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A stability-indicating reversed-phase high performance liquid chromatography method for simultaneous assay of two corticosteroids and estimation of their related compounds in a pharmaceutical injectable formulation

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ABSTRACT

Betamethasone Sodium Phosphate and Betamethasone Acetate are the two corticosteroids active pharmaceutical ingredients (APIs) that are present in the injectable formulation, Celestone Chronodose® Injection. It is extremely challenging to develop a Quality Control friendly HPLC method to separate all the potential impurities and degradation products of the two APIs from each other using a single HPLC method. A novel stability-indicating reversed-phase HPLC (RP-HPLC) method using two oxo-cyclic organic modifiers in the mobile phase was developed and validated. This method can separate a total of 32 potential impurities and degradation products from the two APIs and also from each other. Peak symmetry and separation efficiency were enhanced by using two chaotropic agents (trifluoroacetic acid and potassium hexafluorophosphate) in the mobile phases of this method. The stability-indicating capability of this method has been demonstrated by analyzing aged and stressed degraded stability samples of the drug product. This method uses an ACE 3 C18 (15 cm × 4.6 mm) HPLC column. The method was validated per ICH guidelines and proved to be suitable for routine QC use.

1. Introduction

Celestone Chronodose® Injection is a sterile aqueous suspension containing two corticosteroid active pharmaceutical ingredients (APIs). One API, namely Betamethasone Sodium Phosphate (BSP), is highly water soluble and the other API, namely Betamethasone Acetate (BA), is highly insoluble in water. The clinically effective compound of the two APIs is their metabolite Betamethasone (BTM; compound **14** in Fig. 1). Each milliliter (mL) of Celestone Chronodose® Injection contains 5.7 mg of Betamethasone, as 3.9 mg of BSP (in solution) and 3 mg of BA (in suspension) in an aqueous solution. The two APIs in Celestone Chronodose® Injection drug product are potent anti-inflammatory, antirheumatic, and antiallergic agents and are highly effective in the treatment of corticosteroid-responsive disorders. Corticosteroids suppress inflammation by reducing the production of hormone-like substances in the body that activates specific immune system which causes inflammation. Celestone Chronodose® is also used to prevent respiratory distress syndrome in premature infants. Prompt therapeutic activity is achieved by BSP, which is absorbed quickly after injection. Sustained activity is provided by BA, which is highly insoluble and becomes a repository for slow absorption, thereby controlling symptoms over a prolonged period.

The dosage regiments of this drug product are extremely low because the two APIs in Celestone Chronodose® Injection are highly potent. Therefore, a robust analytical method with high accuracy and sensitivity is required to determine the assay values of BSP and BA, and to estimate the impurities and degradants in this drug product. Accurate estimation of all the impurities and degradation products (including potency for the API) in any pharmaceutical product is required by the health authorities of various countries. A number of HPLC methods to analyze either BSP or BA in the pharmaceutical formulations or biological fluids are reported in the literature [1–3]. However, to the best of our knowledge, there is no HPLC method reported in the literature for simultaneous determination of the two APIs (BSP and BA) and estimation of their related compounds in any drug product formulations. Therefore, our objective was to develop a single HPLC method that can be used by QC laboratories for routine analysis of BSP and BA including the impurities and degradation products in Celestone Chronodose® Injection.

In this paper, we report a sensitive, rugged, and robust HPLC method for simultaneous identification and assay of the two APIs (BSP and BA) and for the estimation of their 32 known impurities/degradants in Celestone Chronodose® drug product. Chemical

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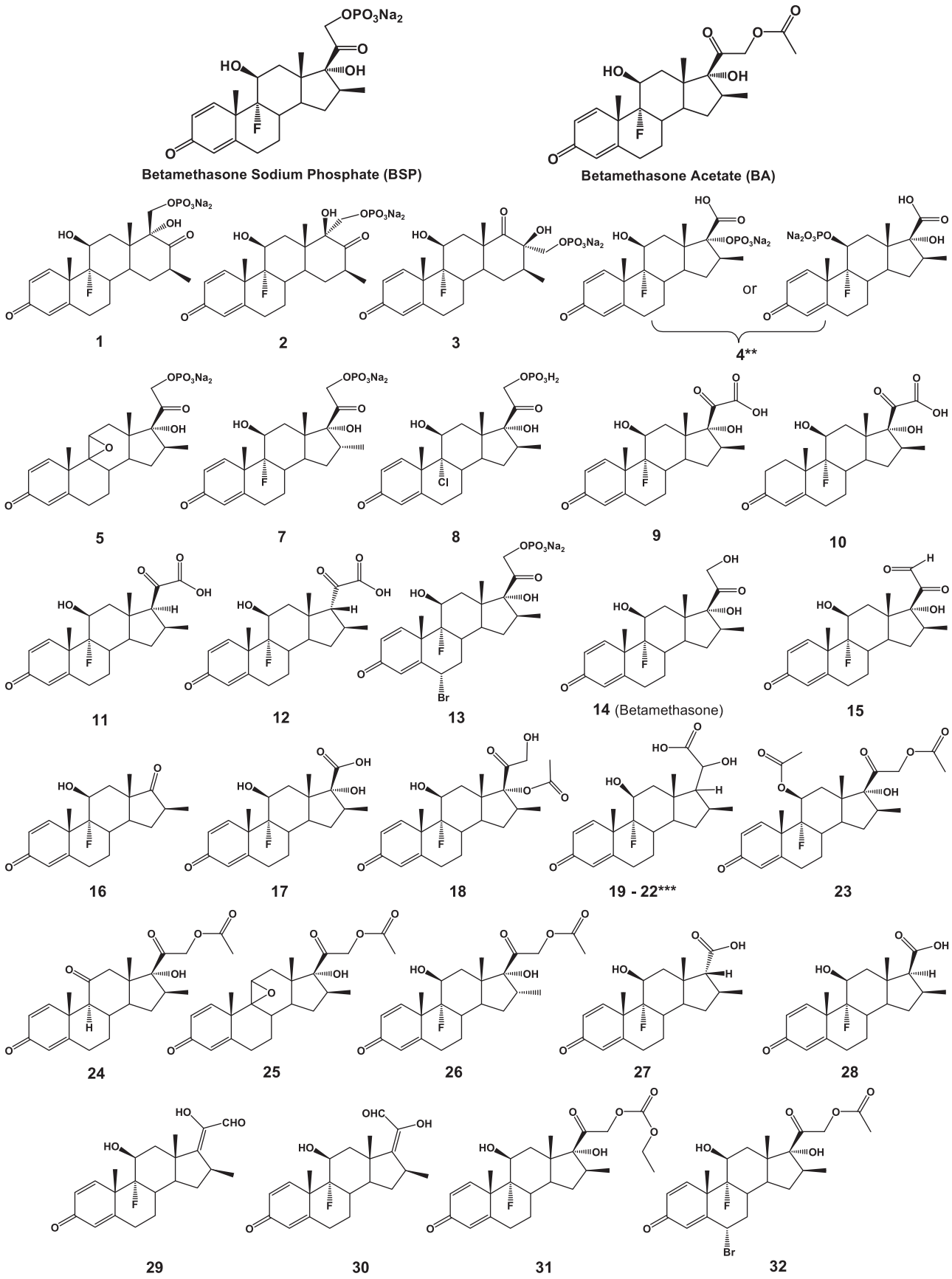

Fig. 1. Chemical structures of BSP, BA, and the impurities/degradants monitored in the new HPLC method. Chemical Structure of compound **6** is not available. (Notes: **Position of the sodium phosphate group in compound **4** could not be determined and can be at either C11 or C17. ***The steric orientation of the bonds at the two chiral centers at C17 and C20 could not be determined for compounds **19**–**22**.).

structures of BSP, BA, and the impurities/degradants of the two APIs are shown in Fig. 1. The 32 impurities/degradation compounds were identified from the API sources and aged product batches, as well as the potential degradation chemistry. Separation of the two APIs and their 32 impurities/degradation compounds from each other is an extremely challenging task. The presence of several isomeric pairs (e.g., BSP and compound **7**, BA and compound **26**) made the task of developing a rugged, sensitive, and reproducible RP-HPLC method even more challenging.

Compared to the commonly used organic modifiers such as acetonitrile and methanol in RP-HPLC, oxo-cyclic organic solvents such as tetrahydrofuran (THF) and 1,4-dioxane provide different and sometime even unique selectivity for certain type of molecules. Tetrahydrofuran has been used and studied as a mobile phase modifier both in reversed-phase and normal-phase chromatography. However, 1,4-dioxane has not been thoroughly studied or used as a mobile phase modifier in RP-HPLC. A few papers reported in the literature used 1, 4-dioxane as the organic modifiers in thin layer, reversed-phase and normal-phase chromatography [4–11]. Chaotropic agents (also known as chaotropic reagents and chaotropes), such as trifluoroacetate, perchlorate, and hexafluorophosphate [12], are large ions or ions with low charge density which have been used to disrupt the three dimensional structure to denature macromolecules such as proteins. In recent years, chaotropic agents were studied in the mobile phase of RP-HPLC for improving the peak shape and also impacting the retention of cationic solutes [13–22]. Although the detailed mechanism of the chaotropic effect in liquid chromatography is not fully understood, there are a number of theories which attempted to explain the nature of the interactions and the mechanism of the chaotropic effect on cationic analytes in liquid chromatography. For example, one model to explain the chaotropic effect is a non-specific "ion association" mechanism dependent on the characteristics of the chaotropic co-solvents [19–22].

2. Experimental

2.1. Materials

The Celestone Chronodose® Injection product, BSP, BA, and the impurities/degradation products were provided by Merck & Co. Inc. (Union, NJ). HPLC grade water, acetonitrile, methanol, and acetic acid, USP/FCC potassium citrate monohydrate, reagent grade perchloric acid (70%), 1 N sulfuric acid, and 1 N potassium hydroxide were purchased from Fisher Scientific (Hampton, NH). HPLC grade tetrahydrofuran (without preservative/stabilizer), HPLC grade 1,4-dioxane, 98.5+% sodium hexafluorophosphate, 99% potassium hexafluorophosphate, 97% pentafluoropropionic acid, 99% heptafluorobutyric acid, and 99+% lithium perchlorate were purchased from Acros (Fair Lawn, NJ). ACS grade sodium citrate dihydrate and 99.5+% trifluoroacetic acid was purchased from Alfa Aesar (Ward Hill, MA). Milli-Q grade water was obtained from an in-house Millipore system (Millipore, Billerica, MA).

2.2. Instrumentation

An Agilent Technologies 1200 Series HPLC system (Agilent, Santa Clara, CA) with a LC Spiderling™ column switching system (Chiralizer™ Services, L.L.C., Newtown, PA) and a Waters 2695 Alliance HPLC systems (Waters, Milford, MA) were used for method development. During the validation, Waters HPLC systems equipped with 2695 separation modules and 2996 photodiode-array detector and/or 2487 dual wavelength UV detectors and an Agilent Technologies 1100 Series HPLC system were used. All HPLC systems were equipped with a column compartment with temperature control and an on-line degasser. Data acquisition, analysis, and reporting were performed by ChemStation (Agilent) and Empower2 (Waters) chromatography software. The ACE 3 C18 column (150 mm × 4.6 mm I.D., 3 μm particle size, 100 Å pore size) was purchased from Mac-Mod Analytical Inc.

2.3. Chromatographic conditions

The aqueous solution for preparing the mobile phases contains 0.5% (v/v) trifluoroacetic acid, 31 mM potassium citrate, and 20 mM potassium hexafluorophosphate, which maintain the pH of the aqueous solution at approximately 3.50. Mobile Phase A consists of 1,4-dioxane:tetrahydrofuran:aqueous solution at 4:4:92 (v/v/v), and Mobile Phase B consists of 1,4-dioxane:tetrahydrofuran:aqueous solution at 14:28:58 (v/v/v). The gradient is linear from 0% to 100% Mobile Phase B in 60 min. The flow rate is 1.0 mL/min and the column temperature is 40 °C. Injection volume was 30 μL and detection wavelength was 254 nm.

2.4. Sample and standard solution preparations

The sample solution was prepared by diluting the Celestone Chronodose® Injection product in water–acetonitrile–acetic acid (70:30:1, v/v/v) to achieve concentrations of BSP and BA as 0.2 and 0.15 mg/mL, respectively. Standard solutions of BSP, BA, and/or their impurities/degradation products were prepared by dissolving accurate and appropriate amounts of the compounds in water–acetonitrile–acetic acid (70:30:1, v/v/v). To determine the linearity of BSP and BA, triplicate preparations of BSP and BA at each of eight concentration levels were carried out, respectively. The eight concentration levels were 0.05%, 0.5%, 5%, 50%, 75%, 100%, 125% and 150% with respect to the BSP (0.2 mg/mL) and BA (0.15 mg/mL) analytical concentration, respectively. The linearity of compound **14** was determined using triplicate preparations of compound **14** at each of the eight concentration levels of 0.05%, 0.1%, 0.25%, 0.5%, 1%, 2.5%, 5%, and 10% with respect to the BA analytical concentration (0.15 mg/mL). The linearity of the other validated impurities/degradation products was determined using triplicate preparations of the impurities/degradation products at each of the six concentration levels of 0.05%, 0.1%, 0.2%, 0.5%, 1%, and 2% with respect to the BA analytical concentration (0.15 mg/mL).

2.5. Calculations

The quantitation of BSP, BA, and their impurities/degradation products was carried out using an external reference standard solution containing BSP and BA at 0.2 and 0.15 mg/mL, respectively. The quantitation of BSP was based on the BSP peak area in the external reference standard solution. The quantitation of BA and the impurities/degradation products was based on the BA peak area in the external reference standard solution. The testing solutions were bracketed between two reference standard solutions and the experimental concentration was obtained from the following equation:

$$\text{Experimental Concentration} = \frac{P_2}{\text{RRF} \times P_1} \times C_1$$

where P_1 = Average peak area of BSP or BA in the adjacent bracketing standards; P_2 = Peak area of BSP, BA, or the impurities/degradation products in test solution; C_1 = Concentration of BSP or BA in bracketing standard; RRF = relative response factor (=1.00 for BSP or BA)

Experimentally, the RRF was determined by the following equation:

$$\text{RRF} \frac{\dfrac{\text{Response Factor of the Individual Impurity/Degradation Product}}{\text{Response Factor of API}}}{\dfrac{\text{Slope of the Linear Regression Curve of the Individual Impurity/Degradation Product}}{\text{Slope of the Linear Regression Curve of API}}}$$

The recovery of each concentration level was then determined by the following equation:

$$\%\text{Recovery} = \frac{\text{Experimental Concentration}}{\text{Prepared Concentration}} \times 100$$

The adjusted retention time ($t'R$) is defined and calculated as:

$$t'R = t_R - t_0$$

where t_R is the peak retention time (in min) and t_0 is the column dead time (in min). In this study, t_0 is determined from the retention time of the solvent front peak.

The resolution factor (Rs) was calculated by ChemStation for Agilent HPLC systems or by Empower2 for the Waters HPLC system.

3. Results and discussions

3.1. Method development

A general strategy to develop efficient and rugged new HPLC analytical methods has been recently reported in the literature [23–25]. The strategy utilizes high-throughput automated state-of-the-art method development technologies and platforms to expedite the selection of the most appropriate HPLC column, to optimize the mobile phase composition, and to fine tune the gradient conditions of the final method in the most efficient manner. This strategy was followed for method development and method optimization activities that are presented in this paper.

3.1.1. Column screening

Method development activities of this project were a very challenging task because of the requirement to separate a huge number of peaks in a reasonable run time. It was critical to identify a suitable HPLC column that can be used for method development activities. To achieve this goal, preliminary column screening studies were conducted using various C18 and C8 stationary phases manufactured by reputable vendors. The screening was conducted by using the LC Spiderling™ automated nine-column switching system. Acetonitrile was used as the initial mobile phase organic modifier. The aqueous solution contained 0.1% TFA. With 0.1% TFA in the mobile phase (pH ~2), all the compounds should be in non-ionized (neutral) form and, therefore, should be adequately retained on the column. The performance of each column was assessed by focusing on separation of the critical pairs (such as compounds **23**/**25** and compounds **31**/**32**) and also on their capability of overall separation of the 34 compounds in the sample.

The column screening results showed that the ACE 3 C18 column provided the best overall separation. This column can separate the two API peaks (BSP and BA) from all the impurities/degradants peaks. Therefore, the ACE 3 column was selected for further method development work because this column has the potential to separate all the impurities and degradation products peaks from each other under appropriate mobile phase conditions. Partial separation of one or both critical pairs (peaks **23**/**25** and **31**/**32**) was achieved by other columns (e.g., SunFire C18 column). However, improvement of separation for both the critical pairs remained unsuccessful under various gradient conditions when the organic modifiers were kept the same. In the successive method development experiments presented in the following sections, it is shown that the two critical pairs (**23**/**25** and **31**/**32**) could be separated by changing the organic modifier in the mobile phase.

3.1.2. Optimization of mobile phase pH value and selection of organic modifier

A number of compounds, such as BSP and compounds **1**–**13**, **17**, **19**–**22**, **27**, and **28**, in this study are ionizable. At different pH values of the mobile phase, these compounds would exert different retention behaviors based on whether they are neutral or ionized at a specific pH. In order to determine the pH value that would provide the best overall separation, four pH values (i.e., 2, 3, 5, and 6) of the aqueous solution in the mobile phases were tested.

Because of such a challenging chromatographic profile and the need to separate a huge number of compounds, it became necessary to explore different organic modifiers to identify the one that would provide best overall selectivity for the new method. Four commonly used organic modifiers in RP-HPLC, namely acetonitrile, methanol, isopropanol (2-propanol), and tetrahydrofuran (THF) were explored under each of the four pH values (i.e., 2, 3, 5, and 6). Two to three gradient profiles were tested for each organic modifier under each mobile phase pH. With the results from these tests, ChromSword® software was used in model-mode option to simulate and predict the best possible separation. The results from the combined studies of the mobile phase pH value and organic modifier showed that no single organic modifier at any given pH was able to provide an overall separation of the compounds in the chromatographic profile. It should be noted that when alcoholic organic modifier (methanol and isopropanol) was used at these pH conditions, the peak of compound **15** became broadened even at very low level. This phenomenon made it impractical to use methanol or isopropanol in the mobile phase and/or in sample diluent. The best separation using a single organic modifier in the mobile phase was obtained from THF at pH of 3. However, the biggest challenge was that the BA peak had interference from compounds **25**, **27**, and **28**.

Combination of two organic modifiers at the same time from acetonitrile, methanol, and THF was also tested. Overall separation using a combination of two solvents could not be achieved. Combination of THF and methanol provided the best separation. BA was well separated from compounds **25**, **27**, and **28**. However, compounds **15** and **18** became interfering with a group of peaks. In addition, compound **15** gave a broad peak due to the presence of methanol in the mobile phase.

From the above results generated during the mobile phase studies, THF showed better separation characteristics compared to acetonitrile and methanol. Therefore, two more solvents from the THF family, namely 1,4-dioxane and 1,3-dioxolane, were selected for further studies. The results of the initial studies showed that neither of the two individual solvents could give an acceptable overall separation of the 34 peaks. However, both solvents were able to provide different separation between the challenging pairs (for example, BA and compounds **25**, **27**, and **28**) than THF was. From these results, assumptions were made that the combination of two different oxo-cyclic solvents might provide the selectivity that was required to obtain adequate separation of all the peaks in this study. Commercial lots of 1,3-dioxolane always contains butylated hydroxyl toluene (BHT) as a stabilizer. Under most of the chromatographic conditions explored, BHT accumulated on the column and tended to elute at the end of the gradient giving a huge hump. Therefore, only 1,4-dioxane was used for all further studies.

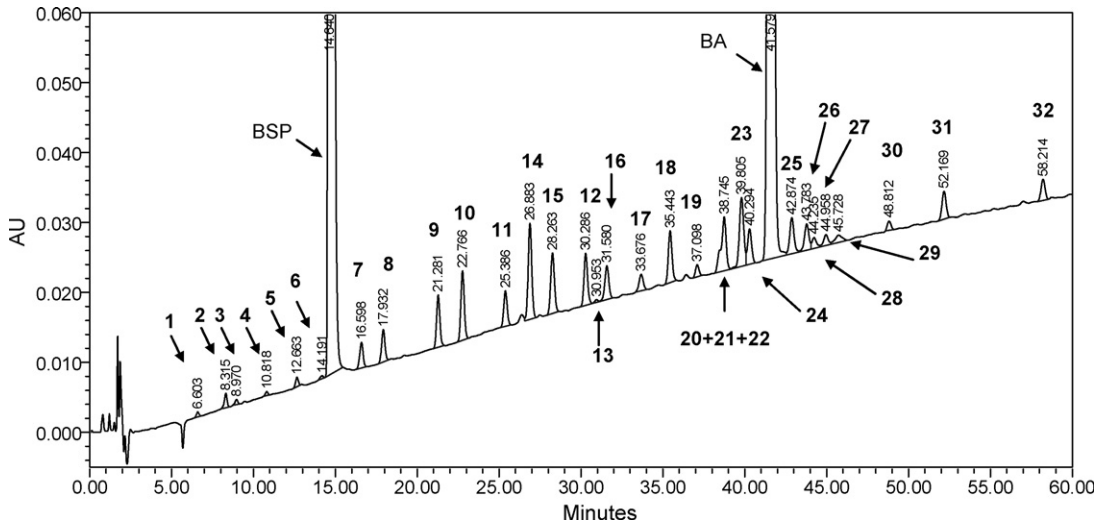

Fig. 2. Chromatogram of an impurity profile mixture obtained by using a gradient of THF from 8% to 24% and 1, 4-dioxane from 5% to 15% in 60 min. An ACE 3 C18 (3 μm, 150 mm × 4.6 mm) column was used at 45 °C, the flow rate is 1.0 mL/min, and the detection wavelength is 254 nm. The pH value of the aqueous solution is 3.

Combination of 1, 4-dioxane and THF was used as the organic modifiers in the mobile phases. After trial runs and optimization, combination of THF and 1,4-dioxane resolved most of the 34 peaks, as shown in Fig. 2.

3.1.3. *Use of chaotropic agents in the mobile phase to improve BSP peak shape and to achieve adequate overall separation*

Desired peak tailing/fronting value for the major peak in a chromatogram should be between 0.8 and 1.5. The tailing factor of the BSP peak under the chromatographic conditions described in Section 3.1.2 was ~2.4. Therefore, it was imperative to investigate and explore various possibilities to reduce the peak tailing of the BSP peak without any significant negative impact on the separation of all other peaks.

The pKa_1 of BSP is ~1.7. Therefore, BSP will be in its ionized form at a mobile phase pH of 3 or above. The unacceptable peak tailing of BSP peak is most probably due to the mutual ionic repulsion occurred between the ionized BSP molecules adsorbed on the column stationary phase surface and the ionized BSP molecules eluted in the mobile phase. Such mutual ionic repulsion can be mitigated by mobile phase additive ions, such as TFA, which compete for the stationary phase surface adsorption. Highly halogenated organic acids (including TFA) have also been widely used as chaotropic agents in RP-HPLC. Therefore, highly halogenated organic acids, such as TFA, pentafluoropropionic acid (PFPA), and heptafluorobutyric acid (HFBA) were tested for potential improvement of the BSP peak shape. Perchloric acid (PCA) and two other inorganic chaotropic salts, namely lithium perchlorate and sodium hexafluorophosphate, were also tested. The results are showing in Table 1.

Data presented in Table 1 clearly demonstrates that all these additives were effective in improving the BSP peak shape to different extents. Although PFPA and HFBA were more effective compared to other additives, it was observed that even the purest form/grade of commercial PFPA and HFBA contained a few impurities that would potentially interfere with certain peaks of interest in the chromatogram. TFA was less effective than PFPA and HFBA in improving the peak shape of BSP, however, it did not contain any interfering impurities. Unfortunately, even with 0.5% (v/v) TFA in the mobile phase the tailing factor of BSP peak was only ~1.6 which is quite far from the most desired value of 1.0. Therefore, it was decided to investigate a combination of TFA and one of the two inorganic chaotropic salts (lithium perchlorate and sodium hexafluorophosphate) at the same time to explore how the BSP peak shape could be improved.

The results presented in Table 1 also reveal that sodium hexafluorophosphate is more effective than lithium perchlorate in improving the BSP peak shape. In order to determine the optimal concentration of the chaotropic salt to be used with TFA, three concentrations (10, 20, and 50 mM) of each salt was tested in the presence of 0.2% (v/v) TFA. At the same salt concentration, sodium hexafluorophosphate was more effective in improving BSP peak shape than lithium perchlorate. For both salts, BSP peak shape/symmetry continued to improve greatly until 20 mM. The BSP peak shape/symmetry only improved slightly at salt concentrations above 20 mM. Therefore, 20 mM sodium hexafluorophosphate was selected as the chaotropic salt in the mobile phase. The observed larger chaotropic effect of sodium hexafluorophosphate compared to lithium perchlorate is consistent with the literature reports of the chaotropic agent strength order: $H_2PO_4^- < HCOO^- < CH_3SO_3^- < Cl^- < NO_3^- < CF_3COO^- < BF_4^- < ClO_4^- < PF_6^-$ [17,20]. Later on, sodium hexafluorophosphate was ultimately replaced with potassium hexafluorophosphate which is much cheaper than the sodium salt, making it more appealing to QC laboratories. The change in the cation of the chaotropic salt did not change any of the chromatographic characteristics (e.g., retention, separation, and peak shape).

In addition to the BSP peak shape improvement, the chaotropic additives also impacted the retention behaviors of the ionized ana-

Table 1
Effect of chaotropic agent additive on the BSP peak shape.

Chaotropic agent	Concentration	BSP peak tailing factor
No	No	2.4
TFA	0.2 (%v/v; 11 mM)	1.8
TFA	0.5 (%v/v; 29 mM)	1.6
PFPA	0.2 (%v/v; 8 mM)	1.6
HFBA	0.1 (%v/v; 8 mM)	1.4
HFBA	0.2 (%v/v; 15 mM)	1.3
PCA	0.35 (%v/v; 35 mM)	1.6
PCA	0.7 (%v/v; 70 mM)	1.4
PCA	2.1 (%v/v; 210 mM)	1.3
$LiClO_4$	50 mM	1.8
$NaPF_6$	50 mM	1.4

Notes: (1) The tests were performed for BSP of approximately 0.4 mg/mL at pH value of 3; (2) TFA, trifluoroacetic acid; PFPA, pentafluoropropionic acid, HFBA, heptafluorobutyric acid; PCA, perchloric acid; $LiClO_4$, lithium perchlorate; $NaPF_6$, sodium hexafluorophosphate.

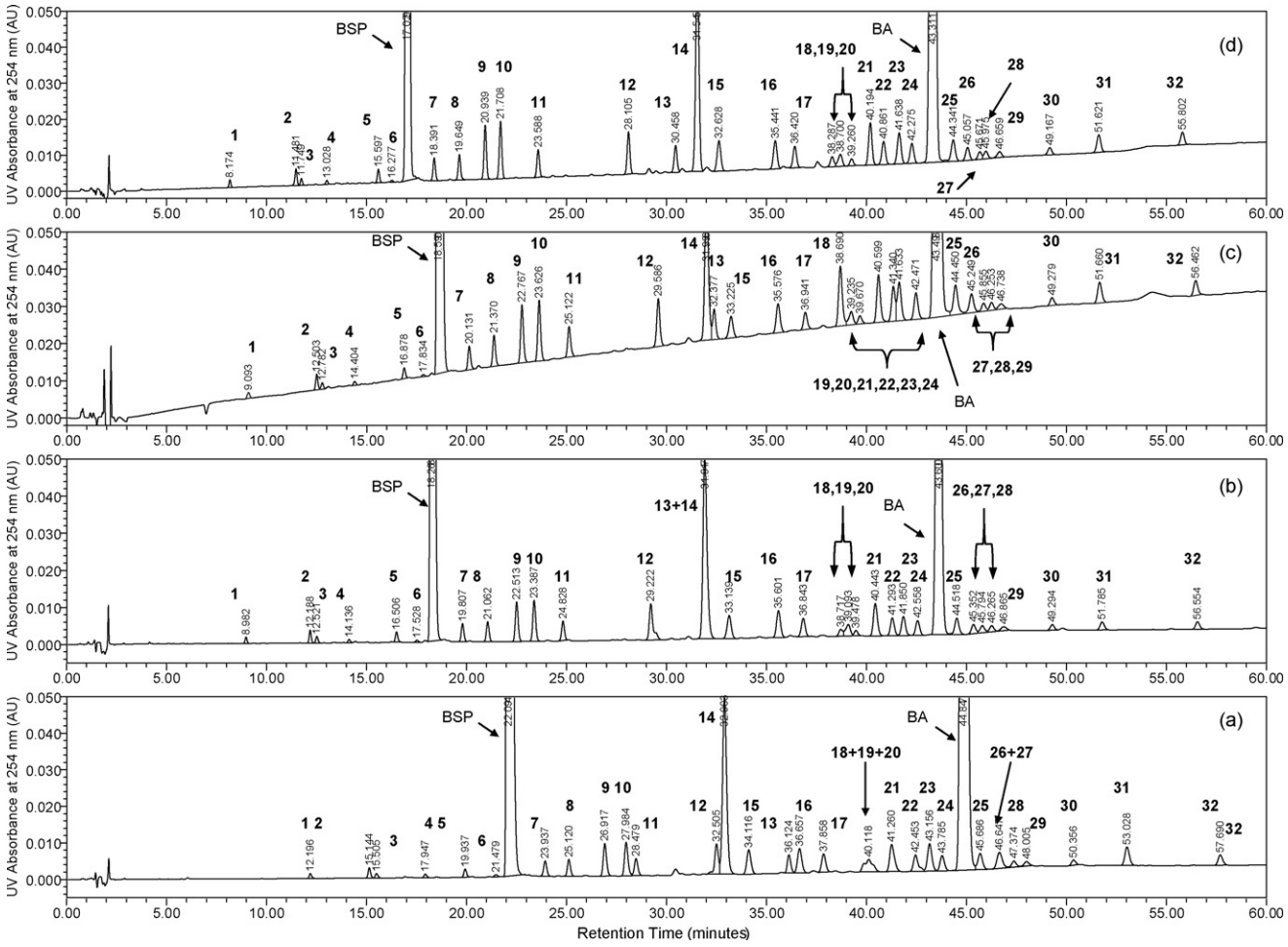

Fig. 3. The representative chromatograms of (a) using no chaotropic additives, (b) using 0.5% (v/v) TFA, (c) using 0.5% (v/v) TFA plus 20 mM lithium perchlorate, and (d) using 0.5% (v/v) TFA plus 20 mM sodium hexafluorophosphate. The chromatographic conditions are listed in Table 1. Not all four conditions used the same profile mixture and, thus, some peak may appear with different responses under different conditions (BSP, Betamethasone Sodium Phosphate; BA, Betamethasone Acetate).

lytes. Representative chromatograms obtained with no TFA and chaotropic additives in the mobile phase, with 0.5% (v/v) TFA in the mobile phase, with 0.5% (v/v) TFA plus 20 mM lithium perchlorate in the mobile phase, and with 0.5% (v/v) TFA plus 20 mM sodium hexafluorophosphate in the mobile phase are shown in Fig. 3. The adjusted retention time ($t'R$) of each of the 34 compounds was plotted against these four conditions as shown in Fig. 4. It can be seen that by using 0.5% (v/v) TFA plus 20 mM sodium hexafluorophosphate in the mobile phase, which improved the peak tailing factor of BSP at the analytical concentration (0.2 mg/mL) to 1.2, the overall separation of BSP, BA, and the 32 compounds of interest were achieved [Fig. 3(d)].

At the selected pH (3.5), the retention of the ionized analytes (as anions), such as BSP and compounds **1**–**13**, was decreased by the addition of the chaotropic agents. Their retention was decreased similarly under the three conditions of chaotropic agent addition so that their $t'R$ "curves" are almost all parallel in Fig. 4. The non-ionized analytes maintained similar retention under all four conditions. The slight decreasing in the retention of the non-ionized analytes can be attributed to the physical masking of the column stationary phase by the chaotropic agents, which slightly reduced the chance for these analytes to interact with the stationary phase. The change in the retention behavior for compound **13** was the most noticeable. It eluted after compound **14** and **15** when there was no chaotropic agent added and eluted closely to compound **14** when 0.5% (v/v) TFA or 0.5% (v/v) TFA plus 20 mM $LiClO_4$ was added in the mobile phase. When 0.5% (v/v) TFA plus 20 mM KPF_6 was added in the mobile phase, compound **13** eluted before compound **14**.

Chaotropic agents interfere with intra-molecular stabilizing interactions mediated by non-covalent forces such as hydrogen bonds, van der Waals forces, and hydrophobic effects. So far, all reported chaotropic effect studies for liquid chromatography have been investigated only for cationic solutes (typically, protonated amines). Chaotropic additives are less polar than water. They can partially or completely break the network of hydrogen bridges (chaotropic order-breaking) and also break the solvation shell surrounding the cations in aqueous media. This leads to their rapid desolvation and in consequence increases hydrophobicity of the analytes resulting in higher retention on the stationary phase [17]. Kazakevich et al. proposed three mechanisms for the chaotropic effect on cationic solutes [19]: (1) *classic ion-pairing*: anions of the additive form essentially neutral ion-pairs with the cationic analytes and the retention of the ion-pairs follows the reversed-phase mechanism; (2) *chaotropic*: counterions disrupt the analyte solvation shell, thus increasing its apparent hydrophobicity and retention; (3) *liophilic*: additive counterions are adsorbed on the surface of the stationary phase, thus introducing an electrostatic component into the general hydrophobic analyte retention mechanism. These mechanisms contribute to the increased retention of cationic analytes in the presence of chaotropic agents.

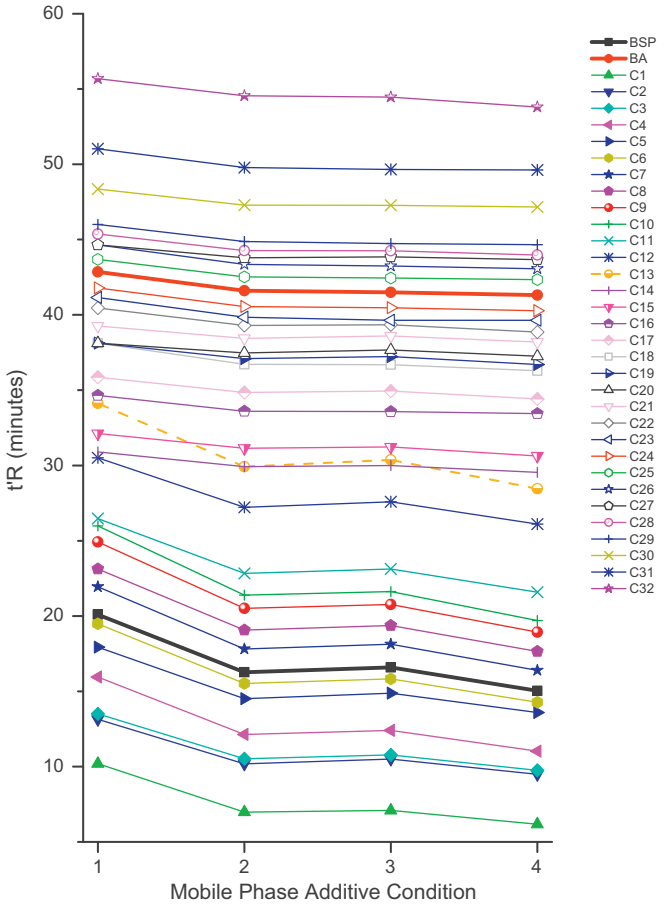

Fig. 4. Adjusted retention times ($t'R$; in min) of the analytes under the four mobile phase additive conditions: (1) using no chaotropic additives, (2) using 0.5% (v/v) TFA, (3) using 0.5% (v/v) TFA plus 20 mM lithium perchlorate, and (4) using 0.5% (v/v) TFA plus 20 mM sodium hexafluorophosphate. The chromatographic conditions are listed in Table 1 (BSP, Betamethasone Sodium Phosphate; BA, Betamethasone Acetate; C*x*, Compound *x*, where *x* = **1**–**32**).

There is no report in the literature on the study of chaotropic effect for anionic analytes under liquid chromatographic conditions. This is the first paper reporting the investigation of the effect of chaotropic agents on anionic analytes under RP-HPLC conditions. More work would be necessary to define the actual mechanism of such effect on anionic analytes. However, the mechanisms described for cationic analytes provide a starting point for the possible mechanism(s) that are involved for anionic analytes.

For anionic analytes under the chromatographic conditions used in our study, "liophobic" factor should be playing a dominant role instead of "liophilic" factor which plays a key role for cationic analytes. The anionic chaotropic ions (e.g., CF_3COO^-, ClO_4^-, and PF_6^-) are adsorbed on the surface of the stationary phase. The electrostatic repulsion between the adsorbed anionic chaotropic ions and the anionic analytes (e.g., ionized BSP) reduces the chance for the analytes to interact with the stationary phase and therefore reduce the retention of the anionic analytes.

For the "chaotropic" factor, the chaotropic anions disrupt the analyte solvation shell. The electrostatic repulsion between the chaotropic anions and the analyte anions pushes the negative charge on the analytes to be delocalized. Without such charge delocalization, the bulk of the anionic steroid analytes in this study is hydrophobic hydrocarbon backbones. The charge delocalization will help the distribution of the electrostatic charge across the whole molecule and therefore increase the hydrophilicity of the whole molecule, which results in the decreasing of the retention of the analytes.

Possibly, these mechanisms can work together with different weights to impose the observed chaotropic effect. It will require more experiments and investigations to fully understand the dominant mechanism(s) that are involved in the chaotropic effect on anionic analytes.

As described by Kazakevich et al. [19], the hexafluorophosphate anion (PF_6^-) has six strong electron-drawing fluoride atoms, which delocalize the negative charge and hence reduce the solvation of the hexafluorophosphate anion. Therefore, the hexafluorophosphate anion has a higher desolvation effect on the analytes in the study and thus higher chaotropic effect than the perchlorate anion (ClO_4^-).

3.1.4. Optimization and finalization of other chromatographic conditions

The optimal pH value of the aqueous solution that was used to prepare the mobile phases was 3.5. At pH 3.5, all the peaks of interest were adequately separated. The pH of 3.5 in the aqueous solution was achieved by mixing 31 mM potassium citrate with 0.5% TFA without any need for further pH adjustment. Addition of sodium hexafluorophosphate has no affect on the pH value of the aqueous buffer solution.

Ideally, the starting mobile phase composition is used as the sample diluent. However, the starting mobile phase composition for this method contained only 8% (v/v) organic modifiers (4% THF and 4% 1,4-dioxane) which is too weak to dissolve adequate amount of BA and the hydrophobic impurities/degradants. Therefore, a higher ratio of organic modifier in the diluent was desired. Acetonitrile of 30% (v/v) was used in the diluent. In order to improve the solution stability of the major compounds, especially BA, 1% (v/v) acetic acid was added to the diluent. Thus, the sample diluent was water–ACN–acetic acid (70:30:1, v/v/v).

The UV absorbance maxima for the BSP, BA and for most of their related compounds are at ~240 nm. However, the UV absorbance of 1,4-dioxane at 240 nm is very strong, which causes a huge negative impact on the detection and quantitation limits of this method. At 254 nm, 1,4-dioxane has significantly less absorbance compared to at 240 nm and did not have significant negative impact on the detection and quantitation limits of this method. Therefore 254 nm was selected as the detection wavelength for this method.

Both THF and 1,4-dioxane have relatively high viscosity, therefore, the flow rate was selected as 1.0 mL/min to ensure the column back pressure would not be too high. At the same time, column temperature was selected as 40 °C, which gave the best overall separation and temperature robustness of the method.

The chromatographic conditions of the final method are listed in the Experimental section. A representative chromatogram of an impurity profile mixture containing all 34 compounds is shown in Fig. 3(d).

3.2. Method validation

Validation of the developed method was performed following ICH guidelines [26,27] with respect to such parameters as linearity, assay accuracy, limit of quantitation (LOQ) and limit of detection (LOD), and precision by two analysts from different laboratories. Validation on specificity, robustness, and solution stability was carried out by the primary analyst. Results of the validation experiments proved that the new HPLC method is suitable for the routine use in the Quality Control (QC) laboratory of pharmaceutical industry.

Table 2
Accuracy/recovery and method precision results.

Compound	Analyst 1 (Laboratory 1) recovery		Analyst 2 (Laboratory 2) recovery		Absolute difference of %RSD[a]
	Average	%RSD[a] (n = 9)	Average	%RSD[a] (n = 9)	
BSP	100	0.3	100	0.3	0.0
BA	99	0.2	100	0.4	0.2
7	99	0.7	100	1.1	0
14	99	1.0	102	3.0	2
16	98	1.1	98	4.7	4
23	99	1.1	97	3.2	2
16	98	1.9	97	5.3	3

[a] RSD, Relative Standard Deviation.

3.2.1. Linearity

To determine the linearity of BSP, triplicate preparations of BSP solutions at each of eight concentration levels were carried out. The eight concentration levels were 0.05%, 0.5%, 5%, 50%, 75%, 100%, 125% and 150% with respect to the BSP analytical concentration (0.2 mg/mL). BA at its analytical concentration (0.15 mg/mL), as well as the product excipients, was spiked into each of the BSP linearity solutions.

Similarly, to determine the linearity of BA, triplicate preparations of BA solutions at each of eight concentration levels were carried out. The eight concentration levels were 0.05%, 0.5%, 5%, 50%, 75%, 100%, 125% and 150% with respect to the BA analytical concentration (0.15 mg/mL). BSP at its analytical concentration (0.2 mg/mL), as well as the product excipients, was spiked into each of the BA linearity solutions.

Based on the availability, five representative impurities/degradation products were selected to be included in the validation, which are compounds **7**, **14**, **16**, **23**, and **26**. Selection of these five compounds covers the whole elution gradient. For example, compound **7** elutes right after BSP, compounds **23** elute before BA and compound **26** elutes after BA, and compounds **14** and **16** elute in the middle of the gradient. For the Linearity study, the five compounds were spiked together in the presence of BSP and BA at their 100% assay levels (0.2 and 0.15 mg/mL, respectively), as well as in the presence of the product excipients. For compounds **7**, **16**, **23**, and **26**, the solutions were at six levels: 0.075, 0.15, 0.3, 0.75, 1.5, and 3.0 μg/mL. Compound **14** (BTM) is the major impurity/degradation product observed in the drug product and, therefore, it was validated in a wider range than other impurities/degradation products. For Betamethasone, the solutions were at eight levels: 0.075, 0.15, 0.375, 0.75, 1.5, 3.75, 7.5, and 15.0 μg/mL.

Linear regression analysis showed a coefficient of determination (r^2) of 1.000 for both BSP and BA (n = 24) and 1.00 for each of the related compounds (n = 24 for compound **14** and n = 18 for compounds **7**, **16**, **23**, and **26**). The y-intercepts obtained from the BSP and BA linearity curves were insignificant, which were within ±0.2% of the respective responses obtained at the assay concentrations. The y-intercepts obtained for each individual impurity/degradant linearity curves were within ±25% of the response of the corresponding impurities/degradants obtained at the 0.000075 mg/mL (LOQ) level.

3.2.2. Limit of detection (LOD) and limit of quantitation (LOQ)

Based on the ICH signal-to-noise approaches [26], the LOQ and LOD for BSP, BA, and the impurities/degradation products were assessed. The LOQ and LOD for BSP were set as 0.05% and 0.02% of the BSP analytical concentration level (0.20 mg/mL), which correspond to 0.1 and 0.04 μg/mL, respectively. The LOQ and LOD for BA and the impurities/degradation products were set as 0.05% and 0.02% of the BA analytical concentration level (0.15 mg/mL), which correspond to 0.075 and 0.03 μg/mL, respectively. The validation showed the signal-to-noise ratios (S/Ns) for LOQ of all the compounds were larger than 10 and the S/Ns for LOD were larger than 3.

3.2.3. Accuracy

The method accuracy was assessed via a recovery study. Known amounts of tested compounds (BSP, BA, **7**, **14**, **16**, **23**, and **26**) were spiked into the solutions in the presence of the placebo ingredients. The solutions were analyzed and these compounds were quantitated. The acquired experimental concentration results of them were compared to the respective calculated theoretical values to derive the recovery. Recoveries of BSP were between 99% and 101% at 0.001–0.01 mg/mL levels and between 99.5% and 100.7% at 0.1–0.3 mg/mL levels. Recoveries of BA were between 96% and 102% at 0.00075–0.0075 mg/mL levels and between 99.3% and 100.7% at 0.075–0.225 mg/mL levels. The recoveries of the impurity/degradant **14** were 97–110% at 0.15–15.0 μg/mL levels. The recoveries of the other four impurities/degradants were 86–103% at 0.15–3.0 μg/mL levels. The average recoveries of BSP, BA, and the five impurities/degradants are shown in Table 2. The assay of BSP and BA, and estimation of their impurities/degradants of the method were demonstrated to be accurate for routine analysis.

3.2.4. Method precision (repeatability and reproducibility)

The method repeatability for BSP, BA, and their impurities/degradants was evaluated through the %RSD (Relative Standard Deviation) of the recoveries on both the primary and the alternate columns. The %RSD for BSP was calculated from recoveries of nine samples [triplicates at the low (0.1 mg/mL), middle (0.2 mg/mL), and high (0.3 mg/mL) concentration levels]. The %RSD for BA was calculated from recoveries of nine samples [triplicates at the low (0.075 mg/mL), middle (0.15 mg/mL), and high (0.225 mg/mL) concentration levels]. The %RSD for the impurity/degradant **14** was calculated from their respective recoveries of nine samples [triplicates at the low (0.00015 mg/mL), middle (0.0015 mg/mL), and high (0.015 mg/mL) concentration levels]. The %RSD for each of the other four impurities/degradants was calculated from their respective recoveries of nine samples [triplicates at the low (0.15 μg/mL), middle (0.75 μg/mL), and high (3.0 μg/mL) concentration levels].

The method reproducibility was evaluated for each compound based on the inter-laboratory difference between the %RSD of recoveries obtained by Analysts 1 and 2 in two difference laboratories.

The method precision results are listed in Table 2, which show that the method has acceptable repeatability and reproducibility.

The method repeatability was further demonstrated by analyzing six sample preparations from on batch of the Celestone Chronodose® Injection product (Product Repeatability study). The average assay values of the BSP and BA peaks were 100.4% with a %RSD of 0.1% and 99.3% with a %RSD of 1.2, respectively. The observed impurities/degradation products are estimated at a range

Table 3
Method robustness variations and retention time/relative retention time results.

HPLC parameter	Variation	RT[a] (min)				RRT[b]		
		BSP[a]	**BA**[a]	**7**	**14**	**16**	**23**	**26**
Procedural condition[c]		16.590	42.566	1.080	0.726	0.817	0.962	1.041
HPLC system	Agilent HPLC	17.524	44.240	1.078	0.727	0.817	0.961	1.041
Column lot	Lot 2	17.221	43.297	1.077	0.729	0.819	0.962	1.040
	Lot 3	17.597	43.951	1.076	0.731	0.821	0.964	1.039
Flow rate	0.9 mL/min	17.866	44.321	1.076	0.734	0.824	0.962	1.039
	1.1 mL/min	16.073	41.525	1.081	0.722	0.813	0.961	1.041
Detector wavelength	252 nm	16.880	42.801	1.079	0.728	0.818	0.962	1.040
	256 nm	16.887	42.816	1.078	0.728	0.818	0.962	1.040
Column temperature	38 °C	17.341	43.470	1.078	0.731	0.819	0.960	1.041
	42 °C	16.475	42.204	1.078	0.725	0.817	0.964	1.040
Injection volume	25 μL	16.885	42.801	1.078	0.728	0.818	0.962	1.040
	35 μL	16.563	42.490	1.080	0.726	0.817	0.962	1.041
Potassium citrate concentration in aqueous solution	28 mM[e]	16.958	43.142	1.077	0.727	0.819	0.963	1.040
	34 mM[e]	16.959	43.136	1.078	0.727	0.819	0.962	1.040
KPF_6[d] conc. in aqueous solution	18 mM	16.662	42.656	1.079	0.725	0.817	0.962	1.041
	22 mM	16.659	42.643	1.079	0.725	0.817	0.962	1.041
Trifluoroacetic acid conc. in aqueous solution	0.45%[e]	16.986	43.191	1.077	0.727	0.819	0.963	1.040
	0.55%[e]	17.212	43.596	1.077	0.726	0.818	0.962	1.040
pH value of aqueous solution	3.4	17.198	43.541	1.078	0.727	0.817	0.962	1.041
	3.6	16.680	42.727	1.079	0.725	0.817	0.962	1.040
Aqueous solution in MP A	4.5:4.5:91	15.343	40.835	1.080	0.717	0.811	0.961	1.042
[Dioxane:THF[d]:AS[d] (v/v/v)]	3.5:3.5:93	17.895	42.705	1.070	0.740	0.827	0.964	1.038
Aqueous solution in MP B	14.5:29:56.5	15.637	41.386	1.082	0.720	0.815	0.962	1.041
[Dioxane:THF[d]:AS[d] (v/v/v)]	13.5:27:59.5	16.163	43.852	1.084	0.713	0.809	0.961	1.042
Dioxane:THF[d] ratio in MP A	3.5:4.5:92	15.498	41.261	1.083	0.720	0.813	0.961	1.041
[Dioxane:THF[d]:AS[d] (v/v/v)]	4.5:3.5:92	16.997	41.992	1.075	0.732	0.822	0.963	1.040
Dioxane:THF[d] ratio in MP B	12.5:29.5:58	17.463	44.945	1.080	0.723	0.814	0.961	1.041
[Dioxane:THF[d]:AS[d] (v/v/v)]	15.5:26.5:58	17.082	43.209	1.079	0.728	0.818	0.962	1.041
Gradient time	54 min	16.117	39.922	1.076	0.734	0.824	0.962	1.039
	66 min	17.647	45.668	1.081	0.722	0.813	0.961	1.042

[a] RT, retention time (min); BSP, Betamethasone Sodium Phosphate; BA, Betamethasone Acetate.
[b] RRT, relative retention time. For compound **7**, the RRTs are calculated against BSP retention times; for compounds **14**, **16**, **23**, and **26**, the RRTs are calculated against BA retention times.
[c] Refer to Table 1 for the procedural conditions. The procedural experiments were performed on Waters Alliance HPLC systems.
[d] KPF_6, potassium hexafluorophosphate; THF, Tetrahydrofuran, AS, aqueous solution.
[e] The pH of the aqueous solution were adjusted to 3.5 ± 0.1 under these variations.

of QL (0.05%) to 0.85% with the %RSD range of 0.2–8.7. The tight %RSD values showed the acceptable repeatability of the method again.

3.2.5. Method specificity

BSP and BA peaks are free from the interferences of sample diluent solvent peaks and excipient peaks.

The method was proven capable of resolving BSP and BA from the key impurities/degradants by analyzing BSP and BA standard solutions, solutions containing the key impurities/degradants, and sample solutions of the injection suspension product beyond expiry, which provided true reflection of the degradation chemistry under the real shelf condition.

The homogeneity and peak purity of the BSP and BA peaks in three representative expired injection suspension product samples was estimated based on a photodiode-array (PDA) scan from 210 to 400 nm. Peak purity results were obtained by comparing the Purity Angle and Purity Threshold calculated by Empower2 PDA software automatically. In each of the three expired product sample, the BSP and BA peaks had Purity Angle less than the Purity Threshold, indicating identical UV spectrum across the peaks.

3.2.6. Method robustness

Variations in HPLC operating conditions were made to demonstrate the robustness of the method. The assay of BSP and BA at their analytical concentrations (approximately 0.2 and 0.15 mg/mL, respectively) and the estimation of five validated impurities/degradants spiked at approximately 0.003 mg/mL were assessed under the procedural condition and each variation. The robustness variations, the retention times of BSP and BA, and the relative retention time of each validated impurity/degradant are listed in Table 3.

The assay values of BSP and BA at different variations of HPLC operating conditions were all within ±2% absolute difference from the procedural condition. The estimations of each impurity/degradant at the 0.003 mg/mL spiking level at all variations of conditions were within ±0.1% absolute difference from the procedural condition. The relative retention times (RRTs) of each related compound relative to BSP or BA in the same chromatogram obtained under all variations remained similar to those from the procedural condition. Under all the robustness conditions, BSP, BA and the eight impurities/degradants were separated from each other.

At the same time, each of the prescribed acceptance criteria of the system suitability was also evaluated. Under every variation of the robustness study, the retention times of BSP and BA were within the retention time suitability ranges set in the test method (17.0 ± 2.0 and 43.0 ± 4.0 min, respectively), the S/Ns of BSP and BA LOQ peaks were both above 10, the tailing factors of BSP and BA peak at assay level (0.2 mg/mL) was within 0.9 and 1.5, and the resolution factor between BSP and compound **7** was larger than 3.0. All system suitability criteria were met under each robustness variation. Therefore, these results clearly demonstrated that this method is very robust.

3.2.7. Solution stability

In the solution stability study, BSP and BA LOQ solution, standard and sample solution (approximately 0.2 mg/mL), and the

product sample solution spiked with the five validated impurities/degradants at approximately 0.003 mg/mL were stored at ambient laboratory temperatures protected from room lights and under refrigeration (at 2–8 °C). The assay of BSP and BA and the estimation of five validated impurities/degradants were carried out on Day 0, Day 1, Day 3, and Day 7.

At each time point under the two storage conditions, BSP and BA assay was within ±2% of the initial (Day 0) result, the absolute difference of the estimation of each impurity/degradant was within ±0.2% compared to the initial (Day 0) results, and the LOQ solution injection gave the signal-to-noise ratios above 10 for both BSP and BS peaks.

Therefore, the BSP and BA standard and samples solutions in 30:70:1 (v/v/v) acetonitrile–water–acetic acid are stable for up to 7 days when stored both at room temperature protected from lab lighting or under refrigeration.

3.3. Batch testing

To demonstrate that the newly developed and validated method would work for the actual injection suspension samples, 32 representative batches of the injection suspension samples, from two manufacturing sites and different ages, were tested with this method. The results showed that the method was appropriate, reliable, and satisfactory for its intended use.

4. Conclusions

The analytical method described in this paper is the first known RP-HPLC method that can separate and simultaneously quantitate the two APIs (BSP and BA) and all of their related compounds in Celestone Chronodose® Injection with a single method. The analytical method is suitable for the identification, assay of BSP and BA, and for identification and estimation of their impurities/degradants in Celestone Chronodose® Injection drug product. Unique selectivity was achieved by using a combination of two oxo-cyclic solvents, namely tetrahydrofuran (THF) and 1,4-dioxane, as the organic modifiers in the mobile phases. Further enhancement of selectivity and BSP peak symmetry were achieved by using chaotropic agents (trifluoroacetate and hexafluorophosphate) in the mobile phases. The goal of separation of the two APIs (BSP and BA) and their 32 impurities/degradation products was successfully achieved.

This is the first paper of chaotropic effect on the retention of anionic analytes under RP-HPLC conditions. Although further work is needed to fully understand the mechanism(s) of anionic chaotropic agents on the impact of retention of anionic analytes, potential mechanism(s) of chaotropic agents has been discussed in this paper.

This method has been demonstrated to be sensitive, linear, accurate, precise, specific, robust, repeatable, and reproducible. Therefore this method is ideally suitable for routine analysis of Celestone Chronodose® Injection drug product in Quality Control laboratories. This method has also been demonstrated to be stability indicating because it can separate degradation peaks from the BSP and BA peaks and accurately quantitate the contents of the injection suspension stability samples.

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