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Journal of Chromatography A, 1163 (2007) 145–156
JOURNAL OF CHROMATOGRAPHY A
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# Efficient method development strategy for challenging separation of pharmaceutical molecules using advanced chromatographic technologies

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Received 22 January 2007; received in revised form 17 April 2007; accepted 18 June 2007
Available online 22 June 2007

**Abstract**

In this paper, we describe a strategy that can be used to efficiently develop a high-performance liquid chromatography (HPLC) separation of challenging pharmaceutical molecules. This strategy involves use of advanced chromatographic technologies, such as a computer-assisted chromatographic method development tool (ChromSword) and an automated column switching system (LC Spiderling). This process significantly enhances the probability of achieving adequate separations and can be a large time saver for bench analytical scientists. In our study, the ChromSword was used for mobile phase screening and separation optimization, and the LC Spiderling was used to identify the most appropriate HPLC columns. For proof of concept, the analytes employed in this study are the structural epimers betamethylepoxide and alphamethylepoxide (also known as 16-beta methyl epoxide and 16-alpha methyl epoxide). Both of these compounds are used in the synthesis of various active pharmaceutical ingredients that are part of the steroid pharmaceutical products. While these molecules are relatively large in size and contain various polar functional groups and non-polar cyclic carbon chains, their structures differ only in the orientation of one methyl group. To our knowledge, there is no reported HPLC separation of these two molecules. A simple gradient method was quickly developed on a 5 cm YMC Hydrosphere $C_{18}$ column that separated betamethylepoxide and alphamethylepoxide in 10 min with a resolution factor of 3.0. This high resolution provided a true baseline separation even when the concentration ratio between these two epimers was 10,000:1. Although outside of the scope of this paper, stability-indicating assay and impurity profile methods for betamethylepoxide and for alphamethylepoxide have also been developed by our group based on a similar method development strategy.


*Keywords:* Method development strategy; Computer-assisted chromatographic method development tool (ChromSword); LC Spiderling 9-port column switching system; Betamethylepoxide; Alphamethylepoxide

## 1. Introduction

The traditional trial-and-error approach is not an efficient process considering the expectation of rapid chromatographic method development in the pharmaceutical industry. Therefore, a systematic approach is needed to efficiently develop a rugged HPLC method that can deliver adequate resolutions for challenging separations.

In this paper, we describe a strategy for an efficient HPLC method development which includes several chronological steps. An analytical scientist will (a) perform a thorough literature search for similar separations, (b) determine the key physicochemical characteristics of the analytes such as UV absorbance, solution solubility, stability, and p$K_a$, (c) use advanced technologies such as computer-assisted method development tool and automated column switching system to carry out mobile phase and stationary phase screening to obtain preliminary/promising chromatographic conditions, (d) examine the data from step c and finalize the HPLC method, (e) identify equivalent columns, and (f) validate the final method and transfer the validated method to quality control (QC) laboratories. The flow diagram of the proposed strategy is provided in Fig. 1. In our laboratory, ChromSword, a computer-assisted chromatographic method development tool, and LC Spiderling, an automated column switching system for quick screening of stationary phases, are generally used to expedite method

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doi:10.1016/j.chroma.2007.06.027

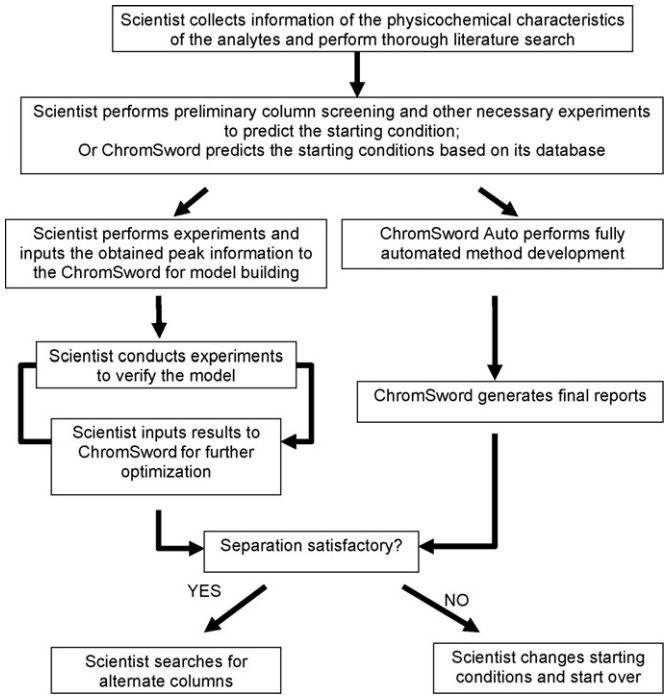


Fig. 1. Flow diagram of the presented method development strategy using advanced chromatographic technologies such as computer-assisted method development tool (ChromSword) and LC Spiderling 9-port column switching system.

development activities. Among various HPLC method development software such as Drylab [1], ACD [2], Waters automated method development system (AMDS), and Perkin-Elmer turbo method development software, ChromSword is one of the tools that are capable of fully automated method development. The software not only can analyze the chromatographic retention data, build retention models, simulate and predict the separation, but can also make the method development almost 100% unattended. The ChromSword auto mode automatically runs the experiments, collects data, performs calculations, evaluates the results, generates new mobile phase conditions and runs the experiments again, repeats the optimization process until no further improvement can be made with the given mobile phase and the stationary phase, and finally generates a method development report. Hewitt et al. have presented one recent example of using ChromSword and an Agilent column switching system for rapid and automated HPLC method development. The authors claimed a saving of 8 days compared to traditional manual method development for a complicated separation [3]. We have found that using ChromSword, in combination with the separation science knowledge of our analytical scientists, HPLC method development time can be significantly shortened and the probability of achieving optimum separation conditions can be enhanced. For example, we developed a method using conventional method development practice (i.e., based on the knowledge and experience of the analytical scientists) to separate two isomer molecules. It took about 2 days for several highly competent analytical scientists to work together to develop a method that provided adequate resolution between the two isomers. On the other hand, the same goal was achieved in just 10 h when we used ChromSword auto. For a simple separation, it is possible that a highly competent analytical scientist would be able to achieve the same goal by spending similar amount of time (or even less) compared to ChromSword. However, for a challenging separation, especially for separation of multiple pairs of peaks, an automated method development approach will be much more efficient than a manual process. Furthermore, the efficiency can be gained from using automated method development tool because the analyst can spend the time on other tasks while let ChromSword do the job unattended. Therefore, it has become a routine practice for our analytical scientists to use this computer-assisted method development tool to develop new HPLC methods.

Two isomers were selected as the representative molecules for the purpose of a proof of concept. Among many difficult separations, a reversed-phase HPLC separation of certain stereoisomers of an active pharmaceutical ingredient (API) can be an extremely challenging task. It becomes even more challenging when baseline resolution is required for the separation of an isomer impurity which is at a concentration level of 0.1% or lower from the API. It is important to monitor isomer impurities because different isomeric forms of an API may have vastly different physiological effects [4–6]. One isomer can be beneficial, while the other isomer might be toxic to human beings. Therefore, it is preferred that the API of a pharmaceutical product is in one pure form instead of mixed isomers. The isomeric pair we chose was betamethylepoxide and alphamethylepoxide (Fig. 2). These two molecules are the key intermediates for synthesizing various steroid APIs such as betamethasone and dexamethasone, betamethasone- and dexamethasone-21-acetate, betamethasone- and dexamethasone-21-phosphate, and betamethasone- and dexamethasone-21-dipropionate, etc. The beta- and dexa- forms of those molecules are epimers with identical chemical structures except for the orientation of the methyl group at the C-16 position. It is critical to have a reliable analytical method to accurately measure the amounts of each of the stereoisomers in the key intermediates because these intermediates are the original source of the undesired isomers in the corresponding APIs. However, it is extremely challenging to develop a rugged, robust, sensitive, and efficient reversed-phase HPLC method that would separate stereoisomers with chemical structure difference as small as betamethylepoxide and alphamethylepoxide. Physicochemical characteristics of these two compounds should be very similar. Therefore, it would be difficult to obtain a mobile phase and a stationary phase that

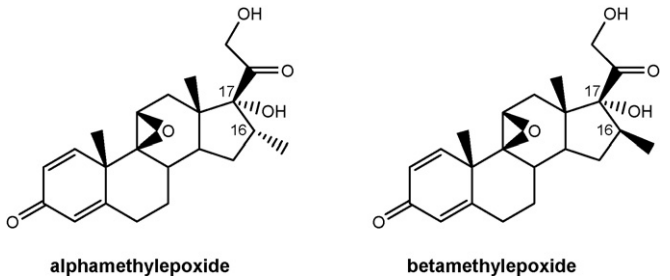


Fig. 2. Chemical structures of alphamethylepoxide and betamethylepoxide.

would provide adequate differences in thermodynamic parameters (entropy, enthalpy, etc.) between these two epimers for a true baseline separation. To our knowledge, there is no report in the literature of a HPLC separation of betamethylepoxide and alphamethylepoxide.

With the aid of the advanced method development technologies, we were able to develop a simple 10-min gradient method on a 5 cm YMC Hydrosphere $C_{18}$ column that separated betamethylepoxide and alphamethylepoxide. The resolution factor of these two epimers was 3.0 that provided a true baseline separation even when the concentration ratio between these two epimers was 10,000:1. Similar separation under the same conditions was also achieved on many other columns from which not only a back-up column to the YMC Hydrosphere $C_{18}$ column could be identified, but also more column choices were provided for separation of molecules with similar structures. It is our belief that the strategy presented in this article can be applied in general to most HPLC method development.

## 2. Experimental

### 2.1. Chemicals and reagents

The reference standards and samples of betamethylepoxide and alphamethylepoxide were provided by Global Quality Services – Analytical Sciences group in Schering-Plough (Union, NJ, USA). All HPLC grade solvents were obtained from Fisher Scientific (Fisher Scientific International, Hampton, NH, USA). Water (18.2 MΩ cm) was obtained using a Milli-Q system (Millipore, Billerica, MA, USA).

### 2.2. Apparatus and HPLC conditions

A Hitachi LaChrom Elite HPLC system (Hitachi High Technologies America, San Jose, CA, USA) equipped with ChromSword method development software (Merck, Darmstadt, Germany); an Agilent Technologies 1100 Series HPLC system (Santa Clara, CA, USA) equipped with a LC Spiderling column switching system (Chiralizer Services, Newtown, PA, USA); and a Waters 2695 Alliance HPLC system (Milford, MA, USA) were used for method development. All HPLC systems were equipped with a column compartment with temperature control, an on-line degasser, and a diode array detector or a dual wavelength UV detector. Different dwell times on different HPLC instruments were taken into account whenever necessary. Data acquisition, analysis, and reporting were performed by EZChrom Elite (Hitachi), ChemStation (Agilent), and Millennium32 (Waters) chromatography software, except for ChromSword simulation. The HPLC columns were purchased through vendors such as Waters, MAC-MOD Analytical, (Chadds Ford, PA, USA), or Phenomenex (Torrance, CA, USA). The analytical wavelength used for detection was 254 nm and samples of 10 μL or 15 μL were injected using auto samplers. A 5 cm YMC Hydrosphere $C_{18}$ column was used for all the experiments except for the column screening in which other columns were also tested.

### 2.3. Mobile phase and sample preparation

The mobile phases were prepared by mixing appropriate amount of HPLC grade acetonitrile, methanol, isopropanol, or Milli-Q water. The mixtures were degassed by sonication for not more than 10 min. During sonication, the mobile phase bottle was loosely capped to prevent losing too much solvent. Vacuum filtration of the pre-mixed HPLC grade solvents was not performed because of potential loss of the more volatile component in the mixture during vacuum filtration. For solvent screening experiments, a 1.0 mg/mL alphamethylepoxide sample solution which contained 0.3% betamethylepoxide (as an existing impurity, not by spiking) was used. For organic modifier screening experiments, isocratic runs using mobile phases containing different mixtures of water and organic modifiers were carried out to obtain the peak parameters for subsequent ChromSword modeling. For ChromSword auto method development, an alphamethylepoxide standard solution at 1.0 mg/mL, a betamethylepoxide standard solution at 0.2 mg/mL, and an alphamethylepoxide sample solution at a 1.0 mg/mL (which contained 0.15% of betamethylepoxide as an existing impurity) were prepared. For linearity/sensitivity study, the 100% analytical concentration of betamethylepoxide or alphamethylepoxide standard solutions were prepared by dissolving (by sonication) approximately 1000 mg of each standard into 1000 mL methanol to achieve a concentration of approximately 1.0 mg/mL. The 10% level betamethylepoxide standard solution was prepared by dissolving 20 mg of the betamethylepoxide standard into 200 mL of the 1.0 mg/mL alphamethylepoxide solution. The other concentration levels at 5%, 1%, 0.5%, 0.1%, 0.05%, and 0.01% betamethylepoxide were prepared by a series dilution using 1.0 mg/mL alphamethylepoxide solution as the diluent. The quantitation of betamethylepoxide was performed using an external betamethylepoxide reference standard. Similarly, for alphamethylepoxide linearity/sensitivity study, the alphamethylepoxide solutions at different concentration levels were prepared by using 1.0 mg/mL betamethylepoxide solution as the diluent. The 0.003% betamethylepoxide or alphamethylepoxide solutions were separately prepared from a dilution of the 10% corresponding betamethylepoxide or alphamethylepoxide solution.

## 3. Results and discussion

### 3.1. Start the method development

During the development of stability-indicating assay and degradation/impurity profile methods for betamethylepoxide or alphamethylepoxide, we had encountered a challenging separation for a pair of epimers: betamethylepoxide and alphamethylepoxide. The separation became extremely challenging when trace amount of betamethylepoxide (0.1% of alphamethylepoxide) had to be separated from alphamethylepoxide because the betamethylepoxide peak always eluted immediately after the alphamethylepoxide peak, which was approximately 1000 times larger in peak size. Although there are quite a few publications on the chromatographic separation

of steroid molecules such as betamethasone and dexamethasone [7–10], there is no report on the separation of betamethylepoxide and alphamethylepoxide. From the chemical structures of the two isomers, it appears that neither betamethylepoxide nor alphamethylepoxide has any functional groups that are easily ionisable. Therefore, mobile phase pH or ionic strength should not significantly affect the retention/separation of these two molecules under reversed-phase chromatography. The cyclic alkane chains of the molecules make them hydrophobic while the hydroxyl groups will potentially provide some hydrogen bonding characteristics. Hence, the method development should be focused on the selection of suitable HPLC columns, optimization of the compositions of organic modifiers in the mobile phases, and fine-tuning of the final elution profile. Typically, the analyte retention time and selectivity also changes as a function of temperature. Therefore, maintaining the column temperature at a constant temperature would provide reproducible retention time and selectivity of chromatographic peaks. A temperature of about 10 °C or more above the laboratory room temperature ensures a consistent temperature control by typical commercially available HPLC column heaters. A column temperature of 35 °C or above is preferred for routine analysis.

The UV detection would be performed at a wavelength of 254 nm because this is the maximum absorbance of betamethylepoxide and alphamethylepoxide. The analytical concentration of betamethylepoxide or alphamethylepoxide sample solution was approximately 1 mg/mL in methanol. It was experimentally observed that the compounds could be easily dissolved in neat methanol by a 10-min sonication at a concentration of approximately 3 mg/mL. Although a mixture of 1:1 water:methanol could dissolve betamethylepoxide and alphamethylepoxide as well, there were some related compounds that were highly hydrophobic and would not dissolve easily in the aqueous organic mixture. As mentioned above, the presented study was part of our method development endeavor for stability-indicating assay of betamethylepoxide, alphamethylepoxide and related compounds, we did not change the sample solvent for the purpose of this presentation. Preliminary tests were performed to make sure that a 20 μL injection of 1 mg/mL betamethylepoxide or alphamethylepoxide methanol solution could be injected into a mobile phase system with much weaker solvents, such as 80% water + 20% acetonitrile. The injections performed for the following method development experiments were then carried out at 20 μL or less.

### 3.2. Identify the best stationary phase(s) via automated column screening

As mentioned in Section 3.1, the success of achieving an adequate separation between betamethylepoxide and alphamethylepoxide depended largely on the identification of the most appropriate stationary phase (i.e., the HPLC column). This step can be the most difficult and time consuming, however, as there are so many commercial HPLC columns available. Column selection should be based on the knowledge, i.e., the physicochemical characteristics, of the sample (especially the key analytes) and the column stationary phase (such as type of bonded phase, bonding type, polarity or functionality, endcapping, carbon loading, hydrophobicity, particle shape, particle size, surface area, ligand density, pore size, hydrogen bonding capacity, trace metals in silica that is used in preparation of the bonded phase, etc.). Although it has been realized that the separation of isomers is possible under reversed-phase chromatography, the exact mechanism is still unknown. One thing seems clear is that the separation of isomers cannot be achieved only by the hydrophobic interaction between the isomer analytes and the stationary phase $C_8$ or $C_{18}$ carbon chains. Surface modification of the stationary phases may play an important role. For example, Snyder and Dolan [11] have pointed out that $C_{18}$ columns made from polyfunctional silanes are more effective in the isomer separations than columns that are made from monofunctional silane. The subtle difference in the hydrogen bonding, dipole–dipole, or other polar/non-polar interactions between the isomers and the stationary phase surfaces, induced by the different stereo orientation of the isomers, must be responsible for the different retention behavior of the isomer molecules. Besides the properties of the stationary phases, column quality such as consistent particle size distribution, lot-to-lot reproducibility, and stability of column bed (column lifetime) must also be considered. Although columns of 15 cm or longer are commonly used, our strategy is to start the method development on 5 cm HPLC columns packed with 2 μm or 3 μm silica particles. It is well known from the Van Deemter equation that the column efficiency increases with decrease in the particle sizes [12]. The reduced particle size, together with a tight particle size distribution, enhances both the interparticle and intraparticle mass transfer by shortening the diffusion distances. Improved mass transfer enhances peak efficiency and peak symmetry, and also allows separations to occur faster, thus shorter columns, higher mobile phase flow rates, and faster gradients can be used without sacrificing resolution and selectivity [13].

Based on aforementioned considerations, a few 5 cm columns were selected for the initial column screening. These columns include YMC-Pack Pro $C_{18}$, YMC-Pack CN, YMC-Pack Phenyl, YMC Basic, YMC Hydrosphere $C_{18}$, TSK Super ODS, TSK Super Octyl, Ace $C_8$, Ace $C_{18}$, Ace $C_{18}$ (300 Å), Waters Atlantis $dC_{18}$, Waters Symmetry Shield, and Waters XTerra MS $C_{18}$. The columns were obtained from four well-established highly reputable HPLC column manufacturers, i.e., YMC (YMC, Japan), TSK (Tosho Bioscience, Japan), ACE (Advanced Chromatographic Technologies, UK), and Waters (Waters, USA). Most of the columns are packed with ultra pure silica particles. As a matter of fact, choosing columns from reputable column manufacturers is a first priority for methods that are meant to be used routinely in QC laboratories. The selected columns contain different carbon chain length, different carbon loading, and different surface functionality. For example, YMC Hydrosphere $C_{18}$ and Waters Atlantis $dC_{18}$ are good for separation of polar compounds under highly aqueous mobile phases; TSK columns are packed with 2 μm particles; ACE column surfaces are base deactivated and are well known for offering good peak shapes, and the Hybrid Particle Technology used in Waters XTerra MS $C_{18}$ column provides an evenly distributed hydrophobicity throughout the particle backbone. Waters Sym-

metry Shield is a polar-embedded column that can offer polar interaction with the polar groups of the analytes. Columns with Phenyl group and Cyano group imbedded stationary phases were also screened because they can provide quite different selectivity from conventional $C_8$ or $C_{18}$ columns. A wide pore ACE $C_{18}$ column with a pore size of 300 Å was also selected for initial column screening. Although wide-pore silica particles are usually used for large molecules such as proteins and nucleic acids analysis, they can also enhance the access of small molecules to the intraparticle surfaces by allowing the steroid molecules, which are not "that" small, to more freely diffuse into and out of the pores. Indeed, the columns in the above mentioned column pool have been successfully used in our group to achieve adequate separation of various steroid compounds.

Column screening was carried out on a LC Spiderling automated column switching system to find out the most promising stationary phase. The LC Spiderling system has 9-column switching capability and can automatically test multiple methods (i.e., mobile phase conditions) on different columns. For conducting scouting runs, the mobile phase A was water and mobile phase B was acetonitrile and the gradient profiles were simple linear gradients. For example, the very first elution was carried out by changing the percentage of acetonitrile from 5 to 100% within 60 min at a flow rate of 1.0 mL/min. Then other elutions such as changing the acetonitrile percentage from 15 to 30% in 20 min, or changing the acetonitrile percentage from 25 to 30% in 14 min were tested to "zoom in" the separation. For this exercise, a concentration ratio of approximately 1:1 between betamethylepoxide and alphamethylepoxide was used to simplify the initial column screening experiments. The sample solution was prepared by spiking the alphamethylepoxide reference standard into a betamethylepoxide sample. The work described in this presentation was part of the development of stability-indicating methods. Therefore, the focus of the scouting runs were to identify columns that would show maximum numbers of peaks and also a good separation between alpha- and betamethylepoxide peaks. Except for the YMC-Pack CN column, all other columns showed more or less separation between betamethylepoxide and alphamethylepoxide ($1.05 < \alpha < 1.09$). However, a careful scrutiny of the total number of peaks, peak shapes, and especially the separation of the small peaks that eluted at the close vicinity of the betamethylepoxide and the alphamethylepoxide peaks revealed that the YMC Hydrosphere $C_{18}$ column has the highest resolution/separation power. This result was not a surprise, though, from a molecular recognition standpoint. The hydrogen bonding capability of the hydroxy group at the C-17 position will be mostly affected by the different orientation of the methyl group at the C-16 position, due to potential stereohindrance. Although the exact surface chemistry of YMC Hydrosphere $C_{18}$ columns is not a public knowledge, it is known that it has a hydrophilic surface for enhanced polar selectivity, in other words, a high hydrogen bonding capability is a reasonably expectation. Therefore, it can be anticipated that this column has the potential to separate betamethylepoxide and alphamethylepoxide, and their related impurities better than most of the other columns. Based on the column screening results, the YMC Hydrosphere $C_{18}$ column was then selected for further study.

### *3.3. Examine different combinations of organic modifiers to determine appropriate mobile phase components*

Although as mentioned above during the initial column screening, we had found there were many peaks that needed to be separated in the betamethylepoxide or alphamethylepoxide samples, initial focus of the method development activities was to separate betamethylepoxide peak from a trace level alphamethylepoxide peak (~0.1% of the betamethylepoxide peak) or vice versa. This was because the initial strategy for any new method development activities should be to identify the pairs of known peaks that would pose the most challenge in achieving baseline separation. The final method can then be built based on the conditions that are suitable for separation of this critical pair with subsequent separation of all other related compounds. To ensure the mobile phase had the largest resolution power for betamethylepoxide and alphamethylepoxide, we started an organic modifier screening on the selected YMC Hydrosphere $C_{18}$ column to determine whether a binary mobile phase system, such as water and acetonitrile or methanol, was adequate, or whether a ternary mobile phase system was necessary.

The commonly used organic solvents in reversed-phase HPLC are acetonitrile, methanol, and to a lesser extent, isopropanol, and tetrahydrofuran (THF). To develop a QC friendly HPLC method, however, the use of THF is not preferred. Fresh HPLC grade THF has to be used to avoid high background absorbance from the trace UV absorbing impurities that are generated by the residual peroxides in old THF (peroxides are also fire and explosion hazards). THF can also attack plastic parts, such as seals, ferrules, tubings, or filters of the HPLC systems. Therefore, we mainly focused the search on finding appropriate combination(s) between acetonitrile, methanol, and isopropanol as the organic modifiers in the mobile phase.

To quickly search for appropriate/optimum organic modifier combinations, we used the model mode of ChromSword. The separation modeling is based on parameters such as peak retention times, half peak widths, and peak areas that are obtained from two or more trial runs. To optimize the volume ratio between organic modifier 1 and 2, the mobile phases are prepared as such that a mixture of water–organic modifier 1 is used as the mobile phase A and a mixture of water–organic modifier 2 is used as the mobile phase B. Then a certain ratio of mobile phase A and B is used to carry out a trial run 1, followed by a trial run 2, and/or more trial runs, which use different ratios between mobile phase A and B. ChromSword then builds a polynomial retention model from which a resolution map is presented. From the resolution map, one can determine the specific organic solvents and their combination that would provide baseline separation between the two peaks (in our case, the betamethylepoxide and alphamethylepoxide peaks).

Three pairs of the organic modifiers were tested, i.e., the combination of acetonitrile and methanol, the combination of acetonitrile and isopropanol, and the combination of methanol

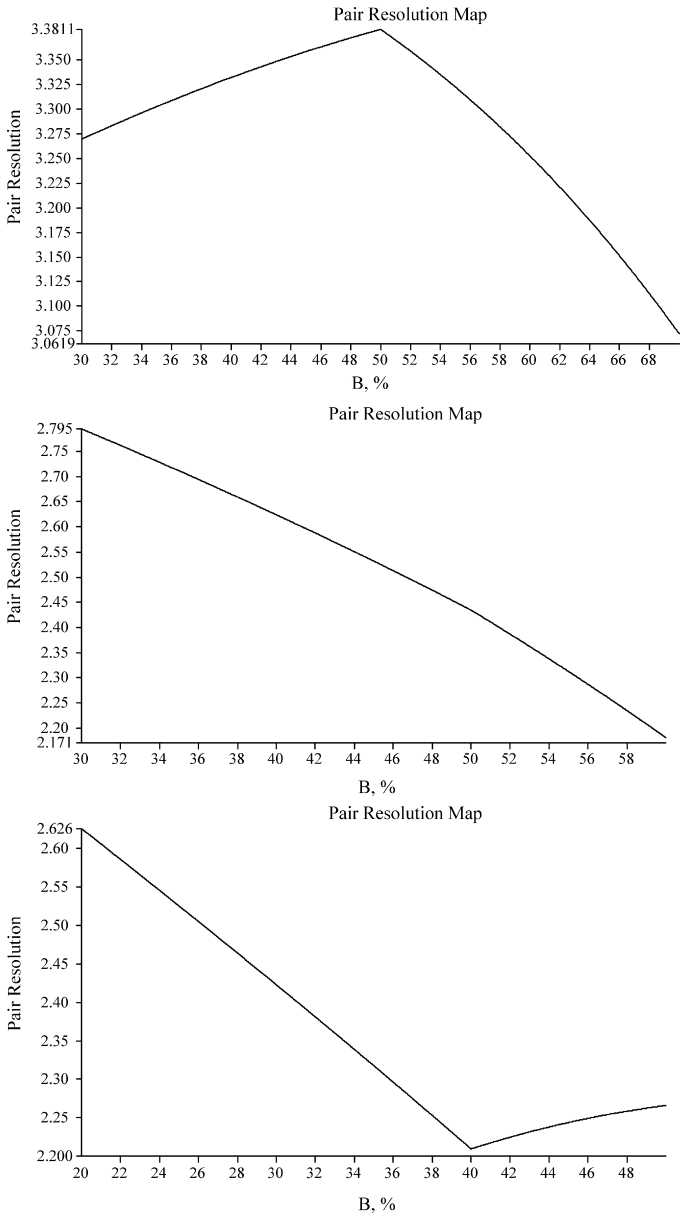


Fig. 3. Resolution map generated from ChromSword during organic modifier screening based on experimental results using acetonitrile and isopropanol (top), or acetonitrile and methanol (middle), or isopropanol and methanol (bottom) as the organic modifier 1 and 2, respectively. The *x*-axis (B%) on the resolution maps refers to the percentage of mobile phase B, which is the mixture of water and isopropanol.

and isopropanol. Fig. 3 shows a resolution map generated from ChromSword. The *y*-axis is the pair resolution values and the *x*-axis is the ratio of the mobile phase B (i.e., the mixture of water–organic modifier 2) in the total volume. From the resolution map, the best volume ratio between every pair of the organic modifiers can be determined. To plot Fig. 3, isocratic runs with the mobile phase A of 80% water + 20% acetonitrile (v/v) and the mobile phase B of 80% water + 20% isopropanol (v/v) were carried out. The highest resolution predicted from the resolution map was achieved when the percentage of mobile phase B was 50%, which corresponded to 10% of acetonitrile and 10% of isopropanol in the total volume. In other words, the volume ratio between acetonitrile and isopropanol was 1:1 (10:10%). At this condition, a resolution between betamethylepoxide and alphamethylepoxide was predicted as high as 3.4 (Fig. 3, top). The resolution map based on the results from the combination of acetonitrile and methanol (Fig. 3, middle) suggested that a higher portion of acetonitrile increased the resolution while methanol suppressed the separation. Within the tested range, the highest resolution was 2.8. The resolution map based on results from the combination of isopropanol and methanol (Fig. 3, bottom) suggested that a higher portion of isopropanol increased the resolution and methanol decreased the separation. Within the tested range, the highest resolution was 2.6. The experimental results also revealed that the peaks were much sharper if the portion of acetonitrile in the mobile phase is increased while the peaks were broader if the portion of isopropanol in the mobile phase was increased, presumably due to poor mass-transfer because of higher viscosity of isopropanol. All the abovementioned solvent screening tests were completed in 4 h.

It was quite evident from the preliminary results that the mixture of acetonitrile and isopropanol could provide the highest resolution. Hence, a ternary mobile phase system using water, acetonitrile, and isopropanol was tested further. At the same time, since acetonitrile appeared promising for achieving adequate resolution and keeping the method simple, a binary mobile phase system of water and acetonitrile was also tested.

### *3.4. Establish preliminary separation conditions using ChromSword auto*

After the selection of a promising column and the mobile phase components, we used the auto mode of ChromSword for further method development. The HPLC system was set-up using water as the mobile phase A and a mixture of acetonitrile:isopropanol at 1:1 volume ratio or neat acetonitrile as the mobile phase B. The YMC Hydrosphere $C_{18}$ column was installed on the Hitachi LaChrom Elite HPLC system at a temperature of 35 °C and the flow rate of 2.0 mL/min was used for this experiment. Reference standard solutions of alphamethylepoxide and betamethylepoxide were prepared in methanol at concentrations of approximately 1.0 mg/mL and 0.2 mg/mL, respectively. The alphamethylepoxide solution was prepared with a higher concentration than the concentration of betamethylepoxide because the alphamethylepoxide peak eluted earlier than the betamethylepoxide peak. The separation of a minor peak from a major peak is more difficult when the major peak elutes immediately before the minor peak. The reference standard solutions were prepared at relatively high concentrations to ensure the peaks of interest were large enough to meet the minimum peak area requirement set by ChromSword to recognize a peak in the sample. ChromSword also required a mixture of these two molecules to generate the optimum final isocratic and/or linear gradient profiles. An alphamethylepoxide sample solution which contained approximately 0.15% level of betamethylepoxide was used as the mixture. After 5 h of fully automated work, ChromSword generated a report that presented a best isocratic method and a best linear gradient method for the ternary mobile phase system. This 5-h work started

with elution tests to verify that peaks could be found in the injections from sample vials that contained alphamethylepoxide and betamethylepoxide, respectively. Then four different isocratic conditions (mobile phase B percentages of 14%, 19%, 20%, and 26%, respectively) were tested individually using both betamethylepoxide and alphamethylepoxide solutions, i.e., total eight injections. After the isocratic trial runs, ChromSword performed three gradient runs including the best linear gradient run based on its modeling. Finally, an isocratic run at 14% mobile phase B was performed using the sample mixture to finish the method optimization. This final isocratic elution gave a resolution factor of 5.8. However, the run time for this condition was relatively long. The retention time of the alphamethylepoxide was more than 30 min and the retention time of betamethylepoxide was more than 38 min. Note that although the predicted resolution map covers the mobile phase B percentages from 5 to 50% (Fig. 4, top), experimentally ChromSword only tested the four mobile phase B percentages between 14% and 26%. The rest part in the resolution map was generated based on its model building. ChromSword was intelligent enough not to test a mobile phase B at 5% which although the predicted resolution was above 7, the HPLC run time might be exceptionally long.

To look for an acceptable separation that could occur within a shorter run time, we adjusted the mobile phase B percentage on the simulated resolution map, i.e., moving the dotted line across the resolution map in Fig. 4 (top). By moving the cursor across the *x*-axis, we could review simulated chromatograms from ChromSword. The chromatogram in Fig. 4 (top) is a simulated separation showing that a resolution of about 3.0 can be achieved between betamethylepoxide and alphamethylepoxide with a run time of less than 10 min. The mobile phase composition for this 10-min run was 79% mobile phase A (water) and 21% mobile phase B (acetonitrile:isopropanol at 1:1, v/v).

Similarly, after spending about 3 h, ChromSword generated a report that presented an optimum isocratic method and a linear gradient method for the binary mobile phase system (water:acetonitrile). The chromatogram in Fig. 4 (bottom) is a simulated separation showing that a resolution of about 2.0 can be achieved between betamethylepoxide and alphamethylepoxide with a run time of less than 10 min.

It was encouraging to find out that a resolution factor of 2.0 was obtained from a simple binary mobile phase system. However, although many textbooks will claim that a resolution of 2.0 is good enough for a robust HPLC separation, this is actually not

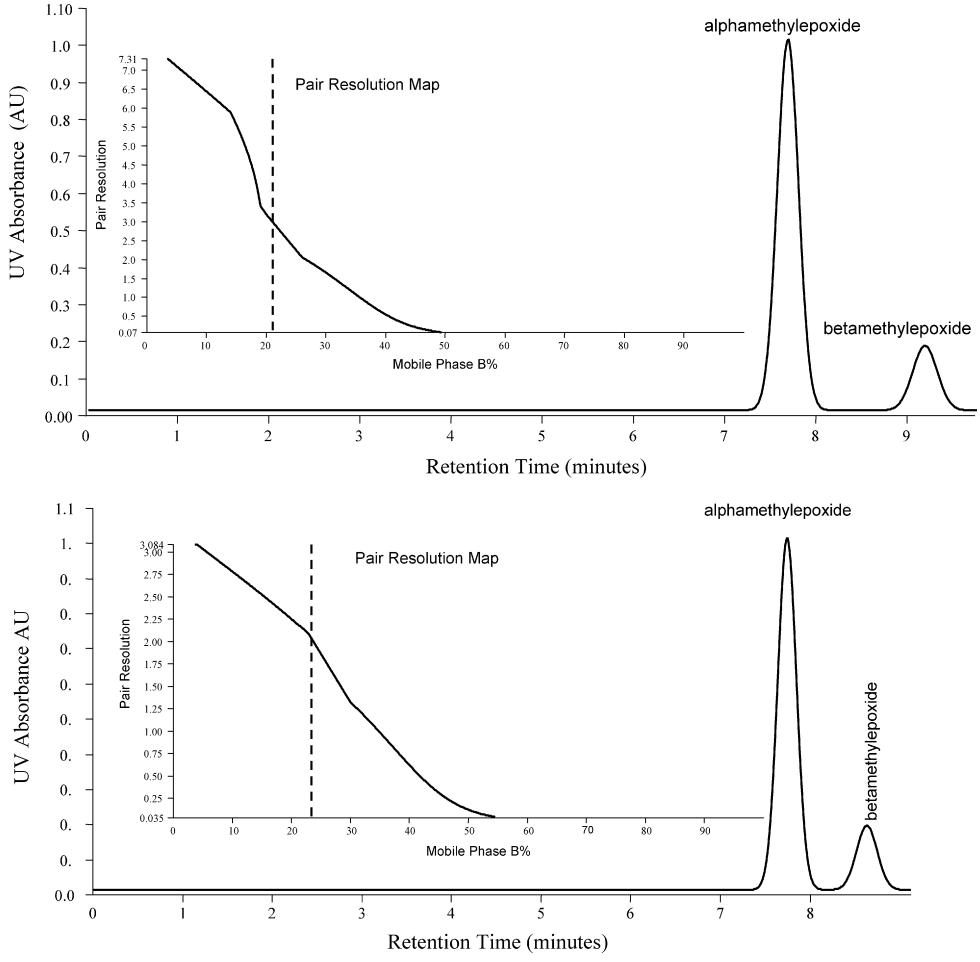


Fig. 4. Simulated resolution maps and chromatograms from ChromSword auto. In the resolution map, the dotted line is the cursor that can be moved horizontally across the resolution map to obtain the predicted resolution (*y*-axis) at corresponding mobile phase B percentage (*x*-axis). The chromatograms represent simulated separation. Top figure: resolution map and chromatogram obtained using 1:1 acetonitrile: isopropanol as the mobile phase B. Bottom figure: resolution map and chromatogram obtained using acetonitrile as the mobile phase B.

that simple and straight forward. Resolution of 2.0 is usually not adequate for a true baseline separation between two peaks which differ dramatically in their peak sizes, especially when the small peak elutes after the large peak [12]. Other factors such as peak tailing, peak width, and different band broadening characteristics of the two peaks would also significantly impact the baseline resolution. A resolution of 2.0 for baseline resolution works well only when the chromatographic behavior of both peaks on the stationary phase is similar and their concentration in the sample is close to 1:1. Because we were developing an HPLC method that must quantitate betamethylepoxide and alphamethylepoxide even when their concentration ratio was 1000:1 or more, a resolution factor much larger than 2.0 was deem necessary. The mixture of acetonitrile and isopropanol at a volume ratio of 1:1 was thus used for further optimization.

Another issue apparent from the resolution map (Fig. 4, top) was that the retention times of the epimers could shift as large as 2.5 min with 1% change in the volume ratio of the mobile phase B. For example, the retention times found for alphamethylepoxide from the resolution map at 19%, 20%, and 21% mobile phase B were 11.3 min, 9.3 min, and 7.7 min, respectively. The retention times found for betamethylepoxide from the resolution map at 19%, 20%, and 21% mobile phase B were 13.7 min, 11.2 min, and 9.2 min, respectively. The findings from ChromSword were verified by the experiments performed on a Waters Alliance HPLC instrument. The experimental results showed retention times of alphamethylepoxide and betamethylepoxide at 19%, 20%, and 21% mobile phase B were 11.2 min, 9.3 min, and 7.7 min for alphamethylepoxide, respectively, and 13.7 min, 11.3 min, and 9.3 min for betamethylepoxide, respectively. The largest retention time shift occurred on alphamethylepoxide which was 2.4 min (13.7 min and 11.3 min).

The best linear gradient elution profile generated by ChromSword auto started at 10% and ended at 69% of the mobile phase B within 13 min. The separation, however, was not very good as can be seen in Fig. 5 (the bottom chromatogram, which was reproduced on a Waters Alliance HPLC instrument based on the ChromSword auto experimental result). During our work, we found that ChromSword auto mode became less effective when separation of a minor peak at concentration level of about 0.1% was attempted from the major peak. From the method development report generated by ChromSword auto, a promising step gradient method was identified in which the mobile phase B concentration was changed from 23 to 31% from 4.5 to 4.6 min. Before 4.5 min the elution was isocratic with 23% of mobile phase B and after 4.6 min the elution was kept at 31% of the mobile phase B. The resolution of this separation was 2.0 but baseline separation was still not obtained for these two peaks (Fig. 5, the top chromatogram, which was reproduced on the Waters Alliance HPLC instrument based on the ChromSword auto experimental result). This observation confirmed that a resolution of 2.0 is not always adequate for a baseline separation between peaks that have significantly different peak sizes.

### 3.5. Finalize the method

As can be seen from the above experiments, there is always some limitation to any advanced technology. Careful and knowledgeable scientists are irreplaceable. What is important is the process by which these tools are used to improve the efficiency of

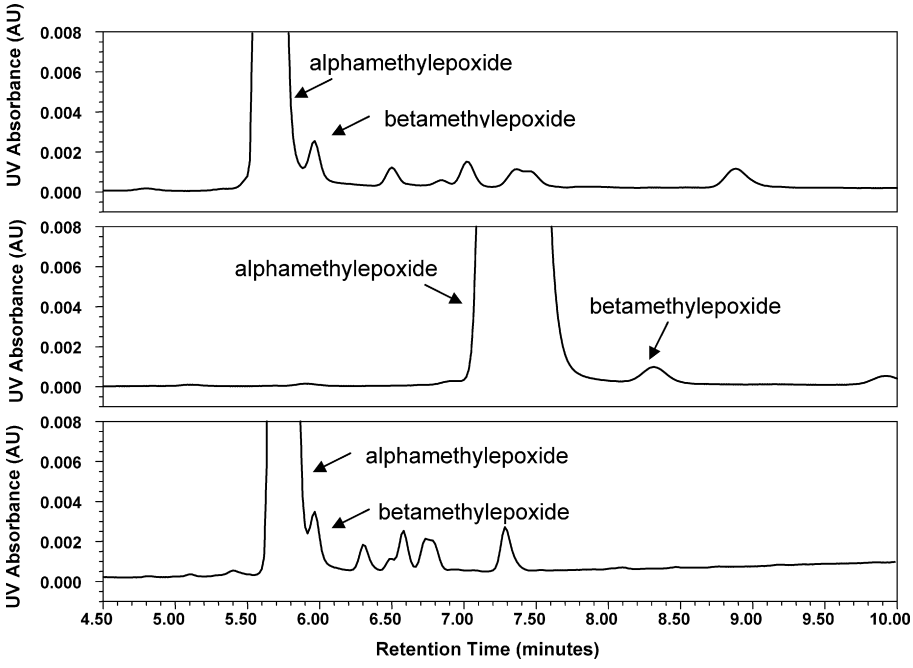


Fig. 5. Overlaid chromatograms of the betamethylepoxide and alphamethylepoxide separation obtained by using the step gradient generated from ChromSword auto (top), the final fine-tuned gradient (middle), and the best linear gradient generated from ChromSword auto (bottom). The sample used for generating the above chromatograms was alphamethylepoxide that contained approximately 0.15% of betamethylepoxide. Other small peaks are from impurities that were not of the interest in this presentation.

bench analytical scientists and to decrease method development time.

To reduce the retention time shifts caused by the variation in mobile phase mixing, one excellent way is to perform partial premixing of the mobile phases. For example, instead of water in mobile phase A and 1:1 acetonitrile:isopropanol in mobile phase B, a 75% water + 25% 1:1 acetonitrile:isopropanol in mobile phase A and 85% water + 15% 1:1 acetonitrile:isopropanol in mobile phase B can be prepared. An on-line mixing of 50% mobile phase A and 50% of mobile phase B will still deliver a total of 80% water and 20% 1:1 acetonitrile:isopropanol. Therefore, even with a 1% change in the mobile phase delivery caused by the on-line mixing, only a 0.1% change in the total amount of the organic modifiers will occur. Therefore, instead of a 2.4-min retention time shift, only a 0.24-min shift might occur. Indeed, when mobile phases have salts, acids, or additives, premixing is necessary in some cases. On the other hand, however, a premixing of mobile phase components creates additional work because more steps required for solution measuring, solvent mixing, degassing, and/or vacuum filtration, and therefore could potentially create the potential for errors from a QC analyst. Since the mobile phase system presented in this work is relatively simple, our approach was to use a gradient instead of an isocratic elution to make the retention time more robust and decrease analyst error in preparing mobile phases.

A fine-tuning of the gradient profile was carried out on the 5 cm YMC Hydrosphere $C_{18}$ column based on the results of the ChromSword auto. Since a 20% mobile phase B could provide a good separation in approximately 10 min, this condition was selected as the gradient starting point. By changing the mobile phase B percentage on the simulated resolution map (Fig. 4, top), we found that a resolution factor of 2.2 could still be obtained at 25% mobile phase B. Therefore, mobile phase B at 25% was selected as the gradient end point. The obtained method was a simple gradient that changed the mobile phase B ratios from 20 to 25% in 10 min. The resulted chromatogram is overlaid in Fig. 5 (the middle chromatogram, generated on the Waters Alliance HPLC instrument) with the chromatograms obtained based on the ChromSword auto results (Fig. 5, top and bottom chromatograms). The resolution factor between betamethylepoxide and alphamethylepoxide was 3.0 in the final method.

The total experimental time spent from solvent combination screening till the finalization of the gradient method was less than 15 h, which included searching the appropriate solvents (4 h), ChromSword auto method development (5 h for ternary mobile phase system and 3 h for binary mobile phase system), analyst fine-tuning the method (30 min), and some instrument set-up time. The achievement of a resolution of 3.0 left some room for improvement of the method efficiency without sacrificing too much resolution, such as reduce the total run time by using more isopropanol, increase the temperature and thus increase the flow rate to reduce the run time to be even shorter, etc. We did not carry out further method optimization as we believe the current presentation is adequate as a proof of concept for our method development strategy.

The robustness of the fine-tuned gradient method was briefly tested by changing the starting or ending mobile phase B percentages, and by applying the gradient at column temperatures of 30 °C and 40 °C. The resulted retention time shifts were less than 0.7 min to the fine-tuned condition from all tested variations (Table 1). Therefore, the gradient elution indeed provided a more robust retention in exchange for a slight scarification of the separation resolution.

The linearity of the peak response was tested by either spiking betamethylepoxide from 0.01 to 10% in the presence of 100% alphamethylepoxide, or by spiking alphamethylepoxide from 0.01 to 10% in the presence of 100% betamethylepoxide. The 100% concentration level corresponded to a 1.0 mg/mL of either alphamethylepoxide or betamethylepoxide in methanol. To demonstrate the method sensitivity, a concentration of 0.01% was selected as the low end of the linearity range. The high end of the linearity was selected at 10% level. Although it is not critical in this presentation to show the exact quantitation limit, a signal-to-noise ratio of 10 or 11 was obtained at a concentration level of 0.003% level. Fig. 6 shows the corresponding overlaid chromatograms, both obtained on a 5 cm YMC Hydrosphere $C_{18}$ column. The mobile phase contained water as the mobile phase A and a mixture of acetonitrile:isopropanol (1:1, v/v) as the mobile phase B. The gradient was carried out by changing mobile phase B from 20 to 25% in 10 min. Only the chromatograms of 0.01–1% are overlaid for visual clarity. Since, the alphamethylepoxide dilution solution contained a certain level of betamethylepoxide as the impurity, the peak areas of betamethylepoxide obtained in the linearity solutions were corrected accordingly for recovery calculation. This is also true for the recovery calculation of alphamethylepoxide as the betamethylepoxide diluent contained a certain level of alphamethylepoxide as an impurity. The chromatograms shown

Table 1
Robustness test results of the final gradient method

| Experimental conditions | Retention time (min) | | Pair resolution |
|---|---|---|---|
| | Alphamethylepoxide | Betamethylepoxide | |
| Mobile phase B from 20 to 25% (35 °C) | 7.1 | 8.2 | 3.0 |
| Mobile phase B from 19 to 25% (35 °C) | 7.7 | 8.8 | 3.0 |
| Mobile phase B from 21 to 25% (35 °C) | 6.5 | 7.5 | 2.9 |
| Mobile phase B from 20 to 24% (35 °C) | 7.4 | 8.6 | 3.0 |
| Mobile phase B from 20 to 26% (35 °C) | 6.8 | 7.8 | 3.0 |
| Mobile phase B from 20 to 25% (30 °C) | 7.9 | 9.1 | 3.0 |
| Mobile phase B from 20 to 25% (40 °C) | 6.4 | 7.4 | 2.9 |

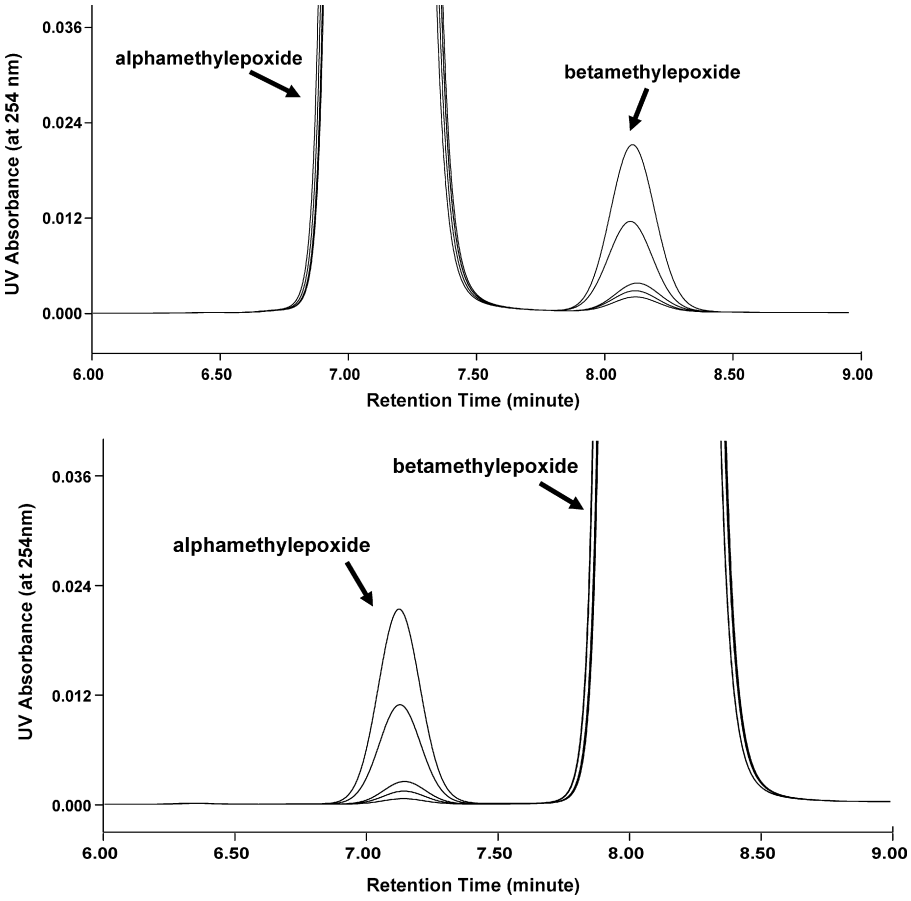


Fig. 6. Overlaid chromatograms obtained from betamethylepoxide linearity study (top) and from the alphamethylepoxide linearity study (bottom). Only the chromatograms obtained in the linearity range from 0.01 to 1% are overlaid for visual clarity. For the top chromatograms the concentrations of betamethylepoxide were 0.0001 mg/mL, 0.0005 mg/mL, 0.001 mg/mL, 0.005 mg/mL, and 0.01 mg/mL, while the concentration of alphamethylepoxide was kept at 1 mg/mL. For the bottom chromatograms the concentrations of alphamethylepoxide were 0.0001 mg/mL, 0.0005 mg/mL, 0.001 mg/mL, 0.005 mg/mL, and 0.01 mg/mL, while the concentration of betamethylepoxide was kept at 1 mg/mL. The unit of the UV absorbance is AU.

in Fig. 6 are raw data without peak area correction. Linear regression analysis reveals that even with the presence of 1.0 mg/mL alphamethylepoxide, the separation between alphamethylepoxide and betamethylepoxide is adequate to ensure a linear response to betamethylepoxide from 0.001 to 0.1 mg/mL with a coefficient of determination $R^2 = 0.99999$. The average recovery of the spiked betamethylepoxide is 96.4% with a standard deviation of 6.4% ($n = 21$), which is good considering the very low concentrations tested. Similar results were obtained from spiking alphamethylepoxide into the 100% betamethylepoxide solution. The coefficient of determination $R^2 = 1.00000$ for the tested range of spiked alphamethylepoxide (0.001–0.1 mg/mL). The average recovery of the spiked betamethylepoxide is 95.1% with a standard deviation of 1.7% ($n = 21$). The slightly better precision of the detection of alphamethylepoxide in the presence of 100% level of betamethylepoxide is expected because the small peak elutes before the major peak. Positive bias from the major peak on the peak area of the minor peak is smaller when the small peak elutes before the major peak. The overlaid chromatograms in Fig. 6 clearly demonstrates the reproducibility of retention times of the alpha- and beta- isomers regardless of the difference of concentrations. Fig. 6 also demonstrates the excellent reproducibility of the resolution between the two stereoisomers.

### 3.6. Search for equivalent columns

For any HPLC method that is meant for routine analysis in pharmaceutical QC laboratories, it is important to identify an equivalent column to the primary column. An optimized separation can be jeopardized even if there is only a little change taking place in the physicochemical characteristics of the primary column. This variation of the primary column does not mean the batch-to-batch variation that can occur in the routine manufacturing of the HPLC columns. As mentioned in Section 3.2, well-established highly reputable HPLC column manufacturers should be selected at the beginning of the method development. Furthermore, the method development is not considered finished until the evaluation of column lifetime and all the necessary robustness studies including testing at least three different lots of each of the selected columns are carried out. The minor variations in the physicochemical characteristics of primary column that is discussed here refers to the changes which occurs during column manufacturing process, such as using silica from

different sources or if surface modification techniques are not 100% reproducible. This can have negative impact in a pharmaceutical QC laboratory because typically all the method details, including the column information are approved by FDA. One way to avoid this situation is to identify and qualify one or more alternative columns using the same HPLC method conditions that are in the original validation package. For this purpose, an equivalent column is a column that provides resolution, selectivity, retention time of all the peaks of interests that are similar to the primary column. The true equivalency can be demonstrated by the fact that the alternate column meets the requirements of system suitability, peak identification, and other critical elements that are set for the primary column.

In this study, the equivalent column search was conducted mainly on the basis of similar stationary phase surface properties such as carbon loading, endcapping, and surface functionality. The selected columns were then screened on the LC Spiderling system using the conditions of the developed method i.e., the chromatographic conditions used to carry out the linearity study on the 5 cm YMC Hydrosphere $C_{18}$ column (see Section 3.5). Two groups of the columns were identified based on the column screening result (Fig. 7). One group of columns can separate the betamethylepoxide and alphamethylepoxide at a resolution factor higher than 3.0. The columns in this group include Waters Atlantis $dC_{18}$ and YMC ODS-AQ columns. The other group of columns are YMC Pack Pro-$C_{18}$, Waters SunFire $C_{18}$, Phenomenex Luna $C_{18}$, and Phenomenex Gemini $C_{18}$, which can provide a resolution factor higher than 2.6 (less than 3.0).

It is beyond the scope of this manuscript to present all the work done on the alternate columns. During method development activities, we have conducted search on the websites such as the USP and PQRI databases (http://www.usp.org/USPNF/columnsDB.html), or other tools/charts provided by column manufacturers, column comparison charts provided by column vendors, or some software that help identify equivalent columns. However, based on our experiences with the molecules of our projects, it is extremely challenging to find a true equivalent column from the databases and charts that would meet all the system suitability criteria and would maintain the selectivity of all the critical pairs compared with the results of the primary column. Therefore, we did a lot of work to identify a few columns which would provide good performances, lot-to-lot reproducibility and stability of the column bed. The names and list of the columns presented in this paper were based on

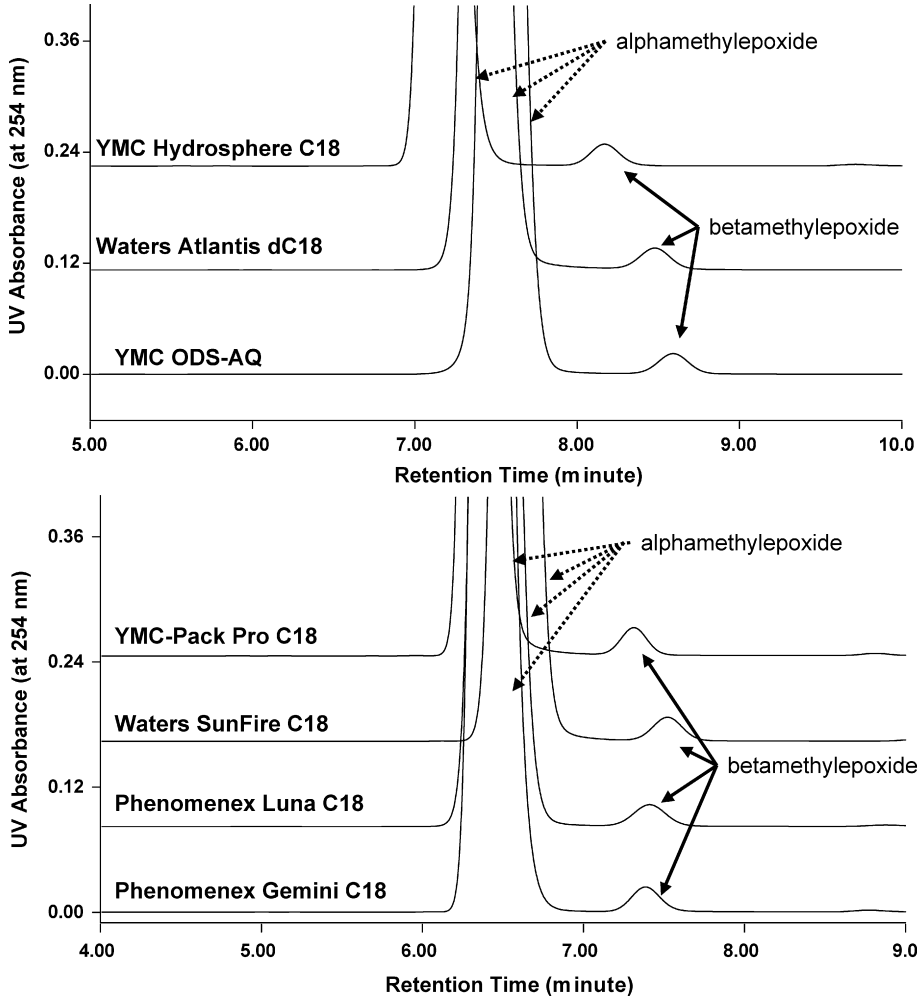


Fig. 7. Overlaid chromatograms of the separation between alphamethylepoxide and betamethylepoxide obtained from various columns. The top chromatograms show separations with a resolution of 3.0, the bottom chromatograms show separations with resolutions between 2.6 and 3.0. The unit of the UV absorbance is AU.

these criteria. The search of the equivalent columns not only can identify an equivalent column, but also can provide more column choices to chromatographers in case other critical pairs need to be separated while the separation between the betamethylepoxide and alphamethylepoxide has to be maintained.

## 4. Conclusions

Use of a computer-assisted method development tool for mobile phase system screening and separation optimization, and an automated column switching system to identify the most appropriate HPLC columns for chromatographic method development has been demonstrated to be powerful and efficient for the development of a new HPLC method. When these advanced method development tools are in the hands of knowledgeable and diligent analytical scientists, the efficiency of HPLC method development can be much enhanced especially when extremely challenging separations are needed. A simple, robust HPLC method for separation of betamethylepoxide and alphamethylepoxide has been developed using the strategy that are described in this paper. This method can accurately quantitate these two epimers even when their concentration ratio is 10,000:1 to each other. Similar separations can be achieved on various columns from different manufacturers. Via this work, highly efficient and rapid method development strategy and process has been presented which can be used as a general strategy for any HPLC method development project. This strategy would significantly save the time of the bench scientists and would also increase the probability of developing an optimum method for intended purpose. As a follow-up of this work, two stability-indicating methods for assay of either alphamethylepoxide or betamethylepoxide samples from commercial lots have been developed based on the similar workflow.

## Acknowledgement

The authors would like to thank all the analytical scientists in Schering-Plough Global Quality Services – Analytical Sciences group for their support of this study.

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