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Integrated high capacity solid phase extraction-MS/MS system for pharmaceutical profiling in drug discovery

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Abstract

A method is described for use in analysis of samples from pharmaceutical profiling of early drug discovery compounds. The method consists of a high capacity autosampler which injects samples into one of two solid phase extraction columns operated in parallel for alternating trapping, washing and elution into a tandem quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) MS/MS mode. A primary method, which is useful for 80–90% of compounds, and a secondary method, which is useful for a majority of the remaining compounds, are described. No analytical HPLC column is used and the analysis rate is approximately 50 samples/h. Specificity is obtained using MRM analysis. Application of the method for high capacity analysis of metabolic stability samples is described.

Keywords: Drug discovery; Stability; High throughput; MS/MS; On-line solid phase extraction; Screening; Pharmaceutical profiling

1. Introduction

Successful pharmaceutical products require activity, selectivity, and pharmaceutical "drug-like" properties [1–3]. The term drug-like properties refers to solubility, permeability, stability, lipophilicity, and toxicity that are consistent with currently marketed drugs and that allow safe delivery of sufficient drug concentration to the therapeutic target. For many years inadequate pharmaceutical properties caused high attrition of drug candidates during pharmaceutical development due to inadequate pharmacokinetics. In response, many pharmaceutical organizations established procedures for testing these properties of compounds in late discovery prior to their promotion to development. This has reduced development attrition by reducing the number of drug candidates with poor properties that are promoted to development.

With this success, the focus has shifted to insuring that discovery compounds possess drug-like properties prior to reaching late discovery. Evaluation of drug-like properties has two major benefits. First, it

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insures that years of discovery work are not wasted on drug candidates that are rejected in late discovery. Also, drug-like properties affect discovery research in a fundamental way, because they affect in vitro and in vivo activity and specificity experiments. For example, if a compound is insoluble, impermeable, or unstable in biological tests, the activity of the compound may be underestimated or the observed activity may be due to a degradant or metabolite. Increasingly, the properties of compounds are being used, along with traditional activity and selectivity properties, to prioritize drug leads and to plan improvement or optimization via synthetic structural modification. Thus, structure–property relationships (SPR) and structure–activity relationships (SAR) are being considered in parallel [1–3] during discovery.

In order for compounds to be selected and optimized in this way, their properties must measured. This function has been termed "pharmaceutical profiling" [2,3]. The following tests are often used: integrity, solubility, permeability, stability, lipophilicity, pK_a, metabolite identification, and CYP450 inhibition. Pharmaceutical profiling provides property data for candidate selection/prioritization during early phases of drug exploration and discovery and for monitoring property optimization by synthetic structural modification.

While measurement of some of these properties can be performed using high capacity UV or fluorescence plate readers in a 96 well format (0.05–0.2 min per sample), some assays, such as stability, require more highly selective analysis methods, due to the presence of interfering components in the complex sample matrices. Typically, HPLC with UV detection produces insufficient sensitivity and selectivity for trace analysis in complex sample matrices and mass spectrometry is commonly used to achieve the sensitivity, selectivity, and throughput needed [4–11]. However, more selective analytical methods typically require much more time (2–10 min per sample) than a plate reader method. For pharmaceutical profiling the cycle time for more selective assays should be on the order of 1 min per sample in order to process the number of compounds and resulting samples that must be profiled in drug discovery. For example, metabolic stability assays often involve the incubation of compounds with liver microsomes in the presence and absence of NADPH at time points, resulting in the generation of at least three samples per compound, or six samples when assays are run in duplicate. Thus, 48 test compounds would generate 288 samples for analysis, for the metabolic stability assay alone. Often selective LC/MS methods require 2–10 min per compound for method development plus 2–10 min per sample for sample preparation and instrumental analysis. Thus, analysis of 288 samples for 48 compounds could require 10–50 h to complete. The analysis method must have a much lower method development and analysis cycle time than this to be useful in drug discovery.

Several groups have reported higher throughput LC/MS-based methods using various approaches to accelerate the analysis. Korfmacher et al. [4] described simultaneous separations in parallel on 2 HPLC columns and the mixing of the HPLC effluents just prior to the MS ion source. This approach doubles the throughput for analysis compared to conventional one-at-a-time LC/MS analysis. Xu et al. [5] described a further enhancement of this approach to perform 8 simultaneous HPLC separations in parallel and combine the effluent just prior to the MS ion source. Parallel chromatography with recombination of column effluents reduces analysis time per sample to as low as 0.25 min. It requires a multi-injector autosampler capable of eight simultaneous injections, plumbing for eight columns operating in parallel, and a manifold for recombination of HPLC effluent from eight columns. Throughput for the eight column instrument is 176 test compounds per day (over 35,000 per year).

An alternative to recombination of column effluents is to use a multiplexing "MUX" ion source. This apparatus sequences between the effluents from four or eight columns operating in parallel, allowing each to enter the electrospray MS ion source for a short interval before moving on to the next column. Deng et al. [6], Morrison et al. [7], and Fang et al. [8] described this approach, which requires the multi-injector autosampler, parallel plumbing fluidics, and a MUX ion source. Throughput is as low as 0.5 min per sample, which is similar to the recombination methods above.

Zheng et al. [9] discussed two methods for accelerated LC/MS throughput: (1) fast chromatography (2 min HPLC analyses) following acetonitrile precipitation sample preparation, and (2) direct sample injection to the MS ion source following solid phase extraction (SPE) sample preparation. Fast chromatography reduces analysis time per sample to as low as 2 min and requires only common HPLC and MS

systems. Direct injection reduces analysis time per sample to as low as 0.25 min and requires solid phase extraction (SPE), but no HPLC column.

Bu et al. [10] described a method for direct injection of a formic acid precipitated sample into a guard cartridge, which traps the test compound. The trapped compound is eluted directly into the mass spectrometer. The method is simple and fast. Analysis times of 3 min per sample injection were demonstrated.

Janiszewski et al. [11] described the development and application of an analytical system that uses a high capacity Gilson autosampler, dual injection valves, sample trapping on short HPLC trapping columns, direct injection into the mass spectrometer ion source, and MS/MS quantitation. This system involves some customized valves and software and reliably provides throughputs of <30 s per sample.

We undertook to implement a high capacity mass spectrometry-based detection system for pharmaceutical profiling applications that incorporated some of the design elements described above. With our limited resources in instrument customization and software programming, we undertook to use standard commercially-available modules. Our throughput needs were on the order of 50–100 compounds per day. The resulting instrument incorporates the following elements:

(1) On-line sample preparation, for removal of salts and proteins that would interfere with electrospray ionization. This is done using alternating parallel solid phase extraction (SPE) columns attached to a multiport valve preceding the mass spectrometer. This design follows the work of Ackermann et al. [12].
(2) A high capacity autosampler capable of maintaining up to 12 plates in a cool, stable environment before injection.
(3) A separate syringe that washes the injection valve to reduce carry-over.
(4) Direct flow injection (no analytical HPLC) into a triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode. This design follows the work of Janiszewski et al. [11].
(5) Automated MRM method development for MS/MS analysis to reduce the resources necessary for method development of 50–100 compounds per day.

Here, we describe the resulting analysis method and its application for high capacity analysis of samples from drug discovery stability profiling. Throughput is on the order of 1.1 min per sample. The hardware is much simpler than some of the options described in the literature (above) and does not require parallel flowing systems, multiple HPLC columns, multiprobe autosamplers, or mutiplexed electrospray ion sources. This makes the system much simpler to operate and maintain. Also, since standard instrument modules and software were used, implementation of the system was rapid.

2. Experimental

2.1. Instrumentation

The integrated instrument configuration is shown in Fig. 1. A model “Twin PAL” autosampler from LEAP Technologies (Carborro, NC) was used. The autosampler was equipped with: (a) six plate holder drawers that each hold two 96 well plates (12 plate total capacity) that were maintained at 10 °C with Pelletier coolers, (b) a 6 port injection valve with a 20 μl loop, (c) a 10 port valve to which the SPE columns were attached, and (d) two rails on which separate injection syringes were held and operated. One of the syringes of the Twin PAL is used to inject 20 μl of sample and the other syringe is used to wash the injection valve. The 10 port valve is configured as shown in Fig. 2, with two Waters (Milford, MA) OASIS® brand HLB columns (2.1 mm i.d. × 20 mm length). OASIS® HLB is polymeric SPE material for sample preparation. The MS/MS system is a Waters Micromass (Beverly, MA) Quatro Micro tandem quadrupole mass spectrometer equipped with an electrospray interface. Mass spectrometer software includes MassLynx (version 3.5), QuanLynx, and QuanOptimize®. The HPLCs are Agilent Technologies (Waldbronn, Germany) 1100 pumps that operate isocratically. In the “primary method” of operation, one HPLC pump provides a mobile phase of 0.1% formic acid at a flowrate of 3.0 ml/min (termed “aqueous mobile phase” in Fig. 2), and the other pump provides a mobile phase of 95% acetonitrile/5% of 0.1% formic acid at a flowrate of 1.0 ml/min (termed “organic mobile phase” in Fig. 2). In the

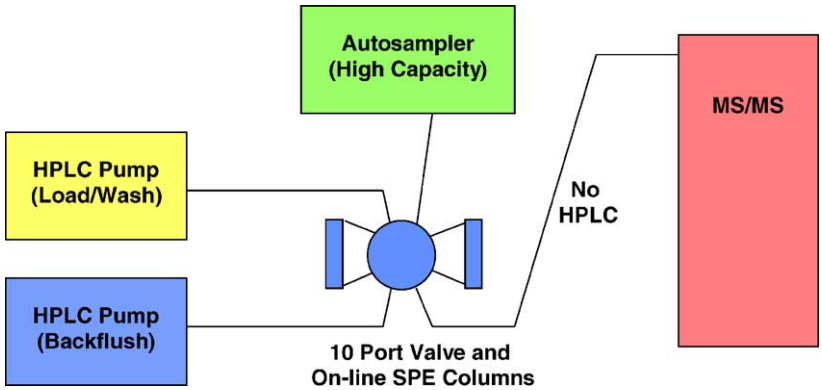

Fig. 1. Diagram of system.

"secondary" method of operation 0.1% triethylamine is substituted for 0.1% formic acid in both mobile phases.

2.2. Methods

2.2.1. Automated MRM conditions selection

Solutions of each test compound are prepared in DMSO and then diluted to 25 μM in 1:1:2 DMSO:acetonitrile:water in a 96 deep well plate. This plate is placed in the autosampler and QuanOptimize® is run to select MS/MS multiple reaction monitoring (MRM) quantitation conditions. Each compound is injected (20 μl) three times through the OASIS® column with elution directly into the MS/MS system using the organic mobile phase. The first two injections are in positive and negative electrospray mode, respectively, and the cone voltage is ramped. The

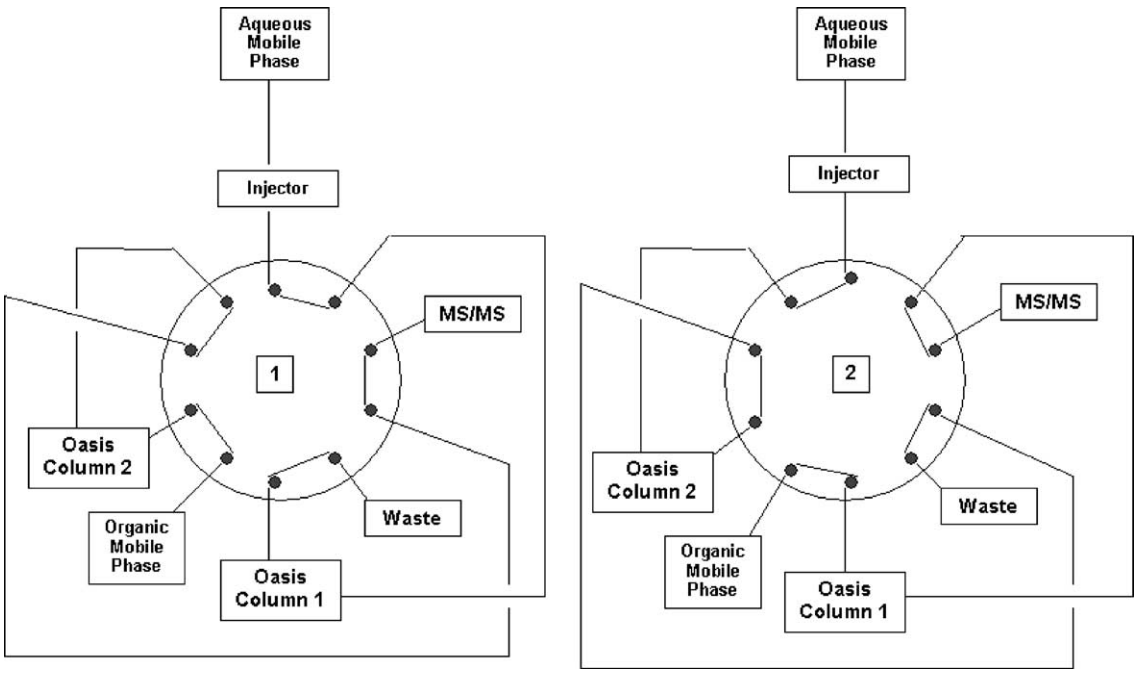

Fig. 2. Diagram of 10 port valve arrangement.

ionization mode and cone voltage producing the highest response is selected. With the third injection, a series of product ion spectra are collected at increasing Q2 collision energies. As a result of this procedure, a set of conditions (ionization polarity, cone voltage, parent/product MRM ion pair, and collision energy) are selected for each compound and stored in the data system.

2.2.2. Metabolic stability incubation

Compounds are incubated by adding 10 μl of 25 μM test compound solutions (above) with 12 μl of 1 mg/ml (protein) rat liver microsomes, 10 μl of 1 mM NADPH, and 220 μl of 0.1 M phosphate buffer (pH 7.4). The "t_0" (time = 0 min) samples are obtained by immediately adding 250 μl of acetonitrile at 0 °C. The "t_{30} + NADPH" (time = 30 min) samples are obtained by incubating the mixture for 30 min on a Boekel Scientific (Philadelphia, PA) model 130000 Jitterbug incubator for 30 min at 37 °C. The "t_{30}-NADPH" (time = 30 min) samples are incubated without NADPH by substituting 10 μl of phosphate buffer (pH 7.4) for the 1 mM NADPH. The incubation is quenched by the addition of 250 μl of acetonitrile at 0 °C, followed by centrifugation for 20 min at 3000 rpm in a Jouan BR4i centrifuge. The supernatant is transferred to fresh 96 well plates for instrumental analysis. Incubations are performed in duplicate. Sample concentration is at 1 μM. The low concentration reduces analysis variability resulting from low test-compound solubility and is more consistent with physiologically relevant exposure concentrations.

2.2.3. Instrumental analysis

Incubated samples in 96 well plates are placed in the plate holder drawers of the autosampler. The autosampler and mass spectrometer software permits sample analysis in any order from any plate in the system. Typically, samples are injected for a given compound in the following sequence: t_0, t_{30} + NADPH, t_{30}-NADPH. A 20 μl aliquot of sample is transferred from the specified well of the plate to the six port valve equipped with a 20 μl sample loop. Once the loop is filled, the injection valve is activated and the sample is swept to the 10 port valve by the aqueous mobile phase. The test compound is trapped on the OASIS® column and the aqueous mobile phase continues to wash the column for 10 s, in order to flush unretained salts and proteins to waste (Fig. 2). The 10 port valve is then activated to the second position and the organic mobile phase elutes the test compound from the column into the MS/MS for 0.5 min. The trapped material elutes from the column in a peak that is 5–10 s wide. The test compound is detected and quantitated using the MRM conditions selected by the automated MRM conditions selection procedure (earlier). The plate position of the next sample is then transferred to the autosampler and it is injected onto the second OASIS® column and analyzed as above. The total cycle time per analysis is 1.1 min.

2.2.4. Quantitation

The t_0 sample is used as the quantitation standard and the "% remaining" after incubation is calculated for the t_{30} + NADPH and t_{30}-NADPH samples by dividing the integrated area of their response by that of the t_0 sample. The software performs the calculation and prints the detected elution peak of each sample so that manual checking can be performed.

2.2.5. Statistical evaluation

The intra-day and inter-day performance of the analysis method was evaluated. Eight drug compounds (Table 2) were each incubated and prepared in quadruplicate in a single 96 well plate using the methods described earlier. The samples were incubated and prepared on 1 day and then analyzed on three successive days. This provided evidence of the intra-day and inter-day performance of the method.

2.3. Materials

Acetonitrile and water were HPLC grade from EM Science (Gibbstown, NJ). DMSO was 99.9% from Aldrich (Milwaukee, WI). All other chemical reagents were from Sigma (St. Louis, MO). Rat liver microsomes were from In Vitro Technologies (Baltimore, MD).

3. Results and Discussion

A typical analysis set for this method involves 45 compounds, two standard compounds (testosterone and buspirone) and a blank. These are analyzed in

duplicate to utilize all the wells of a 96 well plate. The method described here performs both sample extraction and selective quantitation in one integrated, rapid instrumental method.

The MS/MS system is operated in MRM mode. This serves two functions. It provides a specific response for each test compound. Also, the two mass separation stages provide two levels of sample component separation [13,14]. This provides selectivity for the test compound signal compared to background chemical noise. Thus, for this application, analytical grade HPLC separation has been eliminated and the sample is directly eluted into the electrospray ion source of the mass spectrometer.

The QuanOptimize® software greatly simplifies MS/MS MRM method development for each test compound. Manual selection of MRM conditions for each compound would be too time-consuming using manual infusion approaches for method development. A 25 μM solution of each analyte is used to select MRM conditions on the mass spectrometer. This is performed at the same time as the stability experiment incubation and sample preparation are being performed. Thus, when the samples are ready for analysis, the method conditions are ready. Table 1 indicates a typical result from the optimization method. About 1 in 10 compounds fail on the optimization procedure. This is usually due to lack of sufficient ion production by electrospray. In this case, alternate approaches, such as single stage LC/MS or LC/UV, are used.

Table 1
MRM conditions for a set of compounds obtained using the QuanOptimize® automated MRM condition selection procedure

Compound	MRM transition[a]	Polarity[b]	CV[c]	CE[d]
Sulfasalazine	397.2 > 197.2	−	40	25
Oxprenolol	266.4 > 116.1	+	30	20
Verapamil	455.6 > 165.2	+	40	30
Bupivacaine	289.4 > 140.2	+	25	25
Amiodarone	646.4 > 100.4	+	50	40
Floctafenine	405.1 > 331.1	−	25	15
Sulfinpyrazone	403.1 > 125.1	−	25	20
Sulfamethizole	269.1 > 196.1	−	35	20

[a] Parent and product ion masses monitored in Q1 and Q3 of the MS/MS for MRM.
[b] Positive or negative ion polarity selected for MS/MS analysis.
[c] Electrospray ion source cone voltage.
[d] Collision cell MS/MS collision energy.

The SPE method embodies a generic approach, by which approximately 80% of test compounds can be analyzed. A typical peak generated by the elution of the trapped material from the OASIS® cartridge is shown in Fig. 3. The compound elutes in a peak that is approximately 6–8 s wide at the baseline. During development of the method, it was noted that most of the remaining compounds (approximately 20%) eluted with a broad and tailing peak as shown in Fig. 4. Examination of the structures of these compounds indicated that they contained acidic functional groups, either carboxylic acid or phenolic. Substitution of 0.1% triethylamine for the 0.1% formic acid in the organic mobile phase which elutes the analytes from the OASIS® column sharpened the peaks considerably.

Using this method, a 96 well plate of samples is analyzed in less than 2 h. A typical metabolic stability study with 3 plates requires approximately 6 h to complete. Relative quantitation of the "% remaining" is performed by dividing the response of the t_{30} + NADPH, and t_{30} − NADPH samples by the response of the t_0 sample, which is used as a control. Any electrospray matrix suppression of response in the samples, due to co-extracted background components, is compensated for by using the t_0 sample for relative quantitation, which also contains any co-extracted matrix.

Statistical evaluation of the instrumental system is shown in Table 2. Intra-day reproducibility ranged from 2.0 to 26% R.S.D. Inter-day reproducibility ranged from 0.5 to 14% R.S.D. This level of reproducibility is sufficient for early discovery pharmaceutical profiling.

Fig. 5 indicates the specificity of the method. The responses of the system for two compounds incubated at 1 μM concentration and their respective blanks are shown. The 1 μM and blank samples were analyzed using the same MRM conditions. The blank typically has negligible response compared to the sample containing the test compound.

This method provides a rapid and reliable approach for stability profiling in early drug discovery, using a system that is relatively simple to set up and maintain. Late discovery and development typically require a method with greater specificity and higher statistical accuracy and precision. The method described here can be expanded to other discovery pharmaceutical profiling applications which require high capacity and

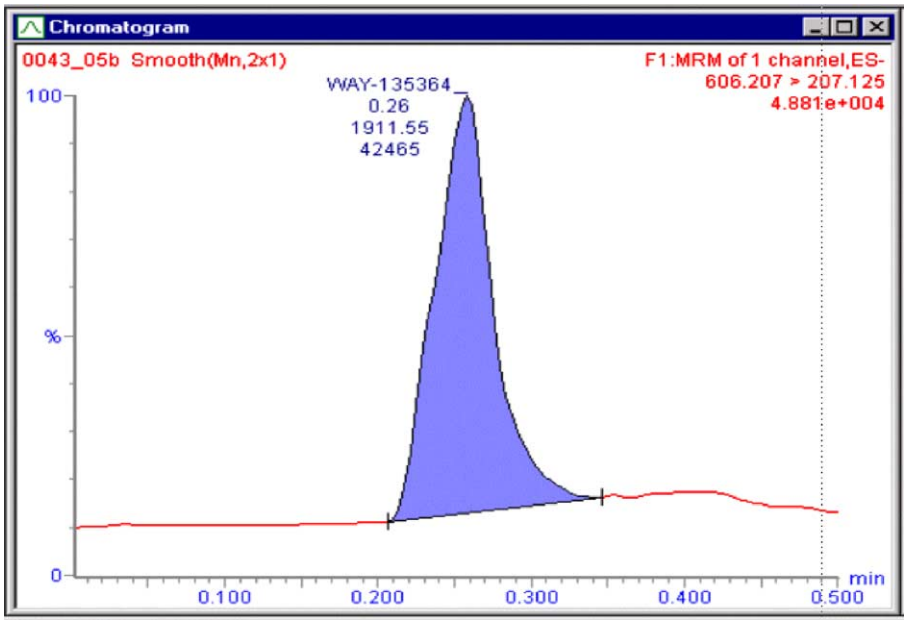

Fig. 3. Midazolam "peak" eluted from OASIS® cartridge with 95% acetonitrile:5% formic acid (0.1%) and detected on MS/MS.

rapid throughput rather than on quantitation within narrow statistical criteria. Compared to the typical LC/MS method with analysis cycle times of 3–10 min per sample, the 1.1 min cycle time of this method permits processing of larger numbers of samples in the same time and lab space. Another advantage of this approach, is that minimal organization resources were required for instrument development and custom software programming. The components used here (HPLC, autosampler, and MS/MS instrument) are equipped with powerful and flexible software that facilitates system integration. In operation, the method requires minimal method development time. The SPE trapping columns last at least 10 days of analysis. The analysis sequence is the same for each compound and the pre-selection of MRM conditions

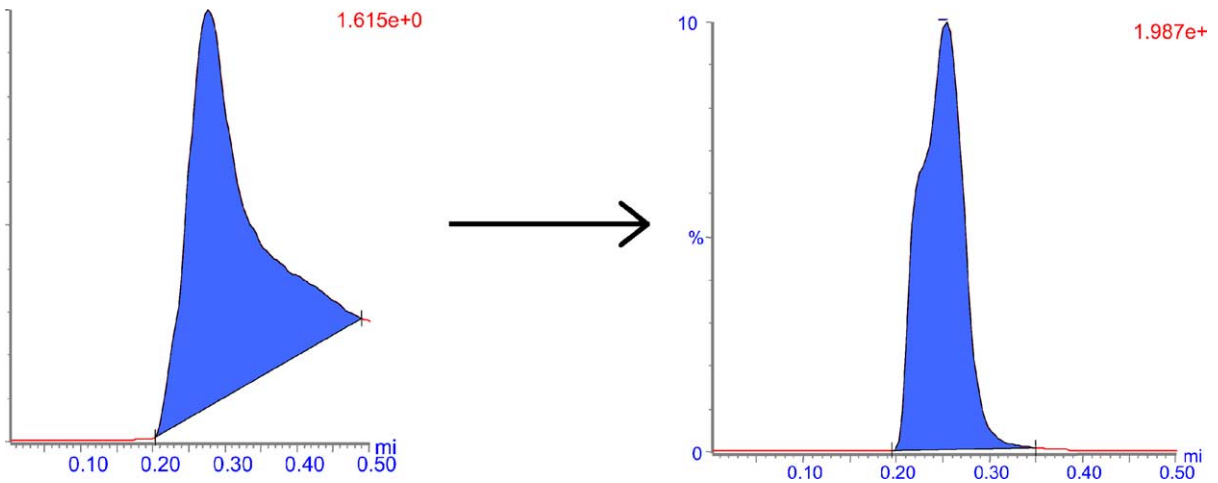

Fig. 4. Improvement of peak shape for an acidic compound with 95% acetonitrile:5% triethyleamine (0.1%) elution solvent.

Table 2
Results for statistical evaluation of the instrumental system using a set of compounds incubate with NADPH

Compound	Day 1		Day 2		Day 3		Inter-day	
	Average[a]	S.D.[b]	Average[a]	S.D.[b]	Average[a]	S.D.[b]	Average[c]	S.D.[d]
Sulfasalazine	84.8	8.26	75.3	16.2	83.5	8.74	81.2	5.16
Oxprenolol	18.8	1.89	17.8	4.57	18.5	3.42	18.3	0.52
Verapamil	31.8	1.89	31.5	2.38	31.8	4.27	31.7	0.14
Bupivacaine	28.3	0.57	28.0	2.65	28.7	2.31	28.3	0.33
Amiodarone	66.8	1.5	67.5	2.38	67.0	2.94	67.1	0.38
Floctfenine	66.5	5.07	65.8	2.63	65.5	7.55	65.9	0.52
Sulfinpyrazone	115	23.9	118	18.7	118	21.6	117	1.23
Sulfamethizole	98.0	6.16	103	5.68	101	2.22	101	2.43

[a] Average percent of drug remaining for four replicates after incubation compared to control.
[b] Standard deviation of percent drug remaining for four replicates after incubation compared to control.
[c] Average of three daily averages of percent remaining.
[d] Standard deviation of three daily averages of percent remaining.

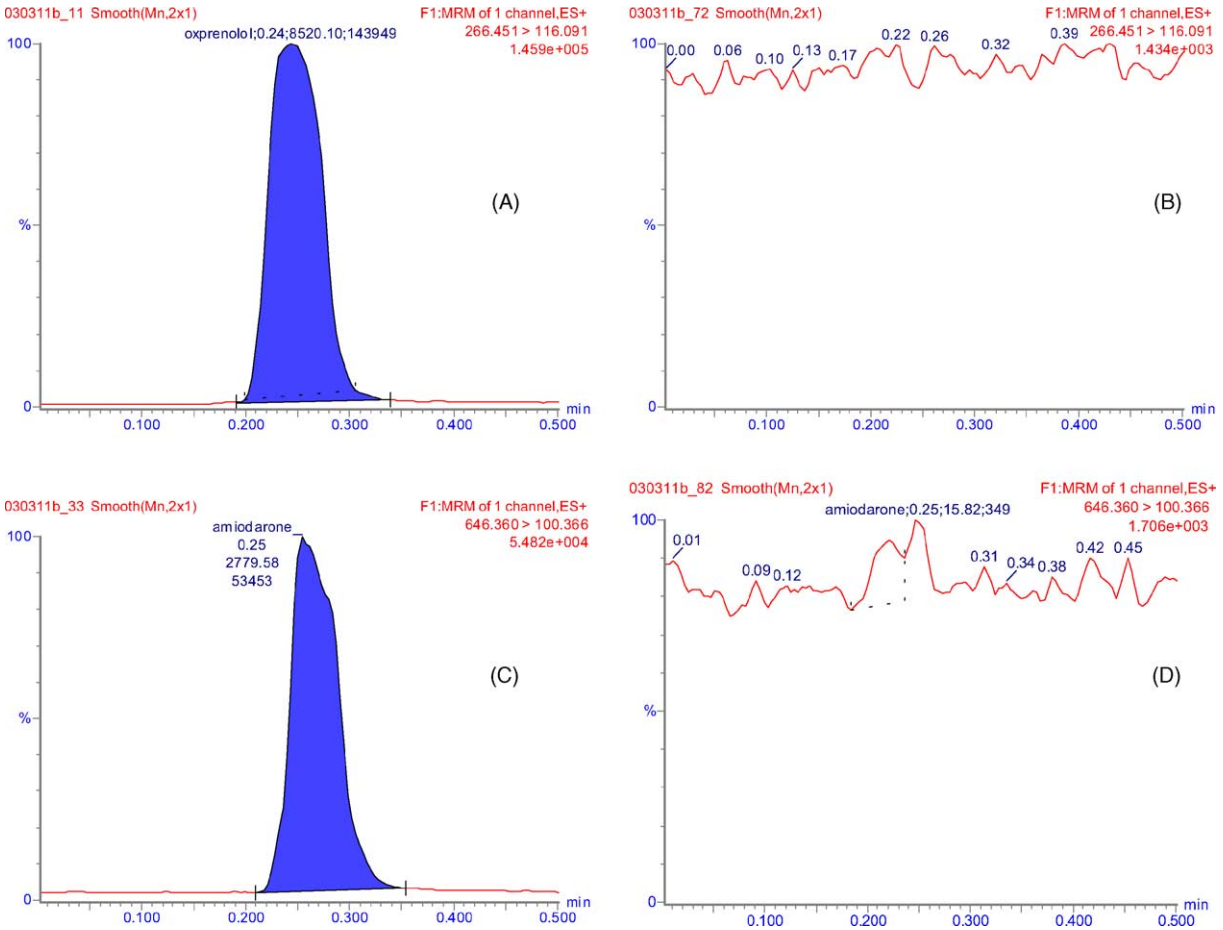

Fig. 5. Typical “peaks” and blanks for two of the compounds in Table 1: (A) oxprenolol at 1 μM, (B) oxprenolol blank, (C) amiodarone at 1 μM, and (D) amiodarone blank.

via an automated protocol saves considerable method development time. The method uses a minimum of steps, which permits rapid turn-around. The minimal number of parts should increase reliability, simplify operation, and reduce maintenance.

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