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Expediting the Method Development and Quality Control of Reversed-Phase Liquid Chromatography Electrospray Ionization Mass Spectrometry for Pharmaceutical Analysis by Using an LC/MS Performance Test Mix

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Mass spectrometry combined with liquid chromatography (LC/MS) has become an important analytical methodology in both pharmaceutical and biomolecule analyses. LC/MS, especially with reversed-phase HPLC (RP-LC), is extensively used in the separation and structural identification of pharmaceutical samples. However, many parameters have to be considered when a new LC/MS method is developed for either separation and structural analysis of unknown mixtures or quantitative analysis of a set of known compounds in an assay. The optimization of a new LC/MS method can be a time-consuming process. A novel kit—LC/MS performance test mix—composed of aspartame, cortisone, reserpine, and dioctyl phthalate has been developed to accelerate the process of establishing a new RP-LC/MS method. The LC/MS mix makes the evaluation and validation of an LC/MS method more efficient and easier. It also simplifies the quality control procedure for an LC/MS method in use.

Electrospray is a soft ionization method[1] suitable to analyzing polar, thermally unstable compounds. Since electrospray ionization mass spectrometry (ESI-MS) was introduced into analytical chemistry as a new methodology for the structural identification of biomolecules and pharmaceutical compounds[2−4] about 10 years ago, it has found a tremendous number of applications in biotechnology and pharmaceutical industries. Since electrospray can serve as an interface to transfer analytes from the mobile phase of reversed-phase liquid chromatography (RP-LC) to a mass spectrometer,[5] RP-LC/MS has become an effective method for fast separation and structural elucidation of compounds in a mixture. The application of RP-LC/MS in pharmaceutical analysis ranges from drug discovery to drug development.[6−10] There are methods to evaluate separation capability and selectivity of RP-LC columns as well as methods to check the performance of a commercial mass spectrometer.[11] However, there is lack of a general method for evaluating the performance of both the chromatography and mass spectrometry of RP-LC/MS simultaneously. In this research, we developed a method, which involves a mixture useful for judging the separation capability of RP-LC and ion detection of ESI-MS simultaneously.

The mixture, LC/MS performance test mix (LPTM), should be appropriate to both RP-LC and ESI-MS. The LPTM would contain compounds with various polarities to represent drug molecules encountered in pharmaceutical analysis. Because octanol−water partition coefficients (Log *P*) could be correlated to membrane permeation properties,[12] the calculated Log *P* (cLog *P*) of a compound was considered in the design of LPTM. Due to the possible diverse polarities in the samples such as those from drug discovery,[13,14] the LPTM would ideally contain a very hydrophilic compound and a very hydrophobic compound. To maintain the simplicity, it would also contain two compounds with very similar polarities to monitor the selectivity of the RP-LC column.

As mass spectrometry is concerned, all the compounds in LPTM should have molecular weights comparable to that of analytes in LC/MS analysis. Since ESI-MS can be operated in either positive ion mode or negative ion mode, LPTM would ideally be usable in both ion detection modes although the switch between two polarities in a mass spectrometer is usually not a difficulty. Therefore, four organic compounds with different

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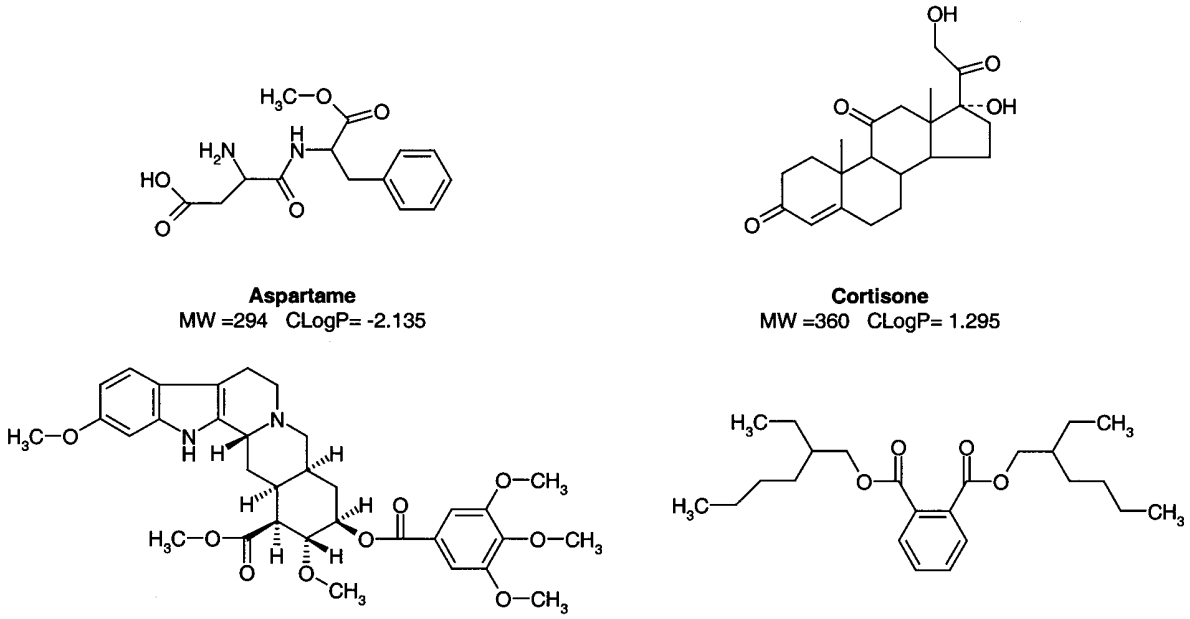

Figure 1. LC/MS performance test mix constituents. LPTM is composed of four organic compounds, aspartame, cortisone, reserpine, and dioctyl phthalate, which have different masses in the range of MW = 200−700 and different polarities in the range of cLog $P = -2$ to $+8$.

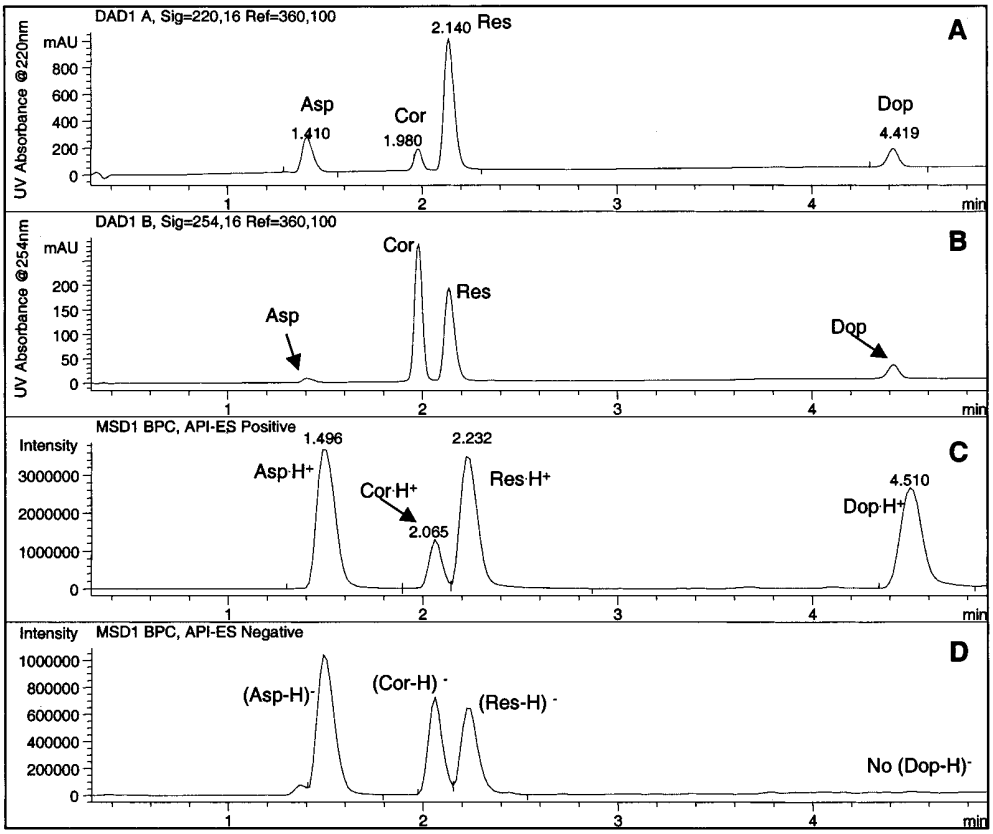

Figure 2. LPTM use in reversed-phase LC/MS. Aspartame (Asp), cortisone (Cor), reserpine (Res), and dioctyl phthalate (Dop) have UV responses at the wavelengths commonly used in HPLC UV detection: (A) a UV chromatogram of LPTM at $\lambda = 220$ nm; (B) a UV chromatogram of LPTM at $\lambda = 254$ nm. The four compounds give mass spectral signals in electrospray ionization. (C) A mass spectral base peak chromatogram of protonated molecular ions or sodium adducts formed in positive ion detection mode; (D) a mass spectral base peak chromatogram of deprotonated molecular ions formed in negative ion detection mode. Conditions: (2 μL of LPTM was loaded on a 30 mm × 3 mm × 3 μm Polaris2000 C18 column installed in the LC/MS system of HP1100 MSD. Mobile phases, (A) 0.05% formic acid in water (v/v) and (B) 0.05% formic acid in acetonitrile (v/v). Gradient, 5−90% B in 3 min. Flow rate 0.6 mL/min; and column temperature 40 °C.)

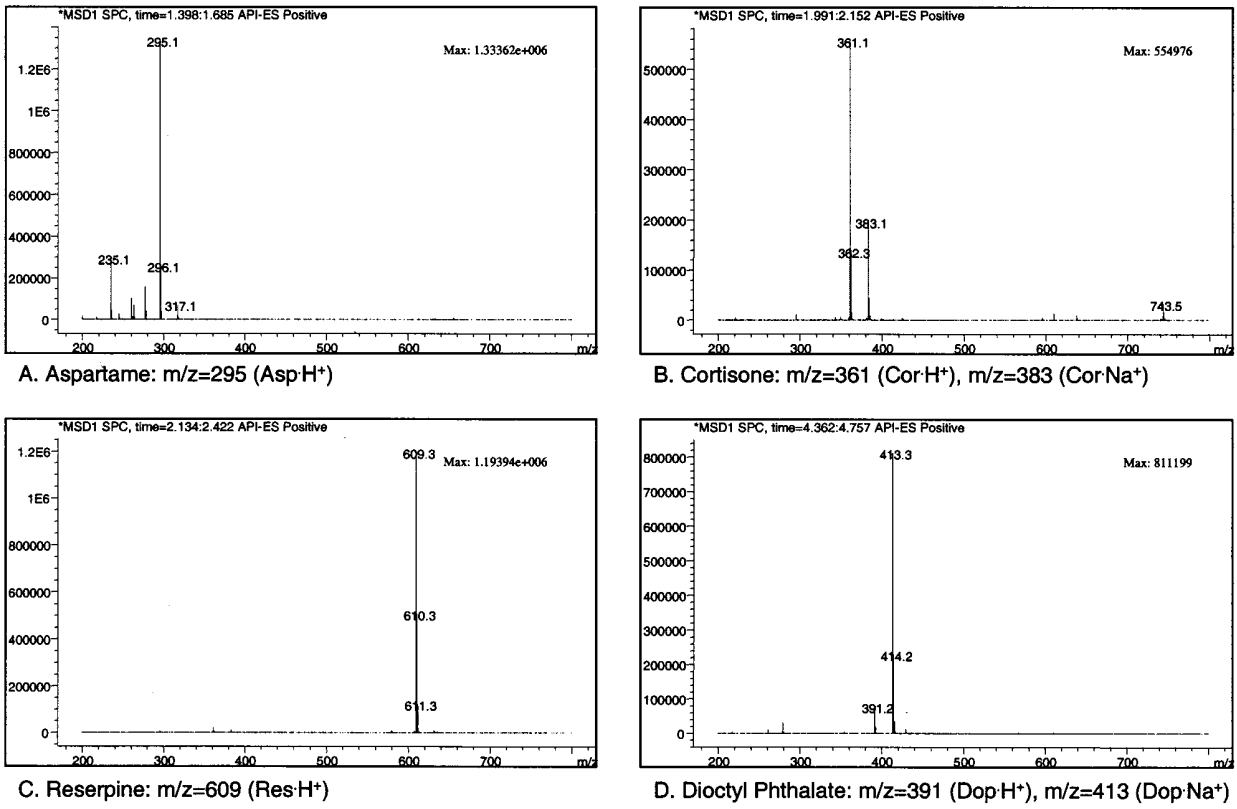

Figure 3. Typical mass spectra of four components in the LPTM: (A) aspartame; (B) cortisone; (C) reserpine; (D) dioctyl phthalate.

hydrophobicities and structural diversity were selected to make up LPTM.

EXPERIMENTAL SECTION

Instrumentation. Experiments were conducted on two types of mass spectrometers, i.e., quadrupole ion analyzer HP 1100 MSD (model G1946A) from Hewlett-Packard and ion trap Finnigan LCQ DECA from Finnigan (San Jose, CA). Both mass spectrometers were connected to an HPLC system individually for on-line separation purposes. The LC/MS system for the HP 1100 MSD consisted of a vacuum degasser (model G1322A), a binary pump (model G1312A), an autosampler, a thermostated column compartment (model G1316A), and a diode array detector (model G1315A) equipped with a long-life deuterium lamp. All devices were from Hewlett-Packard except for the autosampler, which was a CTC PAL system from LEAP Technologies (Carrboro, NC). The LC/MS system of the HP1100 MSD was controlled by ChemStation Rev. A07.01. The autosampler was controlled by Cycle Composer V 1.4.0. from LEAP Technologies. The autosampler and mass spectrometer were controlled by contact-closure. The LC/MS system for the Finnigan LCQ DECA was a hybrid system containing a degasser, a binary pump, and a thermostated column compartment from Hewlett-Packard with the same models as for HP1100 MSD, a CTC miniautosampler from LEAP Technologies, and a Thermoquest UV6000LP UV detector. The system was operated by Xcalibur V.1.1 software from Finnigan Corp.

Reversed-Phase HPLC Columns. Polaris2000 C18 columns packed with 3-μm particles, 30 mm × 3 mm (i.d.) or 50 mm × 3 mm (i.d.), were obtained from MetaChem Technologies (Torrance, CA). Betasil C18 column packed with 3.5-μm particles, 30 mm × 2.1 mm (i.d.), was obtained from Keystone Scientific (Bellefonte, PA). Luna C18 packed with 3-μm particles, 30 mm × 2 mm (i.d.), was obtained from Phenomenex (Torrance, CA). Cartridge columns Zorbax SB-C8 and Zorbax SB-C18 were obtained from MacMod (Chadds Ford, PA). All Zorbax columns in the study were of 3.5 μm in particle size, 30 mm in length, but different internal diameters as described in the text. Targa C18 30 mm × 3 mm packed with 5-μm particles was obtained from Higgins Analytical, Mountain View, CA.

Chemicals. Aspartame (*N*-aspartylphenylalanine methyl ester), cortisone, reserpine, and dioctyl phthalate were reagent grade chemicals from Aldrich Chemical Co. (Milwaukee, WI). Trifluoroacetic acid was a reagent grade chemical obtained from Sigma Chemical Co. (St. Louis, MO). Water, methanol, and acetonitrile were HPLC grade reagents from AlliedSignal, Burdick & Jackson (Muskegon, MI). Formic acid was HPLC grade from EM Science (Gibbstown, NJ). The HPLC mobile phases were prepared by adding 0.05% (v/v) formic acid in water or in acetonitrile.

LC/MS Performance Test Mix. Pure methanol was used to prepare 3528 μg/mL aspartame and 1080 μg/mL cortisone solutions. Methanol containing 0.05% (v/v) formic acid was used to make an 1824 μg/mL reserpine solution. After mixing 1 mL from each of these solutions to make a 3-mL solution containing 1176 μg/mL aspartame, 360 μg/mL cortisone, and 608 μg/mL reserpine, 1.2 μL of dioctyl phthalate was added to the 3-mL mixed solution, which then became the LPTM with a molar ratio 4:1:1:1 for aspartame, cortisone, reserpine, and dioctyl phthalate, respectively.

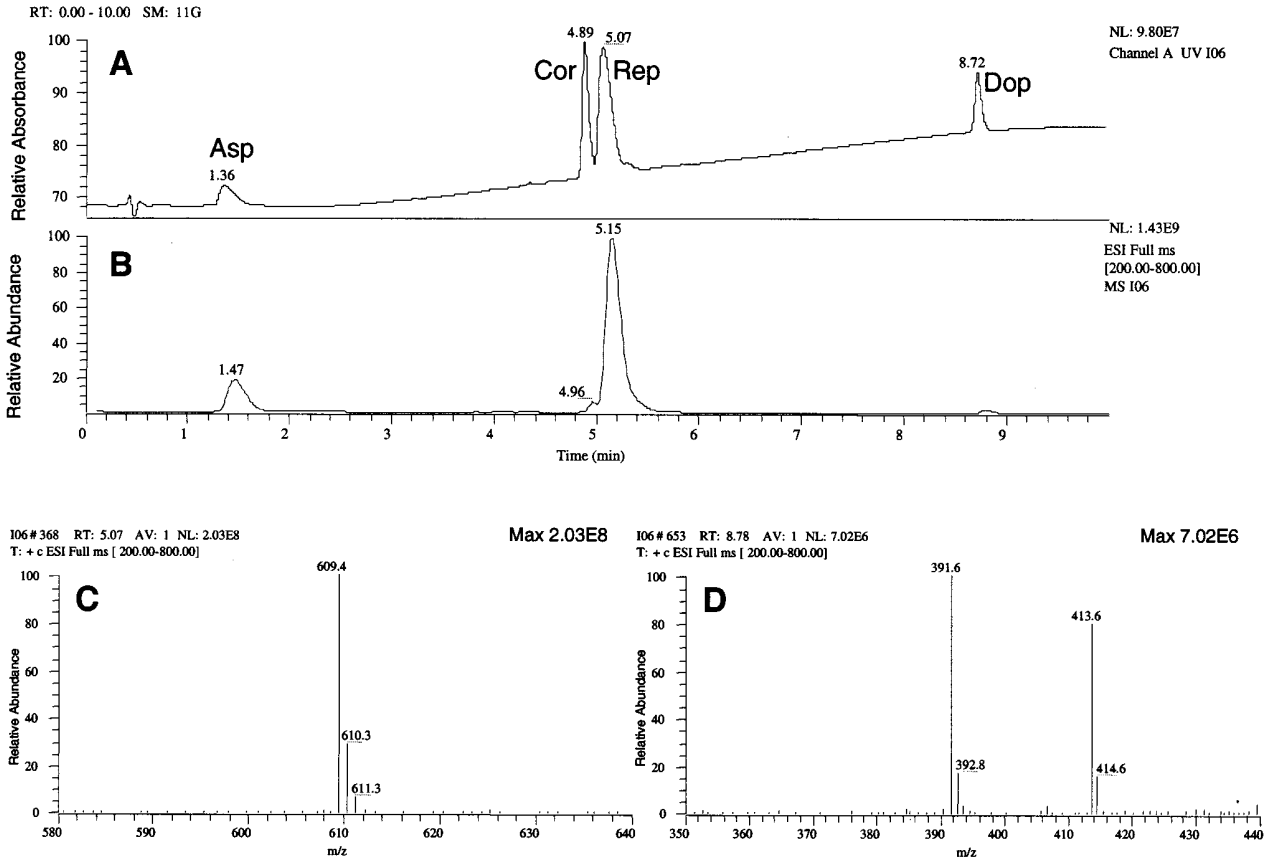

Figure 4. An equivalent of 0.5 μL of LPTM separated by a 30 mm $\times$ 2.1 mm, 3.5-μm Zorbax C8 column and analyzed by the LC/MS of Thermoquest PDA UV detector and Finigan LCQ DECA. (A) UV chromatogram of LPTM; (B) MS chromatogram of LPTM. (LPTM was first diluted with methanol by a factor of 1, and then 1 μL of the solution was loaded on the column); (C) mass spectrum of reserpine peak; (D) mass spectrum of DOP peak.

Reversed-phase test mix containing 5 μg/mL uracil, 700 μg/mL phenol, 600 μg/mL *N*,*N*-diethyl-*m*-toluamide (Deet), and 4000 μg/mL toluene in acetonitrile/water (65:35 v/v) was purchased from Aldrich.

Column test mix containing uracil, acetophenone, methyl benzoate, toluene, and naphthalene was provided by MetaChem Technologies, Inc.

Fmoc-Lys-Boc on Wang resin (0.83 mmol/g) was obtained from NovaBiochem (San Diego, CA). The Fmoc-Lys from a single bead could be obtained free in solution phase by cleavage with trifluoroacetic acid. A single bead was first picked into a 8-mm conical vial purchased from National Scientific Co. (Lawrenceville, GA), and then 25 μL of acid [TFA/H_2O 95:5 (v/v)] was added to the vial. After the bead was immersed in the acid solvent for 1 h, the acid was removed from the vial by vacuum evaporation and the compounds were redissolved in acetonitrile.

A Typical Procedure To Use LPTM. Prior to the use of the LPTM, the LC/MS system was checked to meet the sensitivity and mass accuracy specifications defined by its manufacturer. The diode array detector (DAD) was calibrated and passed tests such as the DAD intensity test, dark current test, and cell test conducted with the diagnosis procedure of ChemStation for HP1100 system. The Thermoquest UV6000 LP detector has similar diagnostics to verify wavelength accuracy and monitor lamp intensity. The HP1100 MSD was calibrated with ESI tuning solution obtained from Agilent Technology (Palo Alto, CA). The Finnigan LCQ DECA was calibrated with PPG standard provided by Finnigan.

The LPTM was used to evaluate an LC/MS method. For generic methods as are used in open access mass spectrometry or high-throughput quality control applications, the LPTM could also provide a sensitivity check for the methods.

Calculated Log *P* (cLog *P*). The calculation of partition coefficients was conducted with software ISIS/Desktop 2.3 provided by MDL Information Systems, Inc. (San Leandro, CA).

RESULTS AND DISCUSSION

I. Features of LC/MS Performance Test Mix. Four organic molecules were chosen to compose the LPTM, i.e., aspartame, cortisone, reserpine, and dioctyl phthalate, shown in Figure 1. These compounds have extremely different polarities as characterized by cLog *P*. The polarity differences suggest that they maybe useful as markers in a chromatogram illustrating the separation capability of a RP-LC column under specific chromatographic conditions. Aspartame is a hydrophilic dipeptide, which would be poorly retained on a RP-LC column in a high-organic mobile phase. Both cortisone and reserpine have similar polarities and they may test the selectivity of the column. Dioctyl phthalate is an ester with extremely high hydrophobicity and should be eluted late from a RP-LC column or by a mobile phase with a high concentration of organic solvent. Dioctyl phthalate could function as a marker to express the end of separation in the

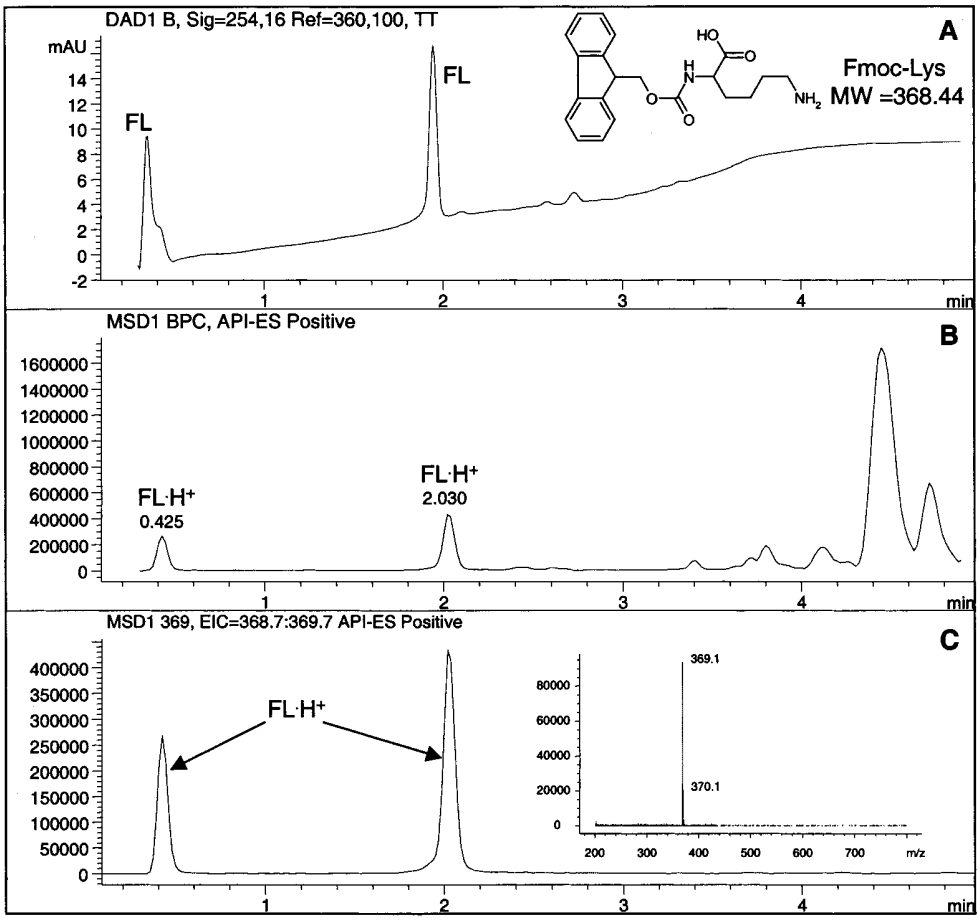

Figure 5. UV and MS chromatograms of Fmoc-Lys from a single bead of Wang resin loaded on a 30 mm × 3 mm × 3 μm Polaris2000 C18 column in 20 μL of acetonitrile. Fmoc-Lys (FL) cannot be effectively retained on the column due to the large volume of organic solvent in the sample and fast gradient: (A) UV chromatogram of Fmoc-Lys at $\lambda = 254$ nm; (B) MS chromatogram of Fmoc-Lys obtained in positive ESI/MS; (C) extracted ion chromatogram (EIC) of the protonated Fmoc-Lys ($FL{\cdot}H^+$ $m/z = 369$) peak detected in MS chromatogram B with an inset of the mass spectrum of the protonated Fmoc-Lys.

chromatogram of a mixture because it would be uncommon to find an organic molecule among pharmaceutical compounds with a longer retention time than dioctyl phthalate.

A typical chromatogram of LPTM is demonstrated in Figure 2. In this example, the loading of four components in LPTM is 2.35, 0.72, 1.22, and 0.78 μg for aspartame, cortisone, reserpine, and dioctyl phthalate, respectively. The four compounds in the mixture have UV absorbance at the wavelengths normally used in pharmaceutical analysis (220 nm in Figure 2A and 254 nm in Figure 2B). While aspartame and reserpine favor shorter UV wavelength, longer wavelength gives cortisone better sensitivity.

All four components in LPTM have their molecular weights in the range of 200−700 amu, which are in the molecular weight range of most small-molecule drugs. In electrospray ionization with a mass spectrometer in positive ion detection mode, aspartame, cortisone, and reserpine will give protonated molecular ions, but dioctyl phthalate would form sodium adducts in addition to protonated molecular ions as shown in Figure 2C. A typical mass spectrum for each of the components in LPTM is demonstrated in Figure 3. When the mass spectrometer is switched to negative ion detection mode, aspartame, cortisone, and reserpine but not dioctyl phthalate will show deprotonated molecular ions signal as shown in Figure 2D. Therefore, the LPTM could be utilized to check the performance of a mass spectrometer as well as the performance of a RP-LC column in LC/MS. In the experiment mentioned above, the LC/MS instrument was equipped with UV detector in addition to the mass spectrometer. If there were no UV detector, LPTM could still be useful to optimize or check LC separation with mass spectrometric detection.

It should be stressed that the observed ion intensities of four compounds involved in the LPTM might vary with the operational settings on different mass spectrometers. When the mass spectrometer is optimized to favor the detection of a specific ion species, the ion intensity ratio of these four compounds could be extraordinary. An example is given in Figure 4, which displays an LC chromatogram of the LPTM collected on a mass spectrometer optimized for the detection of reserpine. The reserpine ion peak obviously dwarfed the ion peaks of the other three components even though they also provided enough sensitivity judged by the fact that only an equivalent of 0.5 μL of LPTM was loaded onto the LC column. In practice, this feature of LPTM can monitor the change of mass spectrometric conditions in operation.

II. A Comparison of LC/MS Performance Test Mix with Other Types of Reversed-Phase HPLC Performance Check Mixes. A number of small organic compounds have been used in the evaluation of RP-LC columns.[11] For example, toluene (MW

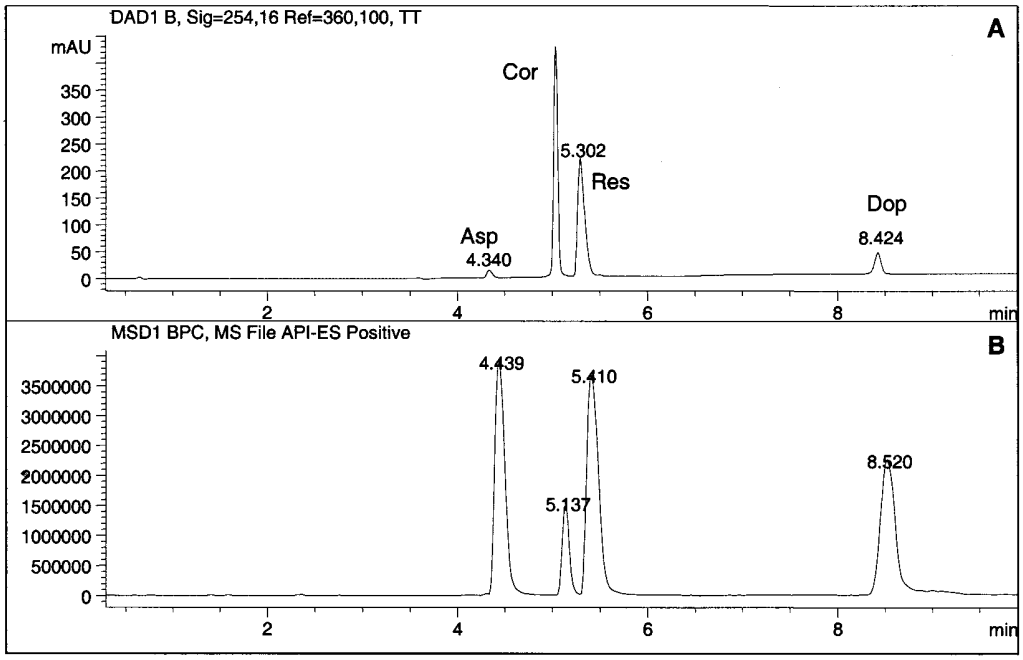

Figure 6. Development of a new RP-LC/MS method to retain the hydrophilic analyte but also elute the hydrophobic analyte with the assistance of LPTM. Conditions: LC column, Polaris 2000 C18, 50 mm × 3 mm × 3 μm; mobile phases, (A) 0.05% formic acid in water (v/v); (B) 0.05% formic acid in acetonitrile (v/v); gradient, 0% B for 2 min, 0−90% B in 4 min, then hold 90% B for 4 min; flow rate, 0.5 mL/min; column temperature, 40 °C. (A) UV chromatogram of LPTM at λ = 254 nm; (B) MS chromatogram created by protonated molecular ions or sodium adducts.

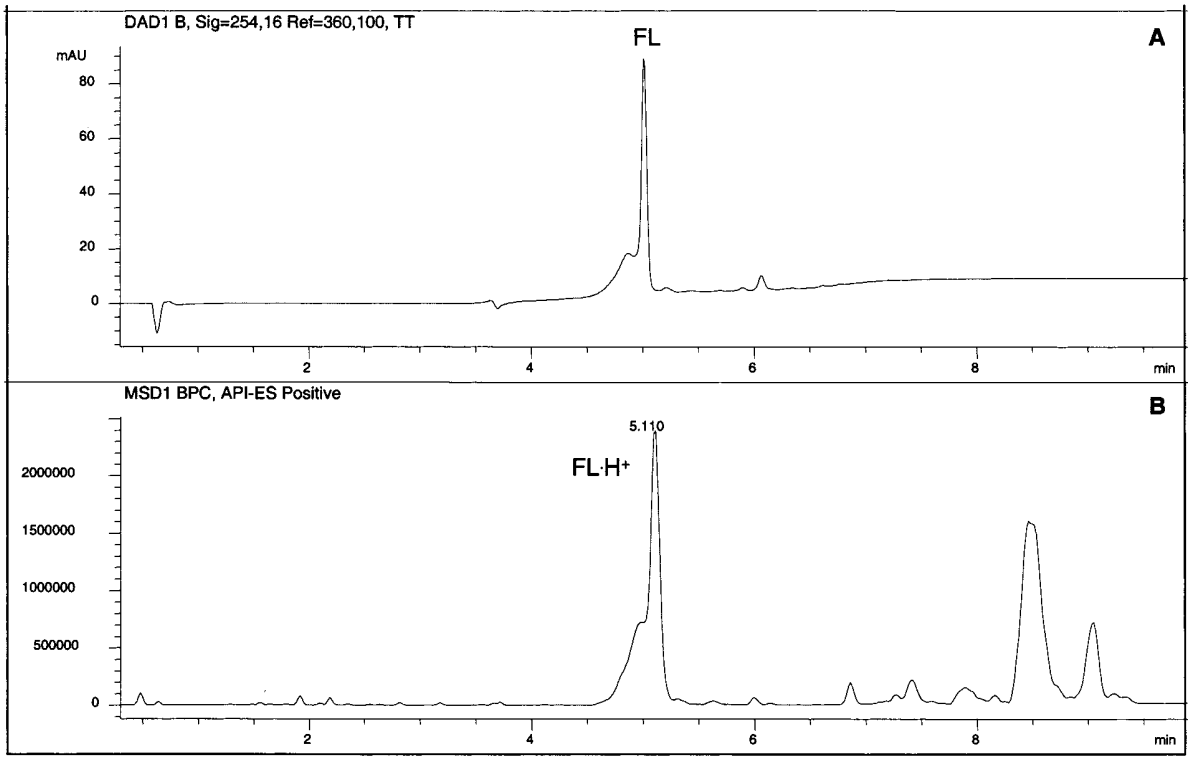

Figure 7. UV and MS chromatograms of Fmoc-Lys from a single bead of Wang resin loaded on a 50 mm × 3 mm × 3 μm Polaris2000 C18 column in 20 μL of acetonitrile with chromatographic conditions listed in Figure 6. Fmoc-Lys can be effectively retained on the column without split peaks. (A) UV chromatogram of Fmoc-Lys at λ = 254 nm; (B) MS chromatogram of Fmoc-Lys obtained in positive ESI/MS.

= 92), phenol (MW = 94), uracil (MW = 112), and Deet (MW = 191) cover a broad range of polarity and have representative functional groups. They can be applied to the evaluation of theoretical plate number of a RP-LC column or its performance. Manufacturers often include a mixture of these compounds in their products for quality assurance purposes. A standard mix of these compounds can also be purchased for checking the performance of a RP-LC column. However, the detection of most of these molecules is incompatible with ESI when the RP-LC column is in use with a mass spectrometer. These small molecules either gave

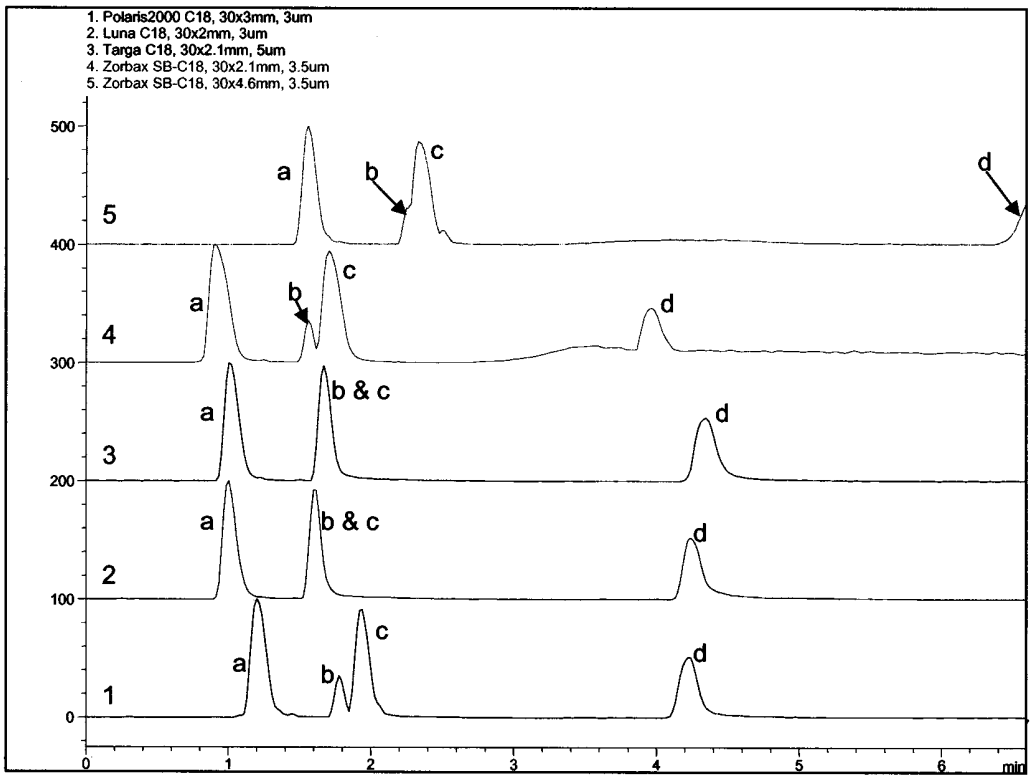

Figure 8. Visual display of the effect of packing material, column dimension, and chromatography conditions on separation of LPTM. It can guide the optimization of chromatography conditions for a specific RP-LC column in use: (a) aspartame; (b) cortisone; (c) reserpine; (d) dioctyl phthalate

a UV signal only or gave a mass spectral signal at low-mass range. Keeping in mind that some mass analyzers such as quadrupoles have mass discrimination, one will prefer that the markers in a mix have molecular weights similar to that of the analyte. For instance, if the drug discovery activity focuses on searching for synthetic drug leads, most members from a combinatorial library will have molecular weights in the range of 500 amu. When an LC/MS instrument or an LC/MS method is to be evaluated, the mass spectral sensitivity demonstrated by the LPTM should be comparable with that of the analyte.

III. Applications of LC/MS Performance Test Mix. 1. Expedite LC/MS Method Development for Pharmaceutical Analysis. In the process of developing an LC/MS method, it is usually best to optimize the method with the analyte in study. Under certain circumstances, it is necessary to develop a generic LC/MS method which can analyze numerous analytes with different chemical properties.[10,13−15] In drug discovery, combinatorial libraries are deliberately designed to include a number of compounds that not only have diverse chemical structures but also have diverse physical properties.[16] The LPTM could provide markers to observe that an LC/MS method can analyze compounds with dramatic variation in polarity.

Drug discovery chemists often apply solid-phase organic synthesis in combinatorial library production. It is often required to analyze the compound(s) cleaved from a tiny single bead in the bead-based solid-phase synthesis. Organic solvents such as methanol and acetonitrile are the common choice to dissolve compound from a single bead. Issues of solubility among diverse library members make the use of weaker solvents problematic. When a library contains highly hydrophilic compounds, the organic solvent will make the compounds unretained on an RP-LC column resulting in split peaks, which disrupt separation in LC chromatography and lower the sensitivity in LC/MS analysis. Fmoc-Lys demonstrates this effect because of its hydrophilicity, as illustrated in Figure 5. In this example, Fmoc-Lys from Wang resin was dissolved in 25 μL of acetonitrile after it had been cleaved from a single bead by trifluoroacetic acid solution. The extracted ion chromatogram of the protonated Fmoc-Lys illustrates that part of the Fmoc-Lys eluted in the void volume with strong solvent injection in a short LC column. A new LC/MS method shown in Figure 6 was developed with the assistance of LPTM, in which hydrophilic compounds are effectively retained on the LC column but hydrophobic compounds are eluted in a reasonable time. It is obvious in Figure 7 that the chromatographic behavior of Fmoc-Lys is much better on this column, and the monomer or residue from the resin material could also be removed from the column for each single bead sample.

Another application is that LPTM could be used to visualize the relationship between separation parameters and RP-LC column properties. It is well known that packing material, particle size, and column geometry can affect chromatography. Working LC/MS conditions for a specific RP-LC column may not work for other similar columns unless adjustments are made in these conditions. For instance, the chromatographic conditions in Figure 8 could work for Polaris2000 C18 with length and internal diameter of 30 and 3 mm, respectively, and particle size of 3 μm. However, the

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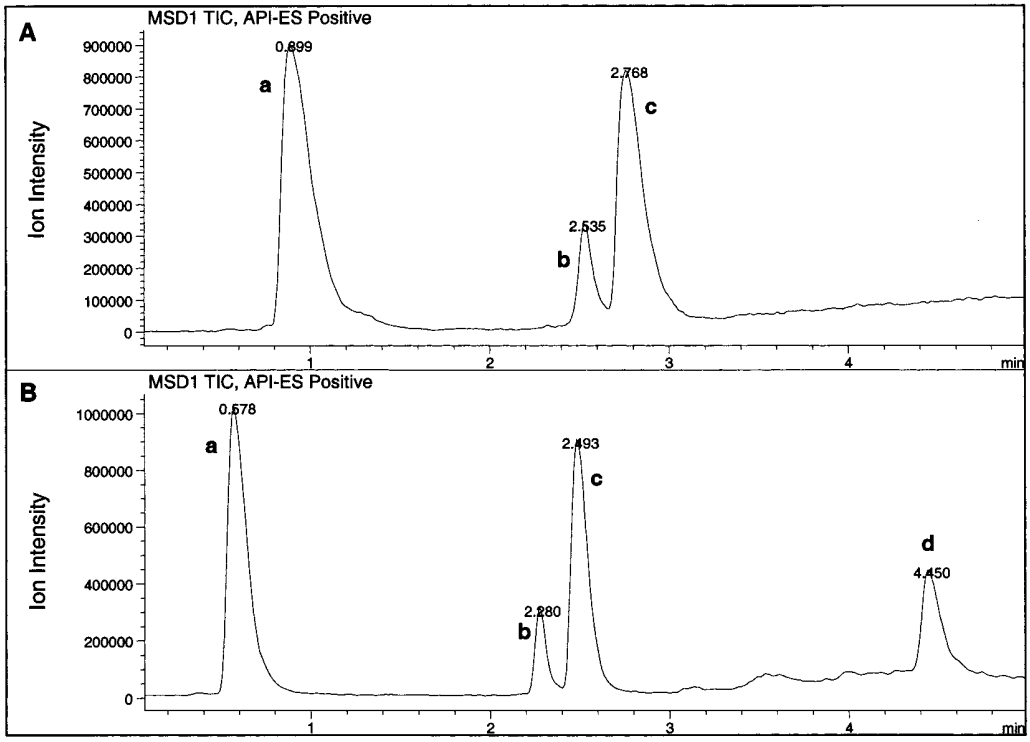

Figure 9. Performance test of LC/MS with LPTM. (A) Mediocre LC/MS in analysis because of the deterioration of the HPLC column: broadened peaks and retention time shifts. (B) Normal performance with a new column under the same LC/MS conditions.

conditions were not optimized conditions for other C18 columns with 30 mm in length. The flow rate was too high and the gradient was too fast for the narrow-bore Luna column and Targa column, which eluted cortisone and reserpine faster. Even for the Zorbax SB-C18 columns with the same length, 4.6 mm i.d. did not separate compounds with medium polarities and also delayed the elution of nonpolar compounds. LPTM could serve as markers to determine a new set of LC conditions for these columns.

2. Monitor the Performance of a RP-LC Column and LC/MS System with LC/MS Performance Test Mix. With the improvement in RP-LC packing material, columns such as Zorbax SB-C18 and Keystones' Betasil C18 have been found to provide excellent lifetimes. LPTM can be used to monitor the performance of RP-LC columns during their service in LC/MS applications. For example, the comparison of two chromatograms of LPTM obtained from the same type of column but at different times could be helpful to track a change in the column property. In Figure 9A, poor performance of a Betasil C18 column led to the injection of 2 μL of LPTM on the column. The LC/MS result helped us identify that the column had deteriorated in comparison to a new column (Figure 9B).

It is usually tedious and time-consuming to perform a thorough diagnosis on an LC/MS instrument, especially when an LC column is involved. The LC/MS chromatograms and mass spectra of LPTM collected immediately after an LC/MS method is established would be helpful for trouble-shooting in future. Whenever there is doubt in the operation of the LC/MS method, the repeat of LPTM analysis with the method can indicate whether the HPLC has a problem in correct delivery of mobile phases or UV detection or whether the mass spectrometer has a problem in ion detection. Thus, LPTM could function as a standard for quality control purposes.

CONCLUSIONS

LPTM contains four components which have different affinities to RP-LC columns. They are aspartame, cortisone, reserpine, and dioctyl phthalate, which have molecular weights in the range of most synthetic drugs. They have strong UV absorbance at the wavelengths commonly used in HPLC analysis and can be easily ionized for detection by ESI/MS in either positive mode or negative mode. The four compounds are chemically stable. Therefore, they can represent organic molecules suitable for RP-LC/MS analysis and serve as markers in LC/MS method development and the performance evaluation of RP-LC columns in LC/MS applications.

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