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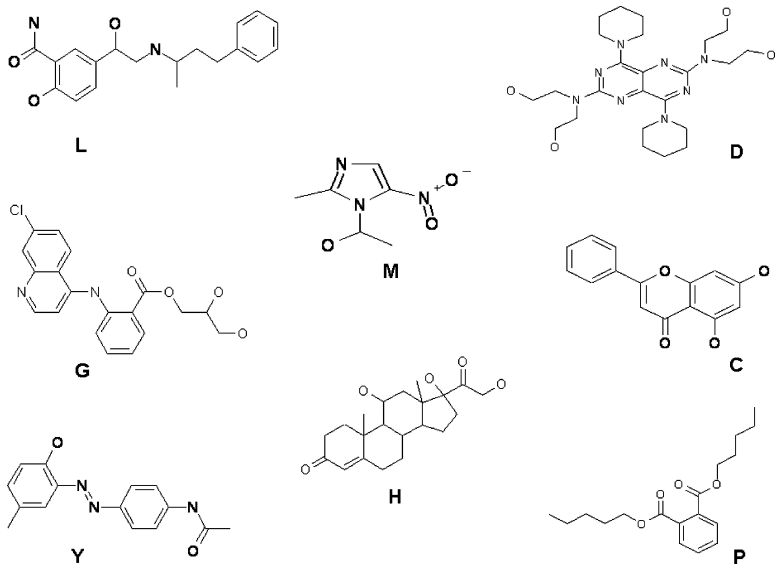

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Enhanced Performance Test Mix for High-Throughput LC/MS Analysis of Pharmaceutical Compounds

Shu Li,[†] Lesline Julien, Peter Tidswell,[‡] and Wolfgang Goetzinger*

ArQule, Inc., 19 Presidential Way, Woburn, Massachusetts 01801

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LC/MS is being used for the routine analysis of small molecules in both the discovery and development stages within the pharmaceutical industry. In drug discovery, LC/MS is relied upon to confirm the identity and assess the purity of chemical entities. To ensure the quality of LC/MS analysis, it is important that the LC/MS system is operating within defined performance criteria. Performance monitoring of the system with a standard compound mix offers many advantages over other alternatives, since it monitors the LC/MS system as an integrated unit under the same working conditions as those used for the analysis of samples. It is also a convenient approach, because the test mix can be injected as part of the automated sequence. Use of a test mix for similar purposes has been described previously (Tang, L.; Fitch, W. L.; Alexander, M. S.; Dolan, J. W. *Anal. Chem.* **2000**, *72*, 5211−5218). To monitor the performance of ArQule's LC/MS operation (with UV and ELS detection) in greater detail, a set of eight compounds was selected from a collection of 137 commercially available "druglike" compounds. The compounds are generally stable and compliant with the rule-of-five criteria. This enhanced mix has a balanced selection of pKa values and covers the typical range of hydrophobicity and molecular masses of pharmaceutical compounds. Moreover, the selected compounds can generally be ionized using ESI and APCI modes with positive and negative polarity. The test mix can be used under formic acid or ammonium hydroxide conditions and with methanol or acetonitrile as an organic modifier. Performance monitoring with the enhanced mix is demonstrated with respect to ionization and mass measurement, as well as changes in gradient profile, flow rate, buffer pH, and ionic strength.

Introduction

LC/MS has become an indispensable tool for many applications in the analysis of small molecules during drug discovery. Separation by LC, identification by MS, and quantification by UV or ELS (or both) in a single injection is a powerful combination for the analysis of crude reaction products as well as final quality control of purified products. The high speed of LC/MS is especially attractive in drug discovery applications as the number of compounds to be analyzed increases. In fact, LC/MS analysis has become such an integral part of drug discovery that today, many downstream decisions depend on these data alone, because other more traditional techniques, such as NMR, presently cannot provide the capacity that is needed in an economical fashion. Potential drug candidates and library compounds are screened only if they are pure enough as measured by LC/MS.[2] For this reason, erroneous LC/MS analysis can lead to resources' being wasted on poor drug candidates or good drug candidates' being excluded from consideration.

To reduce the number and cost of such errors, it becomes necessary to monitor the accuracy of LC/MS analysis. However, due to its high sensitivity, it is not always possible to confirm the LC/MS analysis with other analytical techniques. Thus, to ensure the quality of LC/MS data, it is important to ensure that the system works close to the optimal conditions at all times. To that end, the subsystem of the LC and MS can be individually monitored, and compound mixtures have been suggested to calibrate and tune a mass spectrometer[3−5] or to test the performance of a separation system.[6,7] However, there are disadvantages in this approach, particularly since it does not characterize the LC/MS under the same conditions as the samples. To test the instrument as an integrated unit, a test mix should be injected alongside the sample. Tang et al. introduced a test mix for this purpose,[1] which was adopted by several authors with various modifications.[8,9] The test mix has a balanced selection of acidic (aspartame), basic (reserpine), and neutral (cortisone, DOP) compounds. The application of the mix was demonstrated under various scenarios, expediting method development in addition to monitoring system performance. The objective of this work was to develop a test mix that monitors the performance of the system in greater detail and can be easily used in a high-throughput environment. An ideal test mix for this purpose should be analyzed in the same automated sequence as the sample and should monitor all aspects of the system's behavior as comprehensively as possible. Moreover, the LC/MS data from a system performance check should provide a valuable diagnostic hint in case of an error.

* To whom correspondence should be addressed. Current Address: Amgen Massachusetts, One Kendall Square, Building 1000, Cambridge, Massachusetts 02139. Phone: 617-444-5011. E-mail: wgoetzin@amgen.com.

[†] Current address: Pfizer Global R&D, 140 Eastern Point Rd, Groton, CT 06340.

[‡] Current address: Pfizer Global R&D, 700 Chesterfield Parkway West, Chesterfield, MO 63017.

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To that end, a test mix should behave differently when the system deviates from the optimal working conditions. The suboptimal status of the system can then be discovered from the corresponding differences in the analytical data.

To identify the optimal combination of compounds for the test mix, 137 commercially available, druglike compounds were analyzed on high-throughput LC/MS instruments. The data for over 5000 injections were stored in a database and mined to select a "universal" test mix that can be applied for common reversed-phase chromatography conditions. The mix monitors the LC/MS system in more detail than previously. The subsystems to be monitored include those for detection and separation. For detection, the UV, ELS, and MS signal intensities are monitored. Specifically, for MS, ionization efficiencies in ESI, and APCI, positive and negative modes under different mobile phase media are considered in addition to accuracy of mass measurements. For separation, changes of mobile phase, such as gradient profile (in methanol or acetonitrile), flow rate, pH of mobile phase, and ionic strength are monitored. The test mix is also shown to be helpful monitoring any change in the stationary phase, although it is not selected primarily for this purpose. In addition to system monitoring, the test mix provides first-hand diagnostic information about the system at the time of error. The likely cause of the problem can be discovered using the rich information of the LC/MS data from the test mix. This can be carried out by cross-referencing data from the erroneous system with historical data, data across different systems, and data under different conditions.

Experimental Details

Equipment. Gradient retention data were measured on a Shimadzu HPLC system equipped with a UV detector (SPD-10AVP, Shimadzu), ELSD (Sedex75, Sedere), autosampler (HTS PAL, CTC), and a triple-quadrupole mass spectrometer (QuattroMicro, Waters). UV and ELS signals were acquired through the analog channels of the mass spectrometer and controlled by MassLynx 4.0 software. Solvents were delivered by Shimadzu pumps (LL-10ADVP) controlled by a Shimadzu controller (SCL-10AVP, Shimadzu). The compounds were detected with the UV detector first. The flow was then split, with ~5−10% of the flow then going into the mass spectrometer and the rest into the ELS detector.

The cLogP and logD values were calculated using ACD software (Advanced Chemistry Development). Other physical chemical properties were calculated using Pipeline Pilot (SciTegic).

Chemicals. Methanol, water and acetonitrile were high purity solvents from EMD (OmniSolvent grade). All the test standards were used as received from Sigma-Aldrich (purity >95% in general). Triflouroacetic acid was a Baker Analyzed Reagent from J.T. Baker. Ammonium hydroxide and formic acid were ACS Reagents from Sigma-Aldrich.

Procedure. A CTC PAL autosampler injected 4 μL of samples (2 mM) dissolved in DMSO into the LC/MS system. Dual pumps delivered different percentages of mobile phases A and B at a total flow rate of 3.0 mL/min. Mobile phases A and B were water and acetonitrile, respectively, and were premixed with solvent modifiers, generally 0.1% formic acid for both A and B. The solvent gradient was produced by mixing A and B after the pumps (50-μL mixer). Immediately after the injection, the gradient was started at 5% with no holding time, linearly ramped up to 95% in 1.75 min, held at 95% for 0.15 min, ramped down to 5% in 0.1 min, and then equilibrated at 5% for 0.3 min before the next injection. For acidic and neutral mobile phases, a Sunfire C18 column (Waters) was used; for basic mobile phases, an Xterra MS C18 column (Waters) was used. The columns were 4.6 × 30 mm with 3.5-μm particles. The above procedure was used as a template for all the experiments in this work. One or two settings of the template were modified for each experiment and were explained with the results.

The raw data were processed with the OpenLynx package of MassLynx to generate a report file. The report files were then processed with in-house software to extract or calculate the peak information, such as intensity, peak shape, and retention time. The peak information was then loaded into a local database if the identity of the compound was confirmed by MS.

Results and Discussion

General Considerations. Since the LC/MS instruments under consideration are used to separate, identify, and assess the purity of pharmaceutical compounds, the test mix should represent typical pharmaceutical compounds with respect to molecular weight and physicochemical properties, such as hydrophobicity, and acid and base properties. A single test mix is selected here for all the common methods a system may use for various applications. Compared with multiple mixes for multiple methods, it is not only more convenient but also provides richer information about the system.

1. A Pool of Druglike Molecules. To mimic the usage of the system, the majority of the test mix should be composed of drug or druglike compounds. Thus, ~137 druglike compounds were acquired commercially as the sampling pool, from which a set of compounds with the desired physical−chemical properties was to be selected. Figure 1 shows the distribution of some physical properties for the compound pool. These compounds had molecular weights from 100 to 800, typically from 200 to 500, and clogP from −3 to 9, typically from −1 to 5. Since the mix was to be used heavily in routine operations, several very toxic compounds were not considered. Furthermore, to exclude the compounds that were not chemically stable, stock solutions of these compounds were left on a shelf in amber vials for over 1 year and then diluted to 2 mM for screening by LC/MS. Compounds that decomposed and could not be detected by MS were excluded. The LC/MS data for the remaining compounds, such as retention time; peak width; signal intensity for MS; and UV, LC, and MS conditions, etc., were then stored in a database. The best combination of compounds was then selected by querying the database. This approach also allows us to incrementally improve the test mix in the future when new separation and detection methods are of interest or additional interesting compounds and their data can be incorporated. Once a compound was selected, the chromatogram was checked for traces of decomposition.

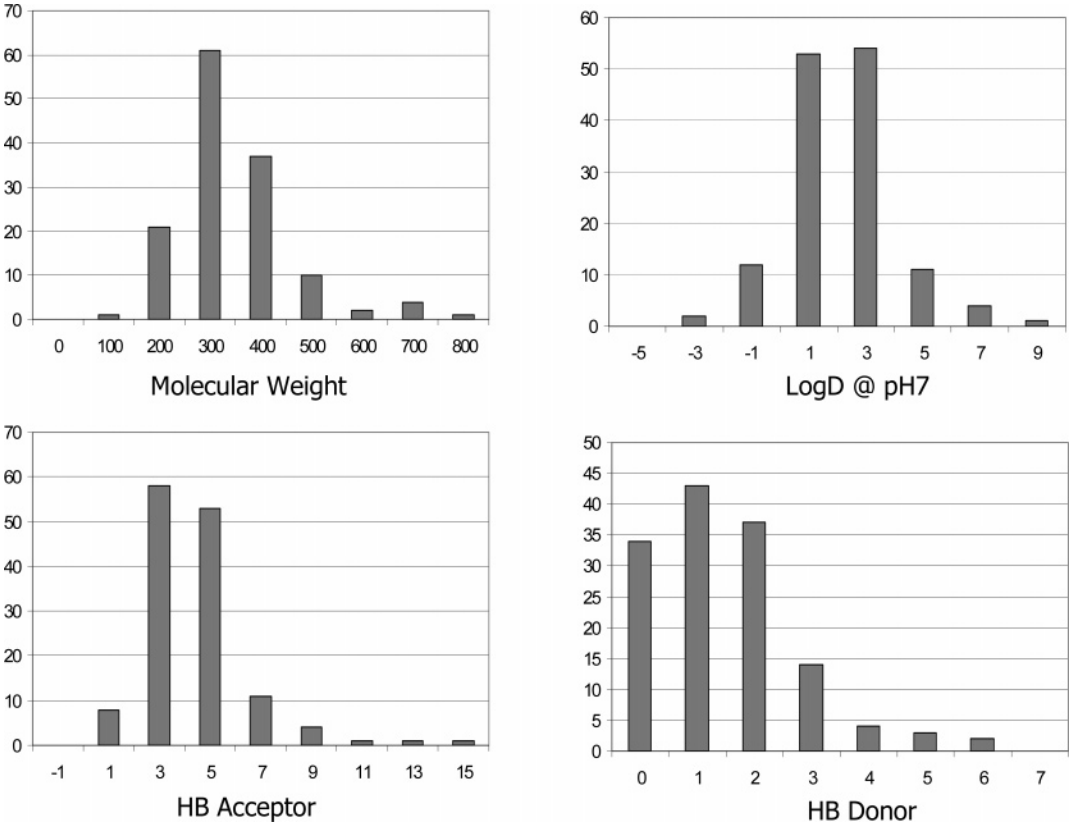

Figure 1. The distributions of selected rule-of-five properties for the 137 compounds.

Table 1. Structure and Properties of the Standard Compounds

compd, abbreviation	MW	acceptors	donors	logD @ pH 7
di-*N*-pentyl phthalate, P	306.2	4	0	5.9
hydrocortisone, H	362.2	5	3	1.4
disperse yellow 3, Y	269.1	4	2	2.9
chrysin, C	254.1	4	2	2.6
labetalol, L	328.2	4	4	0.3
glafenine, G	372.1	6	3	3.5
dipyridamole, D	504.3	12	4	0.6
metronidazole, M	171.1	4	1	−0.1

2. Distribution of Mass, Hydrophobicity and Charge. It is advantageous to keep the test mix simple, as long as the same level of information about the system can be provided. Our intention is to get as much information about the status of the separation and detection system with as few compounds as possible. This will ensure ruggedness of the test and will facilitate data interpretation. A set of eight compounds were identified out of the more than 100 compounds that were tested. Table 1 lists the names of the selected compounds. Figure 2 shows their molecular structures. The structures are diverse, and all the compounds except dipentylphthalate (DPP) and disperse yellow 3 (DY3) are drugs. We found it very helpful to have a colored compound as part of the mixture because this makes the test solution easily identifiable and helps to prevent human errors and potential mix-ups in sample handling. Table 1 also lists the rule of five properties of these compounds. To represent the hydrophobicity of typical drug compounds, our samples were selected to have a logD at pH 7 ranging from ~0 to 6. To monitor the accuracy of mass measurements over the mass range typically encountered in pharmaceutical analysis, the compounds were selected to have unique molecular weights ranging from 150 to 500. Since charge has a large impact not only on the ionization for mass spectroscopic detection but also on liquid chromatography, Table 2 lists the expected charged state of the compounds at different pHs. Charge affects the peak shape or retention time by changing the polarity and, therefore, the distribution coefficient and potentially the loading capacity. Both DPP and hydrocortisone are neutral within the general pH range for reversed-phase separation. The chromatography of these compounds is essentially not affected by the pH or ionic strength of mobile phase. For the basic compounds glafenine and dipyridamole and the acidic compounds DY3 and chrysin, as well as for the zwitterionic compound labetalol, very different chromatographic behavior is expected with changes in pH and ionic strength.

3. The Advantage of a Universal Standard. Since the selected compounds have molecular weights ranging from 150 to 500, mass measurement of these compounds should indicate whether the mass spectrometer is within specification for typical pharmaceutical applications. To that end, the selected compounds should be able to ionize under the most commonly utilized ionization modes. To assess mass spectrometric performance, it is quite common to use compounds that are most amenable to a specific ionization mode. Therefore, a specific subset of compounds could be selected for each ionization mode. A "universal" mix that works for all conditions, in contrast, clearly has benefits with respect to simplicity and convenience.

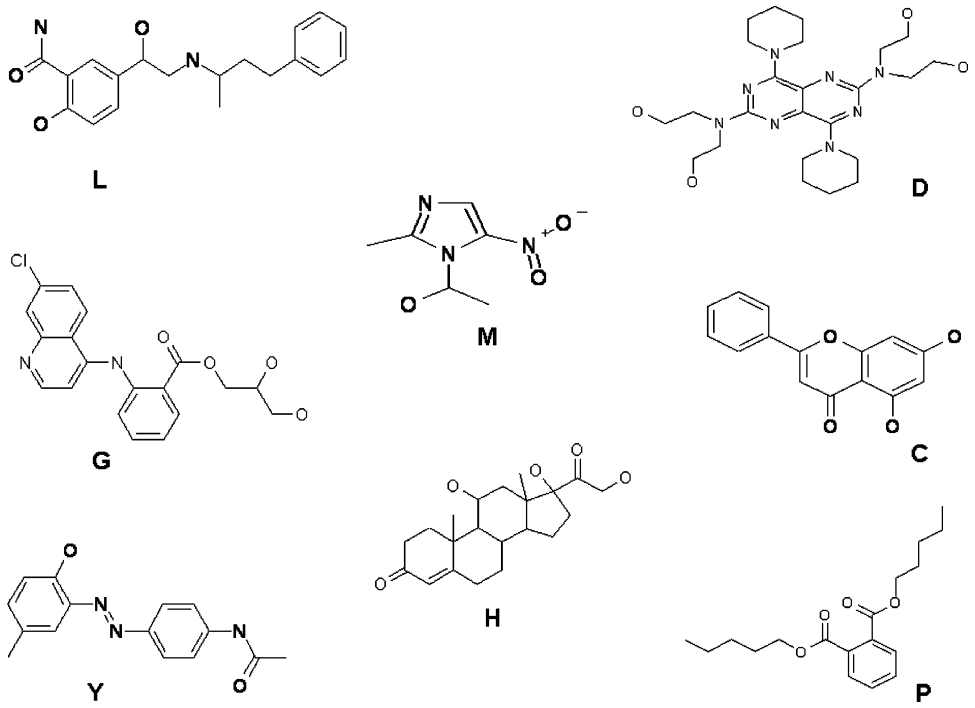

Figure 2. Structures of the selected compounds. Label as in previous table.

Table 2. Charge at Neutral, Acidic, and Basic Conditions

	acidic (pH 3)		neutral (pH 7)		basic (pH 10)	
compd, abbrev	pos	neg	pos	neg	pos	neg
di-*N*-pentyl phthalate, P	0	0	0	0	0	0
hydrocortisone, H	0	0	0	0	0	0
disperse Yellow 3, Y	0	0	0	0	0	1
chrysin, C	0	0	0	1	0	2
labetalol, L	1	0	1	0	0	1
glafenine, G	2	0	1	0	0	0
dipyridamole, D	2	0	0	0	0	0
metronidazole, M	1	1	1	1	1	1

Since the UV detector is always installed at the exit of the column, before the eluent is split into the ELS and MS detectors, it acts as a benchmark and can be used to corroborate the MS data. Therefore, if poor performance is observed in the mass chromatogram but not in the UV chromatogram, the cause of the problem can be located to the postcolumn split or mass spectrometer. Similarly, if poor performance is observed in only one ionization mode, the likely cause can be surmised because the same test mix has been used and can be correlated with data from other methods, detectors, or instruments. If a different test mix were to be used for different ionization modes and a reliable UV chromatogram were not available, then the data would generally be not comparable, and problem identification would be a more challenging task.

The fact that the universal standard is intended to highlight separation as well as detection system integrity has additional implications and advantages. In RP-LC, the elution of completely neutral molecules is determined only by the concentration of the organic component of the mobile phase using a given stationary phase. In contrast, the elution of charged molecules may be affected by factors such as pH or ionic strength of the mobile phase, as well. Comparing and tracking retention times for different types of molecules, including charged compounds, is an immediate check of the integrity of the mobile phase composition and assures a properly conditioned separation system.

Finding such a universal test mix to assess separation and detection system integrity can be challenging. To identify a small subset of compounds that could be used for this purpose, a pool of more than 100 compounds were not only identified and analyzed, but also were run under a number of different separation and ionization conditions. Separation as well as detection data were compiled in a database that now holds data for more than 5000 injections. This database can be expanded as additional separation modes or interesting compounds become available.

Monitoring the Detection System. The test mix should be observable using various detection techniques to monitor the detection system. Since the compounds for the test mix were selected to be UV and ELS active, their signal intensities can be monitored and compared from injection to injection.[10] With the LC/MS/UV/ELSD analysis, compound purity is based on the UV and ELSD chromatograms. The absolute signal response using these detectors is, therefore, important; however, the variation in signal between different molecules is generally not as great as for MS. In contrast, MS identifies the target compound, and if no signal is found, the sample is usually discarded at that stage. For this reason, the following discussion will focus more on the MS signal, because signal variation in MS is more difficult to predict and can vary significantly on the basis of molecular properties.

1. Ion Chromatograms for Different Ionization Modes. Figure 3 shows the BPI (base peak intensity) chromatograms of the test mix eluted with acetonitrile and water modified with 0.1% formic acid. Four chromatograms highlighting the differences in sensitivity between the ionization modes are shown. For example, electrospray is better for some polar

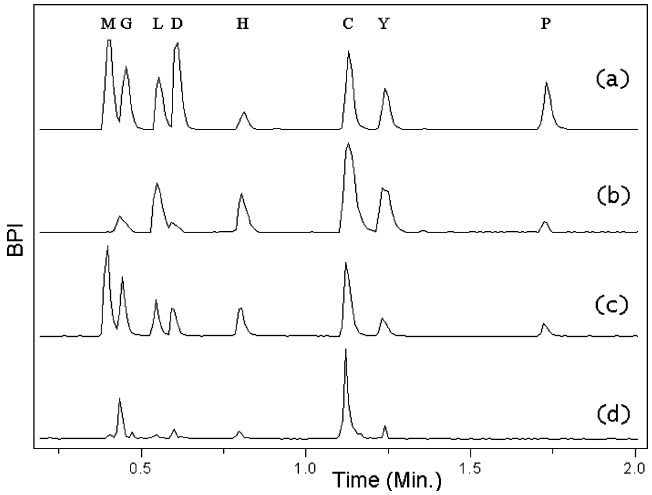

Figure 3. BPI of standard compounds in various ionization modes with an acidic modifier. Both mobile phases A and B contain 0.1% formic acid. (a) = ES^+, (b) = ES^-, (c) = $APCI^+$, (d) = $APCI^-$.

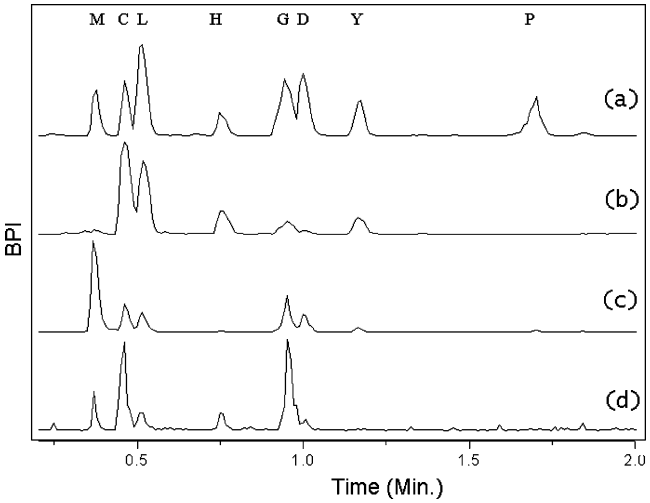

Figure 4. BPI of standard compounds in various ionization modes with a basic modifier. Both mobile phases A and B contain 10 mM ammonium hydroxide. (a) = ES^+, (b) = ES^-, (c) = $APCI^+$, (d) = $APCI^-$.

compounds,[11] but APCI is more sensitive or robust for some other compounds.[12,13] Additionally, positive mode is usually better for proton acceptors, but negative mode is usually better for proton donors.[14] From Figure 3 with positive electrospray, all the compounds had good signal-to-noise ratios. With negative electrospray, all compounds ionized well except for metronidazole and DPP, which are the most hydrophilic and the most hydrophobic compounds, respectively. The signal for dipyridamole was weak relative to others, but it was strong enough to be used for verifying mass accuracy. The last peak matched the retention time of DPP, but the spectrum indicated that there was actually a coeluting impurity, emphasizing the advantage of testing the LC/MS instrument as a whole. With positive APCI, most compounds had good signal intensity, as well. Negative APCI response was the lowest, as compared with the other modes, but most of the compounds were still detectable, and the sensitivity to metronidazole seemed to be higher than in negative electrospray. As in negative electrospray, the detectable compounds lost one proton during ionization, except for DY3, for which the formula mass was observed as the base peak. Since the identity of DY3 was confirmed with the other three modes as the $[M \pm 1]^{\pm}$ ion, the possibility of a coeluting impurity of isobaric molecular weight that ionized only in this mode is small. Additionally, the same effects were observed using orthogonal separation methods. DY3 was believed to ionize by an electron-capture mechanism[15−17] instead of the proton transfer that is usually encountered under negative APCI conditions.

2. Effects of Different Modifiers on Ionization. The efficiencies of ESI and APCI are affected by the type and concentration of modifier. Figure 4 shows the ion chromatograms (base peak intensity) for the test mix using ammonia rather than formic acid as the modifier for the mobile phase. As in Figure 3 (formic acid), all the compounds had good signal-to-noise ratios, but their intensities varied relative to each other. The variation in intensity might be argued to be solely dependent on the different acid/base properties of the compounds, but since ionization can also be affected by factors such as the percentage of organic solvent in the mobile phase and the availability of protons, it could be rather difficult to identify the major factor. The only exception was with hydrocortisone. Using positive APCI, the signal of hydrocortisone seemed to be suppressed with ammonia, as compared to formic acid. Since hydrocortisone is neutral and eluted with the same percentage of acetonitrile in both experiments, the signal suppression might be due to the competition for proton donors with ammonia in the APCI process.[18,19]

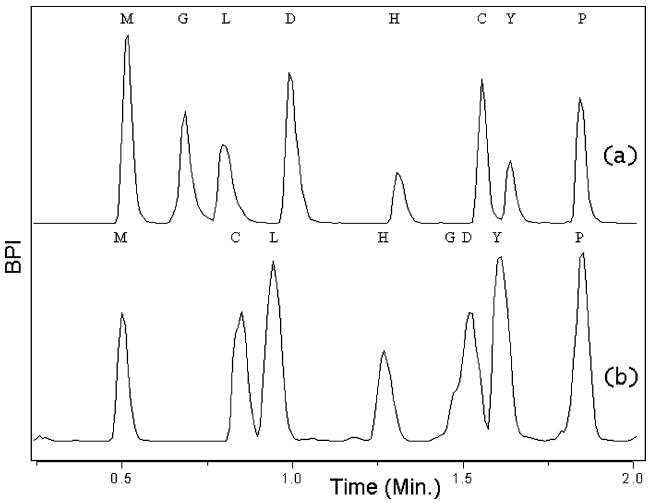

Figure 5. BPI of standards in ES^+ with methanol solvent. The solvent for mobile phase B is methanol instead of acetonitrile. Both A and B contain (a) 0.1% formic acid and (b) 10 mM ammonium hydroxide.

3. Effects of Different Organic Solvents on Ionization. Since ionization is affected by organic composition of the eluent, Figure 5 shows the ion chromatogram (base peak intensity) for the same experimental conditions as Figures 3 and 4, except that methanol instead of acetonitrile was used as the organic solvent in the mobile phase. Only the data for positive electrospray are shown, since no significant additional differences in ionization were observed under all the other conditions. With negative APCI, the formula mass was observed under the base peak for DY3, as before.

Both acetonitrile and methanol were tested because they are commonly used chromatography solvents of different

polarity and H-bonding abilities. Methanol and acetonitrile are known to have different selectivity as elution solvents.[20,21] This may be because of their different solventochromic properties[22] or their different interactions with the stationary phases.[23] As a result, changing acetonitrile and methanol from one to another should be one of the early steps in a strategy proposed for fast method development.[24] In addition to different selectivity, methanol is a nitrogen-free solvent, which makes it compatible with nitrogen-specific detectors, such as CLND.[25−27] On the other hand, mobile phases with methanol might be perceived to be potentially too reactive for some analytes and too viscous for some applications.

Thus, a set of compounds was established that was applicable for ESI and APCI ionization modes. This provides the convenience of monitoring the performance of the system with a single mix for different conditions. In addition, the possibility of cross-referencing among the various chromatograms helps diagnose any point of failure. The previous experiments were carried out under the optimal conditions for these instruments, and a more detailed monitoring of the mass spectrometer and the interface is possible. It has been reported that the breakdown curves of tuning compounds can be used to standardize the collision energy and gas pressure for collision-induced dissociation.[28−30] Using the same principle, compounds can be selected that fragment in-source to various extents at different cone voltages. These spectra can be monitored as a marker for instrument status. For simplicity, however, these aspects are not considered in this work.

Monitoring the Separation System. In the following discussion, a linear gradient from 5 to 95% B is assumed for the elution method. A generic gradient is usually the starting point for method development of unknown samples. In addition, an LC/MS system will usually perform well for other types of gradients or isocratic elution conditions if it does so for a generic gradient.

1. Effects of Flow Rates and Gradient Profiles. To detect a potential variation in flow pattern, the retention times of test compounds need to cover the entire range of the gradient elution. As shown in Figures 3, 4, and 5, the retention times for the selected compounds are spread across the time window of the gradient (0−1.8 min) using either acetonitrile or methanol as solvents and ammonia or formic acid as modifiers. The compounds were selected on the basis of their respective retention times under these conditions by mining the database and looking to represent the physicochemical diversity with regard to molecular weight and polarity, as discussed earlier.

To simulate the impact of a flow variation, due to (for example) a leak or pump malfunction, the nominal flow rate was reduced by 7%. The impact of this change is shown in Figure 6. Under the reduced flow rate, the retention times for all the compounds were shifted by 0.02−0.03 min. This seems like a small change, but given the high reproducibility of retention times and the short elution time that is achieved under these ballistic gradient conditions, it is clearly a deviation and can be easily picked up if the data are compared to a properly run standard. The elution pattern remains largely unaffected by such a change, indicating that

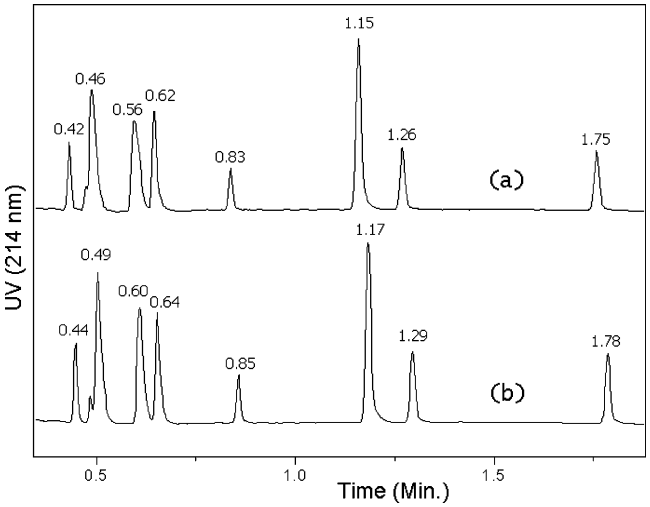

Figure 6. Elution of standards at different flow rates. The retention times are labeled on top of the peaks. Flow rates for various chromatograms are (a) = 3.0 and (b) 2.8 mL/min.

absolute retention times rather than resolution should be monitored to identify such a system deviation. Changing the overall flow rate in a gradient produces less impact than changing the relative composition of the gradient at any given time. This frequently occurs when one of the dual pumps has a malfunction. The effect of such a change can be simulated by running a convex or concave gradient versus a linear gradient, as shown in Figure 7. Clearly, such a change produces an even more dramatic impact and is reflected in a change in the entire elution pattern. The impact is different at various parts of the gradient. This supports our intent to have the elution of test compounds cover the entire range of the gradient so that system inconsistencies can be recognized and are not overlooked if only a single compound or a smaller set is monitored that does not cover the entire elution range.

2. Effect of pH. The pH of the mobile phase can be another source of variability for the separation system. This variation may be introduced by impurities in the reagents, other contamination or operational errors. For high-pH mobile phases that use ammonia or other basic modifiers, absorption of carbon dioxide over time is probably the most prominent reasons for a change in pH. Changes in the pH of the mobile phase can affect the chromatography, especially if the sample is ionizable, and need to be monitored.

When the retention times in Figure 3 are compared with Figure 4, it is seen that several compounds were eluted at approximately the same time, even though the pH of the mobile phase was different (pH 2 vs pH 9), whereas the others eluted at significantly different times. The same can be observed by comparing the top and bottom chromatograms in Figure 5. This is consistent with the computational results in Table 2. The fact that a compound is charged affects not only the ionization efficiency but also the chromatographic behavior, such as retention time and peak shape. Most prominently, charge affects the logD, which has a direct correlation to the retention time in gradient elutions. The larger the logD, the longer the retention time. Figure 8 shows the calculated logD at different pHs for some of the test mix compounds. The variation in logD with pH can be

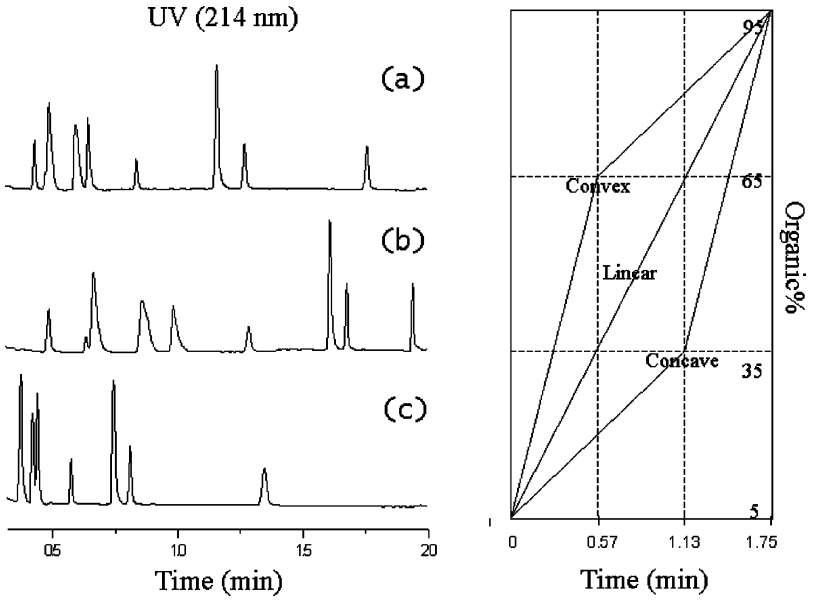

Figure 7. Elution of standards with different gradients. Gradients are (a) linear, (b) concave, and (c) convex.

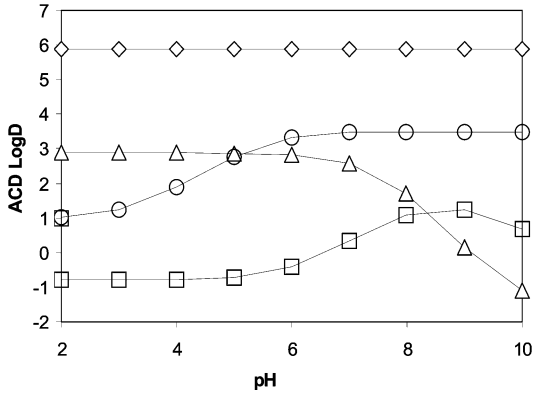

Figure 8. cLogD of selected compounds at various pHs. ◇, DPP; ○, glafenine; □, labetalol; △, chrysin.

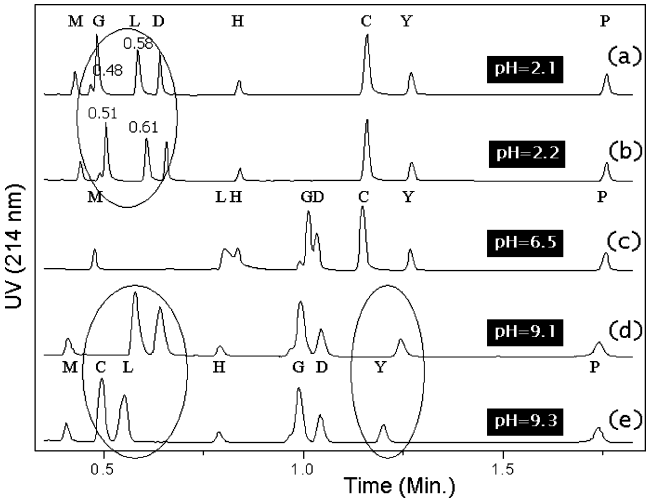

Figure 9. UV [214-nm] chromatograms of the test mix eluted at different pHs. The pH for mobile phase A and retention time for some peaks are labeled on the chromatograms. The modifiers for mobile phases A and B are (a) 0.1% (∼20 mM) formic acid in A and B; (c) 1 mM ammonium acetate in A; (e) 10 mM ammonium hydroxide in A and B. The mobile phases for b and d were prepared from those for a and e, respectively. The mobile phase B is the same, whereas ammonium acetate was added to 1 mM in mobile phase A.

used as an indicator of how sensitive the retention time of the compound is toward a variation in pH of the mobile phase. Figure 9 shows the difference in retention times as the pH of the mobile phase changed by 0.1−0.2 units. At high pH, the retention times of chrysin and labetalol decreased (0.08 min) as the pH increased by 0.2 units. At low pH, the change in retention time with a pH increase of 0.1 unit was less obvious but consistent for glafenine, labetalol, and dipyridamole (0.03 min). Since an aqueous solution of 1 mM ammonium acetate had a pH around 6.5, the middle chromatogram shows that the test mix had a different elution pattern at approximately neutral pH when compared with the acidic and basic conditions. Figure 9 also shows that the retention times for metronidazole, hydrocortisone, and DPP are generally consistent across different pHs, especially on the same column. These compounds may, thus, serve as the reference points for the other compounds and indicate all other aspects of the system are working, for example, flow rate and gradient profiles. These results qualitatively matched the computed logD results.

3. Effect of Ionic Strength. Elution solutions containing very low concentrations of electrolytes can be used on purpose sometimes; for example, to increase the MS ionization. Charge interaction is prominent in these solutions, which have low ionic strength. When a compound is charged, its retention time and peak shape[31,32] may be affected by ion exchange[33] in the process of separation.

Figure 10 shows the chromatograms of the test mix eluted with a mobile phase of low ionic strength. When unmodified water and acetonitrile was used, neutral compounds had good peak shapes, but the peak shapes for the basic and zwitterionic compounds were very poor. Adding an electrolyte is known to improve the peak shape.[7] With only a minor increase in ionic strength, the peak shapes were dramatically improved for basic compounds. Although not shown, the same effect was observed experimentally when sodium chloride was used instead of ammonium acetate, discounting the effects of the weak buffering capacity of ammonium acetate at this pH. Thus, the broadened peak at low ionic

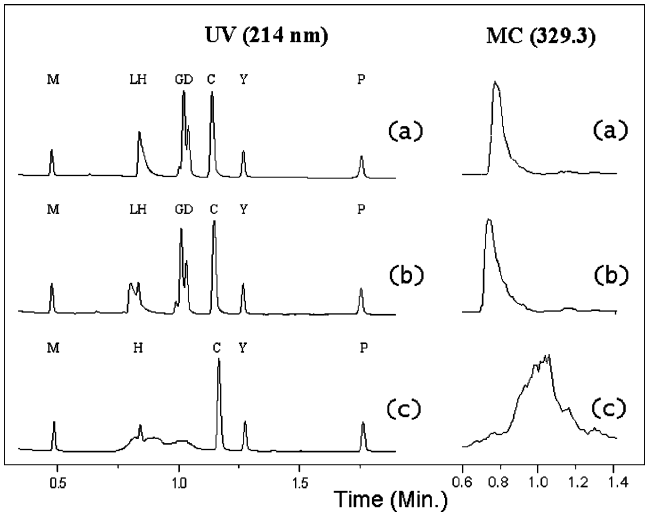

Figure 10. Test compounds eluted with mobile phases of different ionic strengths. Left side is the UV [214-nm] chromatogram; right side is the extracted ion chromatogram for labetalol [329.3]. Ammonium acetate was added in A with final concentrations of (a) 2; (b) 1; (c) 0 mM (no additives).

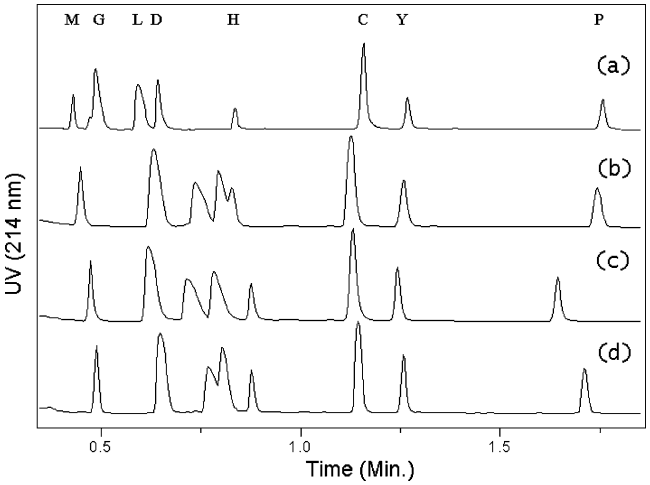

Figure 11. Test mix eluted from different columns. Columns are (a) Sunfire-C18, (b) Xterra-C18, (c) Zorbax SB-C8, and (d) ACE-C8.

strength may be due to the decreased loading capacity for the adsorbed charged analytes.[34,35] Figure 10 shows that electrolytes at concentrations as low as 1 mM in aqueous phase might improve the peak shapes for charged analytes. Experimentally, the pH of the aqueous phase was measured as 6.5, but it was not strictly controlled.

4. Effect of Stationary Phase. Many test mixes have been suggested to characterize stationary phases, and some of them can be used to indicate the degradation of a column.[7] However, they are of limited applicability for our approach, because they do not monitor the change in the mobile phase and the performance of the detectors at the same time, or their elution methods are not compatible with MS. The test mix was initially developed on two stationary phases, which were widely used within our laboratory. It is well understood that the chromatographic behavior of a molecule is determined by its interaction with the mobile phase and stationary phase together. Elution times and selectivity for some of the test compounds are, therefore, expected to respond to a change of stationary phase.

Figure 11 shows the responsiveness of the compounds to some of the different stationary phases commonly used for high-throughput analysis and method development. Different stationary phases are sometimes chosen to resolve coeluting chemicals. To maximize the difference in selectivity, stationary phases may use different ligands, such as phenyl, cyano, or perflouro derivatives. These ligands are different in their hydrophobicity, steric resistance, and electron donating or accepting abilities and, thus, have different selectivity toward different types of molecules.[36−39] Stationary phases nominally composed of the same ligands may have differences in selectivity, as well, due to the different steric resistance, hydrogen bonding, and cation-exchange capacity.[40] This is what can be observed from Figure 11. Since all the columns are C18 columns, the change of selectivity or peak shape must be due to the different silica gel and binding chemistry. The elution orders for the compounds are the same for all the columns; however, their relative retention times or selectivity is different. The most obvious changes in the retention times can be observed for the basic compounds, glafenine, labetalol and dipyridamole. These compounds also have the largest differences in peak shapes from column to column. The Sunfire column has the sharpest and the most symmetric peak shape for the basic compounds, followed by the ACE column, whereas the Zorbax and Xterra columns have broader and less symmetric peak shapes. This highlights the advantage of the database approach for the selection of test compounds. All that is needed is to identify which parameter is to be measured with a compound and which characteristics the compound should have. If a compound cannot be resolved on a new stationary phase that is being evaluated, a replacement compound can quickly be identified and the mixture adjusted to be able to provide the same level of information and system suitability data for any separation method.

Conclusion

A set of compounds suitable for performance monitoring of an LC/MS/UV/ELSD method was selected. The injection of a mixture of these compounds under routine operating conditions is able to provide comprehensive characterization of the separation and detection system's integrity. Changes in the spectra or chromatograms of the mix from previously observed results highlight a potential suboptimal status of the system, such as interface failure, poor mass accuracy, change in mobile phase, flow rate, and profile, as well as degradation of the stationary phase. The mix is applicable to multiple ionization modes for mass spectrometry in conjunction with the most common mobile phases for reversed-phase separations. Selection of the test compounds from a large set of samples that were evaluated was based on interrogation of a database. When different separation conditions are to be explored, a quick replacement of an individual compound is easily possible. Injection of the test mix is a powerful tool to monitor LC/MS system integrity for the routine analysis of druglike compounds in a high-throughput environment.

References and Notes

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