Quantitation of Trace Betamethasone or Dexamethasone in Dexamethasone or Betamethasone Active Pharmaceutical Ingredients by Reversed-Phase High-Performance Liquid Chromatography

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Abstract

Adequate separation is essential for the quantitation of trace amounts of dexamethasone that are typically found in betamethasone active pharmaceutical ingredients and vice versa. In this paper, we describe three simple and efficient high-performance liquid chromatography methods from which true baseline separations between betamethasone and dexamethasone are achieved even when the concentration ratios between these two epimers are larger than 2000:1. One method is developed on a 5 cm ACE C_8 column that uses water and acetonitrile as the mobile phase and 20mM β-cyclodextrin as the mobile phase additive. The resolution factor between betamethasone and dexamethasone is 3.3. The second method is developed on a 10 cm ACE C_8 column that uses water and acetonitrile as the mobile phase, in which the resolution factor between the epimers is 2.7. The third method is developed on a 10 cm ACE C_8 column using water and tetrahydrofuran as the mobile phase, in which the resolution factor between the epimers is 3.1. Preliminary validation studies are carried out for the second and third methods.

Introduction

Betamethasone, dexamethasone (see Figure 1 for the chemical structures), and their ester derivatives are synthetic glucocorticoids used as anti-inflammatory or immunosuppressive agents, and are used in treatment of allergies, arthritis, asthma, etc (1–3). The beta- and dexa- forms of these molecules are epimers with identical chemical structures except that the orientation of the methyl group at the C-16 position is in the opposite direction from the plane. Despite minor spatial differences in structures, different isomeric forms of an active pharmaceutical ingredient (API) may have vastly different physiological effects (4–6). One isomer can be beneficial, while the other isomer might be toxic to human beings. It is preferred that the API of a pharmaceutical product is in one pure form instead of containing mixed isomers. Therefore, a reliable analytical method must be developed to accurately quantitate each of the stereoisomers in either betamethasone or dexamethasone API.

The development of a rugged, robust, sensitive, and efficient reversed-phase high-performance liquid chromatography (RP-HPLC) method that can separate stereoisomers with chemical structure differences as small as betamethasone and dexamethasone is challenging (7–10). The physicochemical characteristics of these two compounds are very similar (11,12). Therefore, it would be difficult to obtain a mobile phase and a stationary phase that could provide adequate differences in thermodynamic parameters (entropy, enthalpy, etc.) between these two epimers for a true baseline separation. Previously, separation of betamethasone and dexamethasone has been attempted by normal-phase and RP-HPLC (7–10). Derivatization was carried out prior to the normal-phase separation (10). Partial separation of the two isomers was obtained with resolutions of approximately 0.9 to 1.5 under reversed-phase conditions (7–9). However, a resolution factor of 1.5 is only adequate for baseline separation of two peaks with similar sizes (13).

Therefore, the objective of our work was to achieve a baseline separation of betamethasone and dexamethasone when one is another's impurity (i.e., concentration ratio of 1000:1 or more to each other). We were able to develop three different methods, and some preliminary validation studies (linearity, recovery, accuracy, limit of quantitation, and robustness) were conducted for two out of the three methods, which will be described in

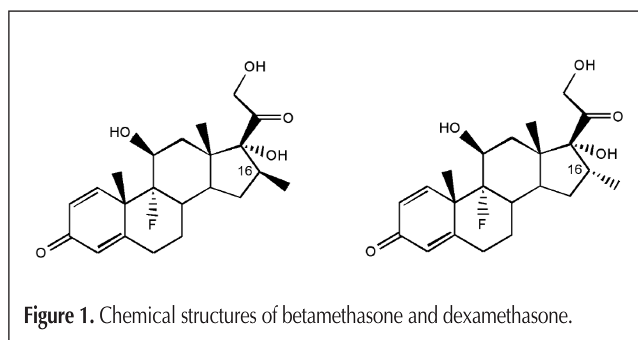

Figure 1. Chemical structures of betamethasone and dexamethasone.

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detail in the Results and Discussion section. Only preliminary validation studies were performed because it was not our intention to perform a full validation for the methods described in this paper. These methods were developed during our endeavor to develop a stability-indicating method for betamethasone and an estimation of its related compounds. A full validation was performed on the final betamethasone assay and impurity/degradation profile method, and the development and validation study will be reported elsewhere.

During the method development, we utilized a chromatographic method development tool with artificial intelligence (ChromSword) (14) to expedite the method development process. Among many computer-assisted HPLC method development software such as Drylab (15), ACD (16), Waters Automated Method Development System (AMDS), and Perkin Elmer Turbo Method Development software, etc., ChromSword is one of the tools that is capable of fully automated method development. ChromSword auto mode runs the experiments, collects data, evaluates the results, performs calculations, makes decisions, optimizes the separation, and generates the results almost 100% unattended. From these results, ChromSword generates a new mobile phase condition, runs the sample, and repeats the process until no further improvement can be made with the given mobile phase and stationary phase. Using this artificial intelligence for HPLC method development, one can save significant amount of time and enhance the probability of achieving a better separation condition compared to a 100% conventional mode of method development (i.e., manual trial-and-error approach based on chromatographer's knowledge and experiences) (14).

Experimental

Chemicals and reagents

The reference standards and samples of betamethasone and dexamethasone were provided by Global Quality Services–Analytical Sciences Group in Schering-Plough (Union, NJ) or purchased from USP (Rockville, MD) or Sigma-Aldrich (St. Louis, MO). β-Cyclodextrin was purchased from Acros Organic (Morris Plains, NJ). All HPLC-grade solvents were obtained from Fisher Scientific (Hampton, NH). Water (18.2 MΩ.cm) was obtained using a Milli-Q system (Millipore, Milford, MA).

Apparatus and HPLC conditions

A Hitachi LaChrom Elite HPLC system (Hitachi High Technologies America, Inc., San Jose, CA) equipped with ChromSword method development tool (Merck KGaA, Darmstadt, Germany) and a Waters 2695 Alliance HPLC system (Milford, MA) 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. Data acquisition, analysis, and reporting were performed (except ChromSword simulation) by EZChrom Elite (Hitachi) and Millennium32 (Waters) chromatography software. The HPLC columns were purchased through vendors such as Waters Corp., MAC-MOD Analytical, Inc. (Chadds Ford, PA), Thermo Fisher Scientific, Inc. (Waltham, MA), or Phenomenex (Torrance, CA). The analytical wavelength used for detection was 240 nm and samples of 10 µL were injected using auto samplers. The preliminary linearity and robustness data presented in this paper were obtained on 10 cm ACE C_8 columns with different lot numbers, and different Waters HPLC instruments were used.

Mobile phase and sample preparation

The mobile phases were prepared by mixing, either on-line or off-line, appropriate amounts of HPLC grade acetonitrile, methanol, isopropanol, THF, or Milli-Q water. The mixtures were degassed by sonication for no 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 components in the mixture during vacuum filtration. A betamethasone standard solution at 1.0 mg/mL spiked with 0.01 mg/mL dexamethasone was prepared for experiments of solvent screening. A betamethasone standard solution at 1.0 mg/mL, a dexamethasone standard solution at a 0.1 mg/mL, and the betamethasone standard solution at 1.0 mg/mL spiked with 0.01 mg/mL dexamethasone were prepared for ChromSword auto method development. For betamethasone linearity/sensitivity study, a 1.0 mg/mL dexamethasone standard solution was prepared as the diluent by dissolving (sonication) approximately 1000 mg of dexamethasone standard into 1000 mL methanol. The 100% level betamethasone stock solution was prepared by dissolving 100 mg of the betamethasone standard into 100 mL of the 1.0 mg/mL dexamethasone diluent. The other concentration levels at 10%, 1%, 0.5%, 0.1%, 0.05%, and 0.01% of betamethasone were prepared by a series dilution using 1.0 mg/mL dexamethasone solution as the diluent. The quantitation of betamethasone was performed using an external betamethasone reference standard prepared at 1.0 mg/mL. Similarly, for the dexamethasone linearity/sensitivity study, the dexamethasone solutions at different concentration levels were prepared by using 1.0 mg/mL betamethasone solution as the diluent. To determine the signal-to-noise ratios of betamethasone or dexamethasone, the standard solutions were separately prepared at the appropriate concentration levels. The 2.5mM, 5mM, 10mM, or 20mM β-cyclodextrin aqueous solutions were prepared by dissolving approximately 2.25, 4.5, 9, 18, and 36 g β-cyclodextrin in 800 mL Milli-Q water, respectively, stirred, and heated to dissolve. The flow rate was 1.0 mL/min for all the experiments using β-cyclodextrin as the mobile phase additive.

Results and Discussion

Considerations for the method development

From the chemical structures of the two epimers, it appears that neither betamethasone nor dexamethasone has any functional groups that can be easily ionized. Therefore, mobile phase pH or ionic strength should not affect the retention and/or separation of these two molecules under RP-HPLC. Hence the method development should be focused on the selection of suit-

able HPLC columns, optimization of the mobile phase compositions, and fine-tuning of the final elution profile. Although typically the analyte retention time and selectivity will change as a function of temperature as well, the practical usable temperature range is not very wide for methods that are intended for routine analysis. A temperature of approximately 10°C or more above the laboratory room temperature ensures a consistent temperature control by typical commercially available HPLC column heaters.

The column selection should be based on the surface properties of the stationary phases. Although it has been realized that the separation of two isomers is possible under RP-HPLC, the exact mechanism of the separation is not very clear. However, it is clear that the separation of the two isomers cannot be achieved only by the hydrophobic interaction between the analytes and the stationary phase (e.g., C_8 or C_{18} carbon chains). Therefore, surface modification of the stationary phases may play an important role. For example, Snyder et al. have pointed out that C_{18} columns made from polyfunctional silanes are more effective in the isomer separations than columns with C_{18} chains that are made from monofunctional silanes (17). The subtle difference in the hydrogen bonding, dipole-dipole, or other polar/nonpolar 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 two isomers.

The commonly used organic solvents in RP-HPLC are acetonitrile, methanol, and to a lesser extent, isopropanol and tetrahydrofuran (THF). Among these four solvents, THF can be very different from acetonitrile and alcohols in terms of the elution strength, hydrogen bonding capability, and chromatographic selectivity. In fact, the mobile phase consisting of water and THF had been used to separate betamethasone and dexamethasone by Izquierdo-Hornillos et al. (18). However, THF has to be used carefully because aged THF could have high background absorbance due to the trace UV-absorbing impurities that are generated by the residual peroxides in old THF. Therefore, at the initial stage of the method development, we did not select THF in our study and mainly focused on the search for appropriate combination(s) between acetonitrile, methanol, and isopropanol that would be used as the organic modifiers in the mobile phase. The factor to be determined was whether a mixture of any two of the three organic solvents had to be used for achieving a desired separation, and if so, at what mixing ratio.

Screening of HPLC columns and organic modifiers

More than 10 HPLC columns (50 × 4.6-mm i.d.) were screened. The selected columns were ACE C_8, ACE C_{18}, ACE C_{18} (300 Å), ACE 3 phenyl, TSK-Gel Super-ODS, TSK-Gel Super-Octyl, TSK-Gel Super-phenyl, YMC-Pack Pro C_{18}, YMC Hydrosphere C_{18}, Thermo Fluophase PFP, and Thermo Fluophase RP C_{18}. The selected columns cover a wide range of stationary phase surface properties, such as carbon chain length, carbon loading, and surface functionality. Most of the selected columns are packed with ultra-pure silica particles. TSK columns are packed with 2 µm particles. ACE columns are base deactivated and are well-known for offering good peak shapes. Short chain (C_8) stationary phases were selected based on the consideration that less diffusion is required for the steroid molecules to reach to the surface under the carbon chains, where the secondary interaction takes place. The phenyl columns are quite different from the conventional C_8 or C_{18} columns due to potential π–π interactions. The greater dipole of the carbon–fluorine bond versus the carbon–hydrogen bond makes the perfluorinated stationary phase unique in the retention of polar and halogenated compounds (18). The perfluorinated phases have also been shown to have shape selectivity for positional/geometric isomers (19). The YMC-Pack Pro C_{18} column possesses a unique endcapping procedure utilizing Lewis acid–Lewis base chemistry. The YMC Hydrosphere C_{18} column can be used under 100% aqueous conditions for the separation of polar compounds, which indicate a strong potential for hydrogen bonding interaction with the analytes. A wide pore ACE C_{18} column with a pore size of 300 Å was also selected for the column screening. Although wide-pore silica particles are usually used for analysis of large molecules such as proteins and nucleic acids, 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.

For organic modifier screening, combinations of acetonitrile, methanol, and isopropanol were examined on each selected column. To search for the appropriate organic modifiers and their mixing combinations, we used the model mode of ChromSword. The computer modeling is based on imported 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: 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 betamethasone and dexamethasone peaks.

The results from the column and organic modifier screening experiments suggested that the 5-cm ACE C_8 was the most promising column, and the most promising organic modifier was neat acetonitrile. The presence of alcohols in the mobile phase negatively affected the resolution between betamethasone and dexamethasone. The best separation occurred under an isocratic condition when the mobile phase consisted of water–acetonitrile at 85:15 (v/v). However, under this isocratic condition, the retention times were approximately 30 min for betamethasone and dexamethasone at a flow rate of 2.0 mL/min, while the resolution factor achieved was only approximately 1.8.

Improving the separation using cyclodextrin as the mobile phase additive

To improve the resolution between betamethasone and dexamethasone on the 5-cm ACE C_8 column, we used β-cyclodextrin as the mobile phase additive. Cyclodextrins are well known as inclusion-complexing agents for both small and large

molecules (20). The interior of the macrocyclic structure is hydrophobic while the exterior is water-compatible due to the existence of many hydroxyl groups. Among the commonly available native cyclodextrins (i.e., the α-, β-, and γ-cyclodextrins), the β-cyclodextrin and γ-cyclodextrin have been largely used as mobile phase additives in chromatography as chiral selectors for the separation of various isomers, including structural, diastereomeric, and enantiomeric molecules (21). In fact, the interactions between steroids and various cyclodextrins have been studied in detail by many research groups (22). The most popular hypothesis on the mechanism of separation in the presence of cyclodextrin is that the inclusion occurs primarily at the A- and B-rings of the steroids, which determines the binding strength of the inclusion complex. The hydrogen bonding interaction occurring between the secondary hydroxyl groups of the cyclodextrins and the hydroxyl groups of the steroid molecules can create the binding selectivity between the steroid isomers. For example, the γ-cyclodextrin has been shown to less selectively bind betamethasone or dexamethasone than β-cyclodextrin does, presumably due to the larger diameter of the secondary hydroxyl rim which is 8.3 Å for γ-cyclodextrin and 6.5 Å for β-cyclodextrin, respectively (23). The smaller diameter of the β-cyclodextrin restricts the entry of the C- and D-rings of the steroids into the cyclodextrin cavity more than γ-cyclodextrin does, and thus provides a higher probability of hydrogen bonding interaction between its secondary hydroxyl groups and the hydroxyl group at the 17-position of the D-ring of the steroids. This hydrogen bonding interaction can be affected by the orientation of the methyl group at the 16-position, and therefore the complexation can be different between β-cyclodextrin-betamethasone and β-cyclodextrin-dexamethasone. Indeed, the apparent association constants (K_f) of β-cyclodextrin-betamethasone complex and β-cyclodextrin-dexamethasone complex are reported as 27 and 22, respectively, in an acetonitrile–water mixture (35:65, v/v), while the K_f values for γ-cyclodextrin-betamethasone complex and γ-cyclodextrin-dexamethasone complex are 212 and 215, respectively (23). Although the absolute K_f values of the β-cyclodextrin-steroid complexes are much smaller than those of γ-cyclodextrin-steroid complexes (which indicate a weaker interaction due to the size restriction) the relative difference in the binding is approximately 19% in the former ones versus only 1% in the latter ones. In other words, the binding selectivity is greater in β-cyclodextrin-steroid complexes.

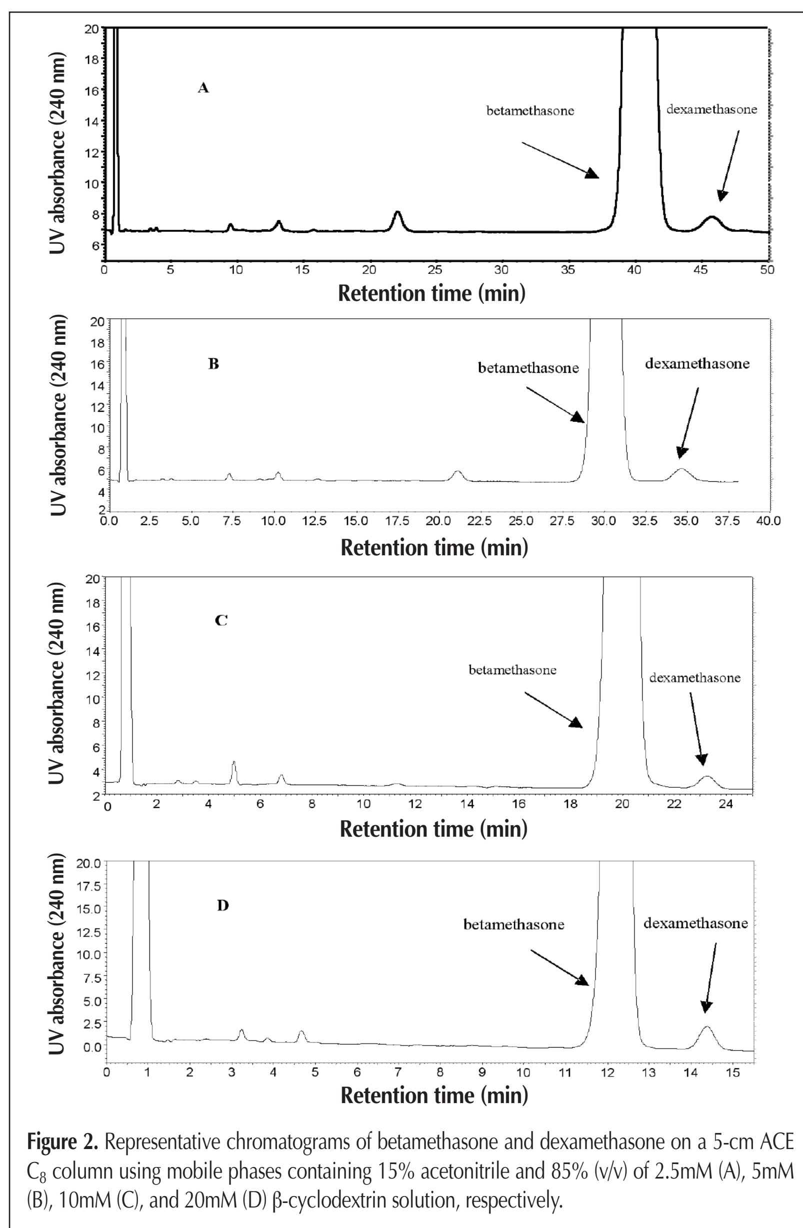

Figure 2. Representative chromatograms of betamethasone and dexamethasone on a 5-cm ACE C_8 column using mobile phases containing 15% acetonitrile and 85% (v/v) of 2.5mM (A), 5mM (B), 10mM (C), and 20mM (D) β-cyclodextrin solution, respectively.

Figure 2 shows the separation achieved when the mobile phase consisted of a mixture of 2.5mM, 5mM, 10mM, or 20mM β-cyclodextrin aqueous solution, respectively, and acetonitrile at a volume ratio of 85:15. As expected, the addition of β-cyclodextrin greatly improved the separation between betamethasone and dexamethasone. The retention time of betamethasone and dexamethasone and the separation resolution between these two isomers were largely affected by the β-cyclodextrin concentration. The resolution factor was 3.3 when the concentration of β-cyclodextrin was 20mM, while the resolution factor reduced to 2.4 when the β-cyclodextrin concentration was 2.5mM. The retention times became shorter with higher β-cyclodextrin concentrations. These observations are consistent with literature reports that an increase in β-cyclodextrin concentration would significantly decrease the retention of the analytes and meanwhile improve the separation resolution (24). The complexation occurs in the mobile phase and the complexes are carried out through the column faster than the analytes in the absence of the complexation, due to a much less retention of β-cyclodextrin.

Up to this point, we have demonstrated that an adequate resolution can be achieved for the separation of betamethasone and dexamethasone by using β-cyclodextrin as the mobile phase additive. After that, as part of the method development for separating other betamethasone-related compounds, we

moved forward to develop a gradient elution. Because there were some highly hydrophobic impurities in the betamethasone samples tested, the acetonitrile content in the gradient was changed from 15% (v/v) to approximately 50% (v/v). Owing to the limited solubility of β-cyclodextrin in water (approximately 2.2 g/100 mL at 30°C, which is equivalent to a 19.8mM solution) (25), the concentration of β-cyclodextrin was selected at 10mM for that study. The corresponding retention time of betamethasone was approximately 20 min with a flow rate of 1.0 mL/min. Peak distortion, however, appeared when the gradient was applied. The betamethasone peak started broadening after approximately 15 injections and peak splitting finally occurred. To quickly check the column longevity, we performed 30 injections under the isocratic condition (i.e., mobile phase consisted of water–acetonitrile at 85:15 [v/v] with 10mM β-cyclodextrin as the additive). No peak broadening or distortion was observed. Because cyclodextrin is typically used in isocratic elutions in which the water content is kept as high as possible in the mobile phase, the peak distortion observed during gradient elution was presumably due to a slow equilibration of the cyclodextrin between the mobile phase and stationary phase under the constant change in the acetonitrile concentration. Because of the peak distortion, although good separation between betamethasone and dexamethasone was achieved, we did not perform any quantitation studies using this method. Instead, we moved the method development forward to develop a method that was not only suitable for the quantitation of betamethasone and dexamethasone, but also from which a betamethasone impurity/degradation profile method could be developed.

Separations achieved on a 10-cm ACE C_8 column

Although limited improvement in resolution can be obtained by just increasing the column length, because the resolution factor obtained on the 5-cm ACE C_8 column with water–acetonitrile as the mobile phase was close to 2.0, further method development was carried out on a 10-cm ACE C_8 column. As the HPLC column and mobile phase components had already been screened and selected, what needed to be optimized was the elution profile. For that purpose, the ChromSword auto option was used. ChromSword auto is the most powerful operation mode of this chromatographic method development tool. At a given column and mobile phase solvent, ChromSword auto will control the instrument and automatically run the experiments, analyze the results, and optimize the mobile phase conditions until no further improvement in the separation can be achieved. During the automated method development exercise, the artificial intelligence searches for the best isocratic mobile phase condition and the best linear gradient. For our purpose, we have found that the information from the isocratic runs is more useful than the separation from the linear gradient. In the gradient run, ChromSword auto looks for a separation that can provide a resolution of approximately 2.0. However, a resolution factor higher than 2.0 is necessary for a true baseline separation of a minor peak from a major peak when the concentration of the minor peak is approximately 0.1% or lower compared to the major peak.

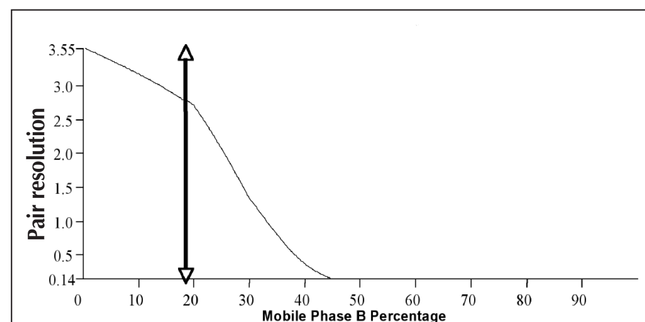

Figure 3. Pair resolution map simulated by the ChromSword auto. The arrow is a cursor that can be moved horizontally across the resolution map to obtain the predicted pair resolution (*Y*-axis) between betamethasone and dexamethasone at the corresponding mobile phase B percentage (*X*-axis).

The HPLC system was set up using water as the mobile phase A and acetonitrile as the mobile phase B. The 10 cm ACE C_8 column was installed in the column chamber at a temperature of 35ºC, and the flow rate of 2.0 mL/min was used for this experiment. Reference standard solutions of betamethasone and dexamethasone were prepared in methanol at concentrations of approximately 1.0 and 0.01 mg/mL, respectively. The reference standard solutions were prepared at relatively high concentrations to ensure that the peaks of interest were large enough to meet the minimum peak area requirement that is needed by ChromSword auto to recognize a peak in the sample. After 7.5 h of fully automated work, the ChromSword autogenerated a report that presented a best isocratic method in which the mobile phases consisted of 80% of water and 20% of acetonitrile (v/v). At this mobile phase composition, the resolution factor between betamethasone and dexamethasone was 2.8, and the HPLC run time was approximately 20 min. A simulated resolution map from the automated runs is shown in Figure 3. Interestingly, although the predicted resolution map covered the mobile phase B percentages from almost 0% to approximately 45%, ChromSword auto only experimentally tested the mobile phase B percentages between 18% to 35%. The other parts in the resolution map were generated by this artificial intelligence method development tool based on its model building. The ChromSword auto was intelligent enough not to test a mobile phase B at, for example, 10% because although the predicted resolution was higher than 3.0, the real HPLC run-time might be unacceptably long. The arrow in Figure 3 is the cursor that can be moved horizontally across the resolution map to obtain the predicted resolution (Y-axis) at the corresponding modifier (acetonitrile) percentage (X-axis). By moving the cursor across the X-axis, we could review simulated chromatograms from ChromSword. However, we found that the retention times of the betamethasone and dexamethasone could shift as large as 5 min with one percent change in the volume ratio of acetonitrile (i.e., from 20% to 19% or 21%). Experimental results also confirmed this finding. Although it is well known that the isocratic retention depends on the solvent strength and type, the strong impact observed of the solvent strength on retention of the steroid compounds makes the isocratic methods not very suitable for routine analysis. Therefore, a semi-manual development of a gradient profile was carried out based on the results of the ChromSword auto. By changing the acetonitrile percentage on the simulated resolution map, we found that using a mobile phase containing

19% (v/v) acetonitrile could provide a resolution of approximately 2.8 within 21 min, and a resolution factor of 2.0 could still be obtained at 25% (v/v) acetonitrile. Therefore, we made the 19% acetonitrile as the gradient starting point and the 25% acetonitrile as the gradient end point. The gradient run time was initially set as 20 min and was finalized to be 18 min after several trial-and-error runs (see Figure 4 for chromatograms). The resolution factor between betamethasone and dexamethasone was 2.7 in this fine-tuned method. The robustness of the fine-tuned gradient method was briefly tested by changing the starting or ending acetonitrile percentages. The robustness tests were performed on a different HPLC instrument with a column from a different lot. The resulted retention time shifts were mostly less than one min by comparing the retention times obtained in the fine-tuned condition (i.e., gradient from 19% to 25%) against those of obtained in other tested conditions (Table I). Under the fine-tuned conditions, the betamethasone or dexamethasone retention time difference obtained on different column lots and different HPLC instruments was less than 1 min (see Figure 4 and Table I). Therefore, this gradient elution provided a much more robust/reproducible retention with only a slight scarification of the separation resolution. Preliminary validation studies were performed to check the linearity, accuracy, recovery, and limit of quantitation. The linearity of the UV responses to dexamethasone was tested by spiking dexamethasone from concentration levels of 0.01% to 100% in the presence of 100% betamethasone (i.e., the analytical concentration of 1.0 mg/mL). Figure 4 shows the corresponding overlaid chromatograms. Only the chromatograms of 0.01%–1% are overlaid for visual clarity. Because the betamethasone dilution solution contained a certain level of dexamethasone as an existing impurity, the peak areas of dexamethasone obtained in the linearity solutions were corrected accordingly for quantitation purposes. The chromatograms shown in Figure 4 show raw data obtained from the 0.01% to 1% dexamethasone linearity solutions without peak area correction. Linear regression analysis reveals that in the presence of 1.0 mg/mL betamethasone, the separation between betamethasone and dexamethasone was adequate to ensure a linear response to dexamethasone within the tested concentration range with a coefficient of determination R^2 = 0.99999. The average recovery of the spiked dexamethasone was 98.4% with a standard deviation of 4.9% (n = 21), which is good considering the wide concentration range tested. The limit of quantitation for dexamethasone was determined to be 0.01% of the betamethasone analytical concentration (1.0 mg/mL), because the signal-to-noise ratio obtained from a separately prepared 0.01% dexamethasone solution was 13 (> 10).

Table I. Robustness Test Results Obtained on the 10 cm ACE C_8 Column Using Water as the Mobile Phase A and Acetonitrile as the Mobile Phase B

Experimental Conditions	Retention Time (min) Betamethasone	Dexamethasone
Mobile phase B from 19%–25%	12.5	13.3
Mobile phase B from 19%–24%	11.9	12.6
Mobile phase B from 19%–26%	13.0	13.9
Mobile phase B from 18%–25%	13.4	14.2
Mobile phase B from 20%–25%	11.3	12.1

However, an unexpected result was obtained when using the same method to test the quantitation of betamethasone in the presence of 1.0 mg/mL dexamethasone API. Figure 5 shows that the betamethasone peak (approximately 0.1%) does not return to the baseline. This phenomenon was observed in all the dexamethasone samples we tested, including dexamethasone that was purchased from Sigma-Aldrich at HPLC grade, USP grade, and Sigma reference standard grade. Apparently, there is some unknown impurity eluted between the betamethasone and dexamethasone peaks. Evaluation of the integration of the betamethasone peak by baseline drop and by baseline skimming revealed that a quantitation error as large as 50% could occur depending on the selected integration parameters.

Therefore, it became necessary to modify the conditions of the current method or to develop another method to accurately quantitate betamethasone in the dexamethasone API. Based on the knowledge gained from the column and organic modifier screening, we knew that the separation conditions developed on

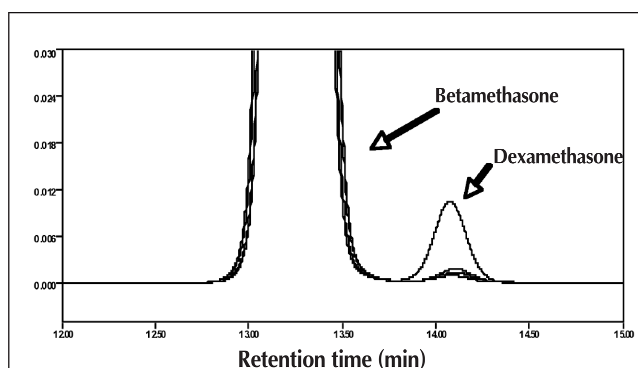

Figure 4. Overlaid chromatograms obtained from dexamethasone linearity study. Only the chromatograms obtained in the linearity range from 0.01% to 1% dexamethasone are overlaid for visual clarity. From top chromatogram to bottom, the corresponding dexamethasone concentrations are 0.01 mg/mL (1%), 0.001 mg/mL (0.1%), 0.0005 mg/mL (0.05%), and 0.0001 mg/mL (0.01%), respectively.

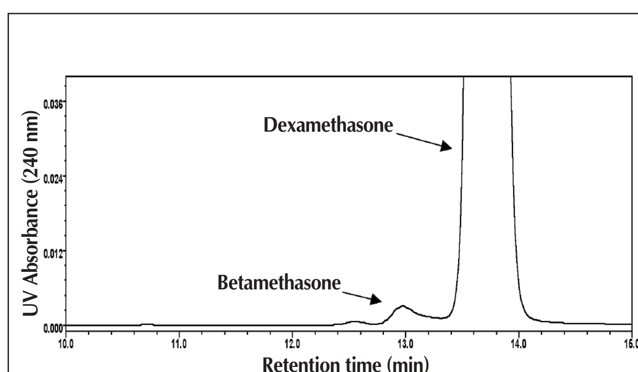

Figure 5. A chromatogram of dexamethasone API obtained at a concentration of 1.0 mg/mL. Betamethasone was at approximately 0.1% level as an existing impurity in the dexamethasone API. The mobile phase consisted of water and acetonitrile.

the 10-cm ACE C_8 column were the best possible method to achieve adequate resolution on the tested columns. At this stage, we decided to study the separation using tetrahydrofuran (THF) as one of the organic modifiers. As mentioned before, THF should not be the first choice. However, THF should be tried as a last resort for a difficult separation because it may provide some interesting results due to its unique properties. With the help of ChromSword, we quickly screened different combinations of THF with other organic modifiers, and the results indicated that the water–THF mixture would be the most effective mobile phase system. By following the same method development flow (i.e., automated ChromSword method development followed by a semi-manual gradient profile fine-tuning), we found a separation between betamethasone and dexamethasone with a resolution factor as high as 3.1. Because of the higher mobile phase viscosity, the flow-rate of the mobile phase was 1.0 mL/min. The gradient was simply to change the THF percentage in the mobile phase from 17% to 20% (v/v) in 20 min (see Figure 6 for chromatograms). The robustness of the method was also briefly tested by changing the starting or ending THF percentages. Again, the robustness tests were performed on a different HPLC instrument with a column from a different lot. The resulted retention time shifts were also approximately one min for the tested variations (Table II). Under the fine-tuned condition (i.e., gradient from 17% to 20%) the betamethasone or dexamethasone retention time difference obtained on different column lots and different HPLC instruments was about 1 min (see Figure 6 and Table II). Therefore, this gradient elution was also demonstrated to be robust and reproducible. The limit of quantitation for betamethasone was determined to be 0.05% of the dexamethasone analytical concentration (1.0 mg/mL) because the signal-to-noise ratio obtained from a separately prepared 0.05% betamethasone solution was 13 (> 10). The higher limit of quantitation was presumably a result of the higher background absorbance of the water–THF system. Therefore, the concentration of 0.05% was selected as the low end for evaluating the response linearity. Figure 6 shows the corresponding overlaid chromatograms. Only the chromatograms of 0.05%–1% are overlaid for visual clarity. Similarly, due to the existence of a small amount of betamethasone in the dexamethasone API, the betamethasone peak areas were corrected for quantitation purposes, while the chromatograms presented in Figure 6 are the raw data without peak area correction. Linear regression analysis reveals that even in the presence of 1.0 mg/mL dexamethasone, the separation between betamethasone and dexamethasone was adequate to ensure a linear response to betamethasone from 0.005 mg/mL to 1.0 mg/mL with a coefficient of determination $R^2 = 0.99999$. The average recovery of the spiked betamethasone was 96.2% with a standard deviation of 5.7% ($n = 18$).

Table II. Robustness Test Results Obtained on the 10 cm ACE C_8 Column Using Water as the Mobile Phase A and THF as the Mobile Phase B

Experimental Conditions	Retention Time (min) Betamethasone	Retention Time (min) Dexamethasone
Mobile phase B from 17%–20%	16.2	17.8
Mobile phase B from 17%–21%	15.5	17.0
Mobile phase B from 17%–19%	16.9	18.7
Mobile phase B from 16%–20%	17.5	19.1
Mobile phase B from 18%–20%	14.8	16.3

Interestingly, when testing betamethasone API analysis using this THF method, we found that there was an impurity peak co-eluted with dexamethasone. Indeed, the compositions of steroid APIs are usually very complicated, so careful examination of the separation is necessary to ensure the quality of the developed analytical method.

As a final remark, we would like to point out that the peak resolution (larger than 2.0), response linearity ($R^2 > 0.999$), and quantitation limit (either 0.01% or 0.05%) of betamethasone or dexamethasone achieved on the 10-cm ACE C_8 column all exceeded or met the requirements set by pharmacopoeia (e.g., USP). Also, although we did not perform a formal column lifetime test, the 10-cm ACE C_8 column could at least sustain more than 50 injections with either water–acetonitrile or water–THF as the mobile phase.

Conclusion

Three simple and efficient HPLC methods for a true baseline separation of betamethasone and dexamethasone are described in this paper. The first method was developed on a 5-cm ACE C_8 column that used 20mM β-cyclodextrin as the mobile phase additive. The resolution factor between these two epimers was 3.3. The second method was developed on a 10-cm ACE C_8 column that used water as mobile phase A and acetonitrile as mobile phase B. The resolution factor between betamethasone and dexamethasone was 2.7. Dexamethasone was accurately quantitated even in the presence of betamethasone API whose concentration was 10,000 times higher. The third method was developed on the 10-cm ACE C_8 column using water as the mobile phase A and THF as the mobile phase B. The resolution factor between betamethasone and dexamethasone was 3.1. For this method, betamethasone was accurately quantitated in the

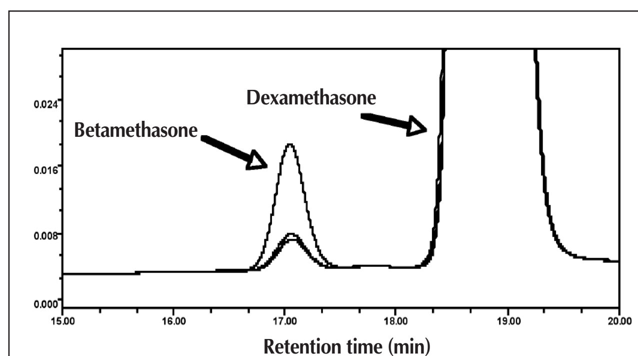

Figure 6. Overlaid chromatograms obtained from betamethasone linearity study. Only the chromatograms obtained in the linearity range from 0.05% to 1% betamethasone are overlaid for visual clarity. From top chromatogram to bottom, the corresponding betamethasone concentrations are 0.01 mg/mL (1%), 0.001 mg/mL (0.1%), and 0.0005 mg/mL (0.05%), respectively.

presence of dexamethasone API whose concentration was 2,000 times higher. From this study, we have demonstrated that a true baseline separation can be achieved for steroid stereoisomers under RP-HPLC conditions. During method development, a chromatographic method development tool with artificial intelligence (ChromSword) was used to optimize the separations. Based on our experiences gained during the method development, we would like to point out that the combination of advanced chromatographic method development tools, and knowledgeable and careful bench analytical scientists, is the key to success in challenging separations.

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