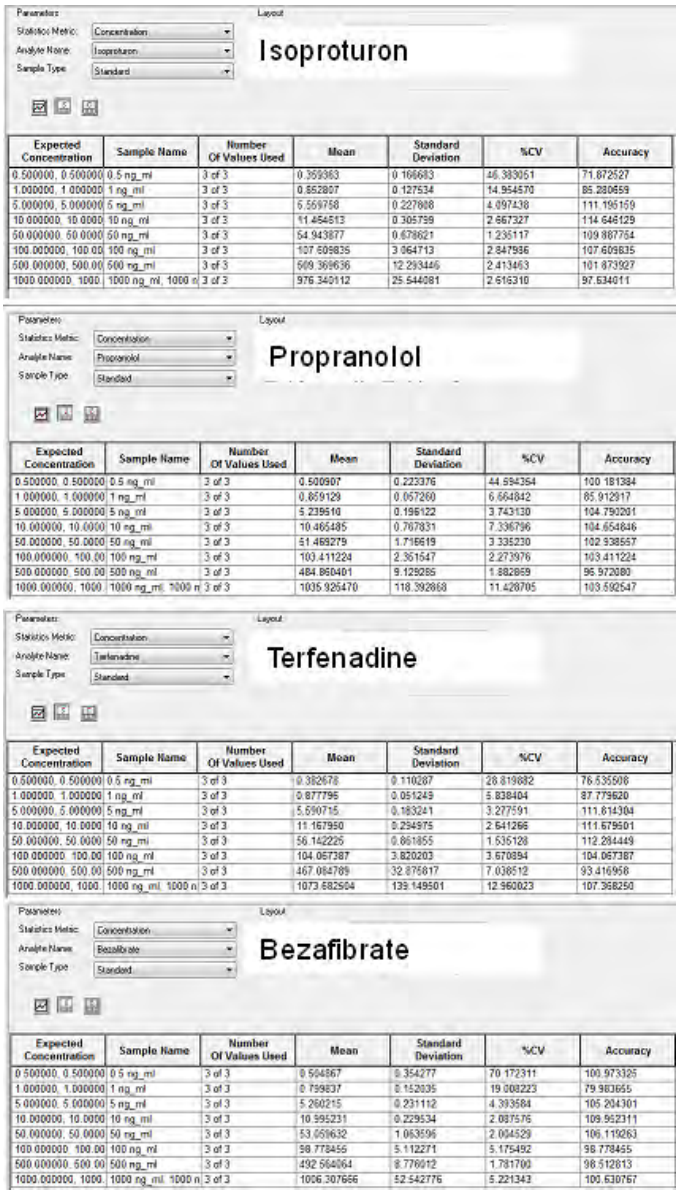


Figure 7. %CV and %Accuracy values obtained for selected drugs, across the calibration curve concentrations



Isoproturon

Expected Concentration	Sample Name	Number Of Values Used	Mean	Standard Deviation	%CV	Accuracy
0.500000, 0.500000	0.5 ng_ml	3 of 3	0.359363	0.166683	46.383051	71.872527
1.000000, 1.000000	1 ng_ml	3 of 3	0.852807	0.127534	14.954570	85.280659
5.000000, 5.000000	5 ng_ml	3 of 3	5.559758	0.227808	4.097438	111.195159
10.000000, 10.0000	10 ng_ml	3 of 3	11.454513	0.305799	2.667327	114.646129
50.000000, 50.0000	50 ng_ml	3 of 3	54.943877	0.678621	1.235117	109.887754
100.000000, 100.00	100 ng_ml	3 of 3	107.609835	3.064713	2.847936	107.609835
500.000000, 500.00	500 ng_ml	3 of 3	509.369636	12.293446	2.413463	101.873927
1000.000000, 1000.	1000 ng_ml, 1000 n	3 of 3	976.340112	25.544081	2.616310	97.634011

Propranolol

Expected Concentration	Sample Name	Number Of Values Used	Mean	Standard Deviation	%CV	Accuracy
0.500000, 0.500000	0.5 ng_ml	3 of 3	0.500907	0.223376	44.594354	100.181384
1.000000, 1.000000	1 ng_ml	3 of 3	0.859129	0.057260	6.664842	85.912917
5.000000, 5.000000	5 ng_ml	3 of 3	5.239510	0.195122	3.743130	104.790201
10.000000, 10.0000	10 ng_ml	3 of 3	10.465485	0.767831	7.336796	104.654846
50.000000, 50.0000	50 ng_ml	3 of 3	51.469279	1.716619	3.335230	102.938557
100.000000, 100.00	100 ng_ml	3 of 3	103.411224	2.361547	2.273976	103.411224
500.000000, 500.00	500 ng_ml	3 of 3	484.860401	9.129285	1.882859	96.972080
1000.000000, 1000.	1000 ng_ml, 1000 n	3 of 3	1035.926470	118.392868	11.428705	103.592547

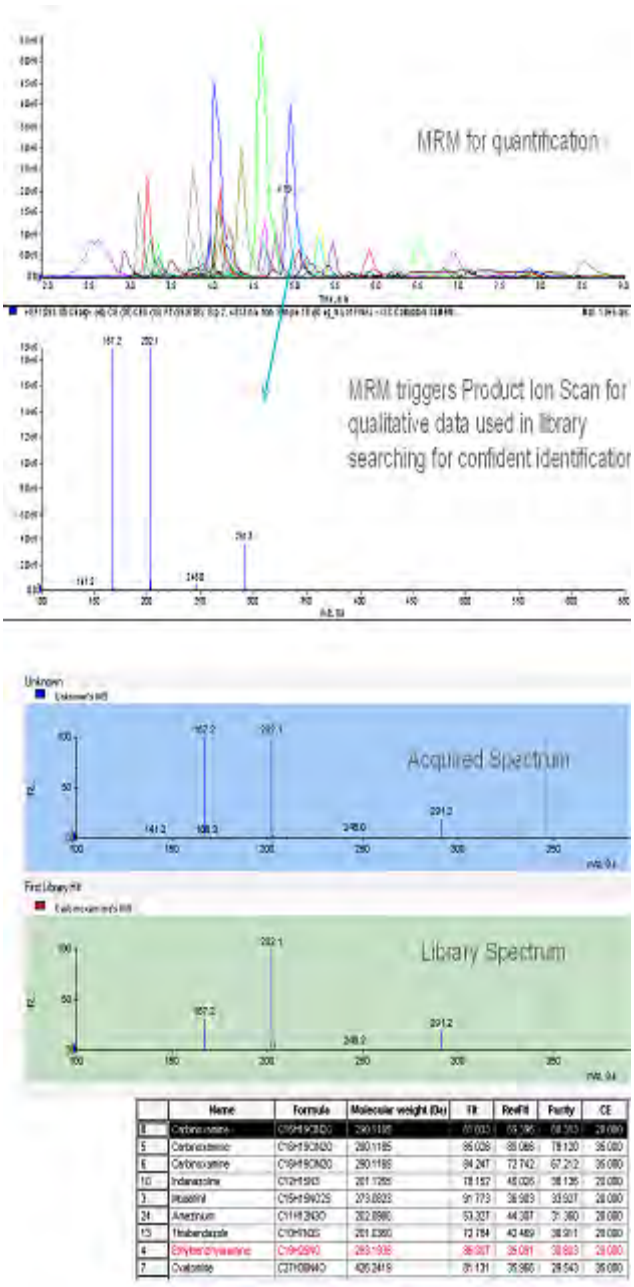
Terfenadine

Expected Concentration	Sample Name	Number Of Values Used	Mean	Standard Deviation	%CV	Accuracy
0.500000, 0.500000	0.5 ng_ml	3 of 3	0.382678	0.110287	28.819682	76.535508
1.000000, 1.000000	1 ng_ml	3 of 3	0.877795	0.051249	5.838404	87.779620
5.000000, 5.000000	5 ng_ml	3 of 3	5.590715	0.183241	3.277591	111.814304
10.000000, 10.0000	10 ng_ml	3 of 3	11.167950	0.294975	2.641266	111.679501
50.000000, 50.0000	50 ng_ml	3 of 3	56.142225	0.861855	1.535128	112.284449
100.000000, 100.00	100 ng_ml	3 of 3	104.067387	3.820203	3.670894	104.067387
500.000000, 500.00	500 ng_ml	3 of 3	467.084789	32.876617	7.038512	93.416958
1000.000000, 1000.	1000 ng_ml, 1000 n	3 of 3	1073.682504	139.149501	12.960023	107.368250

Bezafibrate

Expected Concentration	Sample Name	Number Of Values Used	Mean	Standard Deviation	%CV	Accuracy
0.500000, 0.500000	0.5 ng_ml	3 of 3	0.504867	0.354277	70.172311	100.973325
1.000000, 1.000000	1 ng_ml	3 of 3	0.799837	0.152035	19.008223	79.983655
5.000000, 5.000000	5 ng_ml	3 of 3	5.260215	0.231112	4.393584	105.204301
10.000000, 10.0000	10 ng_ml	3 of 3	10.595231	0.229534	2.087576	105.952311
50.000000, 50.0000	50 ng_ml	3 of 3	53.059632	1.063596	2.004529	106.119263
100.000000, 100.00	100 ng_ml	3 of 3	98.778455	5.112271	5.175492	98.778455
500.000000, 500.00	500 ng_ml	3 of 3	492.564064	8.776012	1.781700	98.512813
1000.000000, 1000.	1000 ng_ml, 1000 n	3 of 3	1006.307666	52.542776	5.221343	100.630767

Figure 8. %CV and %Accuracy values obtained for selected drugs, across the calibration curve concentrations



	Name	Formula	Molecular weight (Da)	Tt	RevFit	Purity	CE
8	Carbinoxamine	C16H19ClN2O	290.1185	89.003	88.196	88.303	29.080
5	Carbinoxamine	C16H19ClN2O	290.1185	86.026	88.086	78.120	35.080
6	Carbinoxamine	C16H19ClN2O	290.1185	84.247	72.742	67.212	35.080
10	Indanazoline	C12H15N3	201.1266	78.152	45.026	38.126	29.080
3	Risedronate	C15H17NO2S	275.0823	91.773	36.983	33.507	29.080
24	Amezinium	C11H13N3O	202.0980	53.227	44.387	31.360	29.080
15	Thiabendazole	C10H7N3S	201.0360	72.784	42.489	38.911	29.080
4	Erythromycin	C37H67NO13	733.4663	86.087	35.091	30.893	29.080
7	Cyclonite	C3H6N6O6	426.2419	81.131	35.960	28.543	35.080

Figure 9. Example of extracted spectra and library search purity score values using an MS/MS library search algorithm

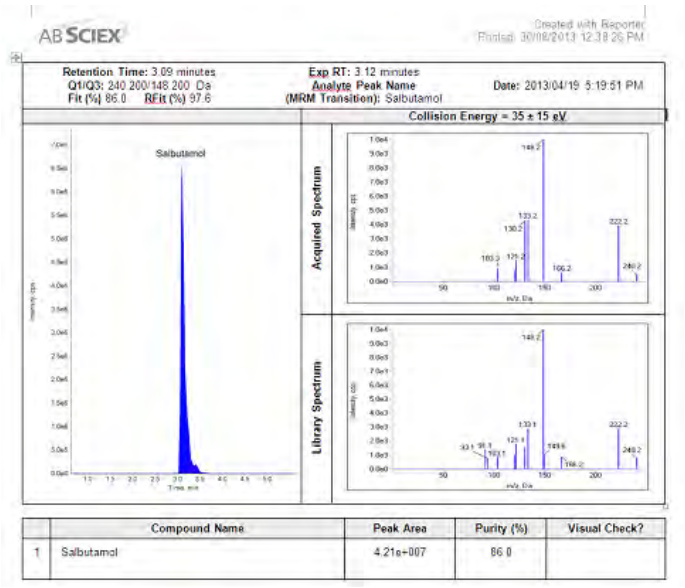


Figure 10. The Cliquid® Software was used to automatically generate a report after automated acquisition, data processing and library searching. An extract from the report is shown, showing the integrated MRM signal of Salbutamol and the comparison of the acquired EPI spectrum and the spectrum from the library.

Conclusions

As a result of this study, we were able to show:

- The Spark Holland PICO system in combination with the SCIEX QTRAP® 4500 LC-MS/MS System, as part of an On-Line SPE LC-MS/MS workflow, allowed the automated injection and cleanup of serum samples for screening and confirmation for over 100 analytes in a single run.
- The QTRAP® allowed high accuracy compound identification by performing full scan MS/MS experiments using the Enhanced Product Ion mode after being triggered from a Scheduled MRM™ Pro Algorithm IDA survey scan with automated library searching capabilities to compare the unknown with a standard library spectrum.
- Quantitative analysis was performed in the same run allowing for both quantification and qualitative data to be collected simultaneously. Linear calibration curves resulting in R2 values 0.99 or greater were achieved for the samples analyzed.
- Analysis for large panel of drugs in serum with automated sample preparation, detection and confident identification in a single injection was achieved.

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