Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 646–654

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development and validation of a stability-indicating RP-HPLC method to separate low levels of dexamethasone and other related compounds from betamethasone

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ARTICLE INFO

Article history:
Received 17 July 2008
Received in revised form 8 December 2008
Accepted 11 December 2008
Available online 24 December 2008

Keywords:
Reversed-phase HPLC
Stability indicating
Method development
Validation
Betamethasone
Dexamethasone

ABSTRACT

Betamethasone (BM) is an active pharmaceutical ingredient (API) or an intermediate which is used to manufacture various finished pharmaceutical products. Betamethasone is also used as a starting material to manufacture other APIs that are related to this steroid family. It is quite a challenging task to separate dexamethasone (DM) peak (the alpha epimer) and other structurally related compounds from BM. A stability-indicating reversed-phase high performance liquid chromatography (RP-HPLC) method has been developed which can separate and accurately quantitate low levels of DM and other related compounds from BM and also from each other. This method was successfully validated for the purpose of conducting stability studies of betamethsone in quality control (QC) laboratories. The stability-indicating capability of this method was demonstrated by adequate separation of DM and all the degradation product peaks from BM peak and also from each other in aged stability samples of betamethasone. A gradient mobile phase system consisting of (A) water:acetonitrile (90:10, v/v) and (B) acetonitrile:isopropanol (80:20, v/v) was used with an ACE Phenyl column (10 cm × 4.6 mm, 3 μm particles, 100 Å pore size) and an ultraviolet (UV) detection at 240 nm.

1. Introduction

Betamethasone (9α-fluoro-16β-methylprednisolone) is a semi-synthetic active pharmaceutical ingredient (API) or an intermediate which belongs to the family of glucocorticoid steroid. It is a strong anti-inflammatory and immunosuppressive agent. It is used to stimulate fetal lung maturation, and to decrease the incidence and mortality from intracranial hemorrhage in premature infants. It is also used in topical pharmaceutical creams to relieve skin irritation [1–3]. In addition to its (BM) direct use as an API in numerous pharmaceutical finished products, BM is also used as a key intermediate for the synthesis of other related steroid APIs, such as betamethasone dipropionate, betamethasone sodium phosphate, betamethasone valerate, etc. Therefore, the control of the purity of BM is critical to ensure the quality of betamethasone related drug products as well as the quality of other steroid APIs that are manufactured by using BM as a starting material.

A thorough literature search revealed no report of any stability-indicating HPLC method for betamethasone. There are two major challenges to develop a stability-indicating RP-HPLC method for BM. First challenge is to obtain a baseline separation of dexamethasone isomer from BM and the second challenge is to obtain separation of a huge number of structurally similar (known and unknown) compounds (including degradation products) from each other and also from BM and DM peaks. The chemical structures of known impurity peaks are listed in Fig. 1. Trace amount of dexamethasone is present in typical commercial lots of betamethasone. As dexamethasone peak elutes immediately after the BM peak, separation of low levels (less than 0.1% compared to BM) of DM from BM peak is another big challenge. The beta- and dexa-forms of these molecules have identical chemical structures except that the orientation of the methyl group at the C-16 position is in opposite direction from the plane. Physicochemical characteristics of these two compounds are very similar [4,5]. Therefore, it is quite challenging to obtain a mobile phase and a stationary phase that would provide adequate differences in thermodynamic parameters (entropy, enthalpy, etc.) between these epimers that must be obtained for a baseline separation. Previously, separation of betamethasone and dexamethasone has been attempted by normal-phase and reversed-phase HPLC [6–9]. Derivatization was carried out prior to the normal-phase separation [9]. The samples used by most of these authors contained approximately 1:1 ratio of BM and DM. Using these sample mixtures, partial separation of the two isomers was obtained with resolutions of approximately 0.9–1.5 under reversed-phase conditions [6–8]. It is important to note that a resolution factor of 1.5 is adequate only for baseline separation of two peaks with similar sizes [10]. To ensure the purity and

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doi:10.1016/j.jpba.2008.12.023

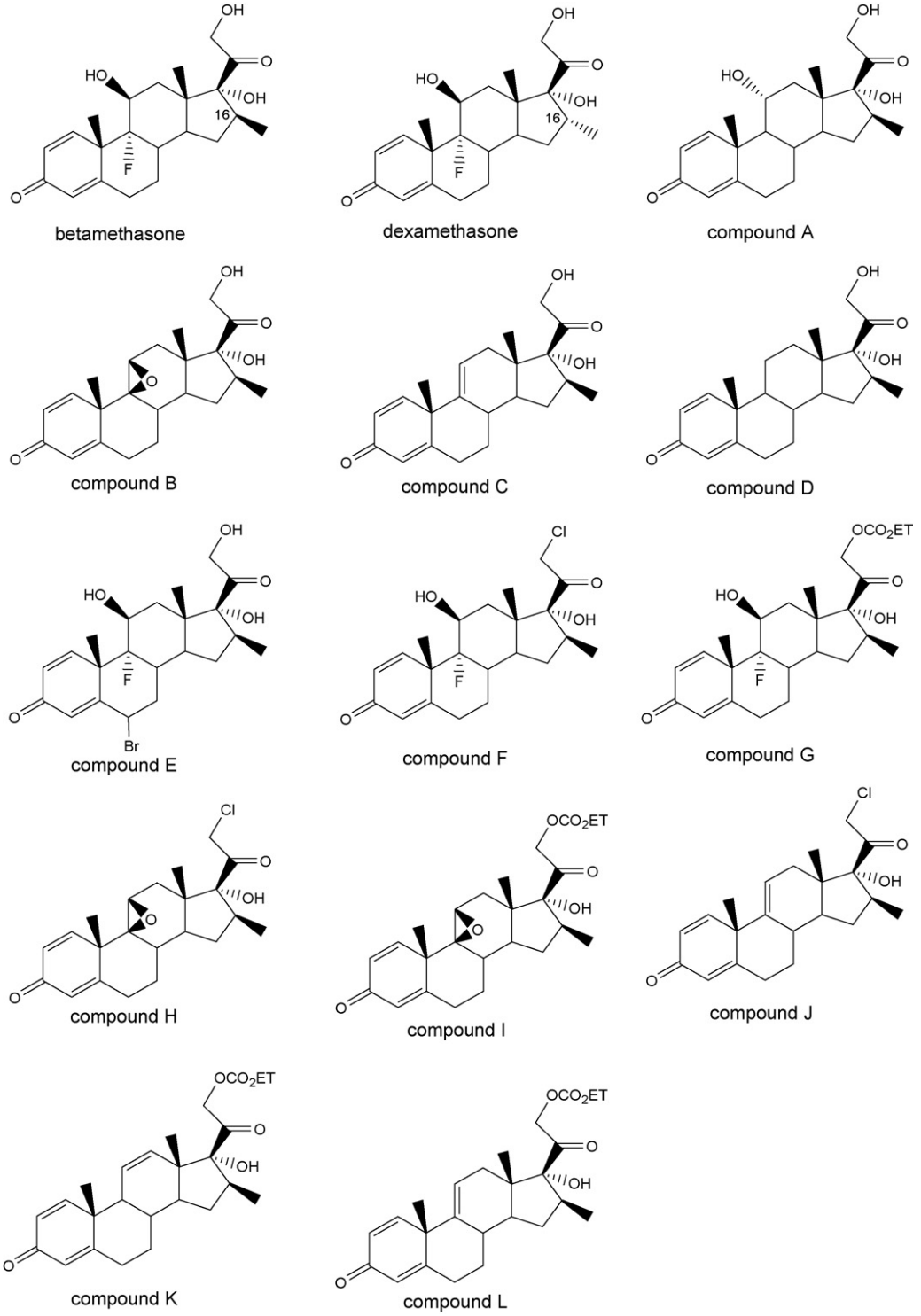


Fig. 1. Chemical structures of betamethasone and some of its related compounds.

the quality of betamethasone, an analytical method is required to be capable to separate all the impurities and degradants from the BM peak (including the α-isomer) and also separate all the major impurity and degradation peaks from each other.

In this paper, we describe a reversed-phase HPLC method for the assay of betamethasone and estimation of its related compounds. This method has been demonstrated to be sensitive, accurate, linear, precise, reproducible, repeatable, specific, and robust, and therefore suitable for routine analysis of betamethasone in quality control laboratories. This method is also demonstrated to be stability indicating because it can separate the degradation peaks from the betamethasone peaks that are present in typical stability samples of betamethasone. From the best of our knowledge via literature search, this is the first known RP-HPLC method that can separate all the related compounds of BM from each other and from BM and is therefore suitable to conduct stability studies of betamethasone.

2. Experimental

2.1. Materials

Betamethasone and related compounds were obtained from Global Quality Services—Analytical Sciences reference standard group in Schering-Plough (Union, New Jersey 07083, USA). All HPLC grade solvents were purchased from Fisher Scientific (Fisher Scientific International Inc. Liberty Lane Hampton, New Hampshire 03842, USA). Water (18.2 MΩ.cm) was collected from a Milli-Q system (Millipore, Billerica, Massachusetts 01821, USA).

2.2. Instrumentation used for method development and method validation

A Hitachi LaChrom Elite HPLC system (Hitachi High Technologies America, Inc.; San Jose, California 95134, USA) equipped with ChromSword method development software (Merck, Darmstadt, Germany); an Agilent Technologies 1100 Series HPLC system (Santa Clara, California 94306, USA) equipped with a LC Spiderling column switching system (Chiralizer Services, L.L.C., Newtown, Pennsylvania 18940, USA); and a Waters 2695 Alliance HPLC system (Milford, Massachusetts 01757, USA) were used for method development. All HPLC systems were equipped with a column compartment with temperature control and an on-line degasser. Data acquisition, analysis, and reporting were performed, except ChromSword simulation, by EZChrom Elite (Hitachi), ChemStation (Agilent), and Millennium32 (Waters) chromatography software. Different Waters HPLC systems in different laboratories were used for method validation, such as Waters 2695 or 2690 HPLC separations modules equipped with a Waters 2996 photodiode array detector or 2487 dual wavelength UV detectors. The HPLC columns were purchased from vendors such as Waters Corp., MAC-MOD Analytical, Inc., or Phenomenex.

2.3. Chromatographic conditions of the final method

The validation of the final method was performed on an ACE Phenyl column (10 cm × 4.6 mm, 3 μm particles, 100 Å pore size) with an ultraviolet (UV) detection at 240 nm. The mobile phase A was prepared by mixing HPLC grade or Milli-Q water and HPLC grade acetonitrile in a volume ratio of 90:10. The mobile phase B was prepared by mixing HPLC grade acetonitrile and HPLC grade isopropanol in a volume ratio of 80:20. The gradient elution was carried out according to the program listed in Table 1. The flow rate was used at 2.0 mL/min and the column temperature was maintained at 30 ± 5 °C. The total chromatographic run time is 40 min with an additional 10 min of column re-equilibration time between each injection. The solution stability samples were analyzed using a photo-diode array (PDA) detector covering the range of 200–400 nm.The injection volume was 10 μL.

2.4. Solution preparations

Sample solutions were prepared by dissolving appropriate amounts of betamethasone or its related compounds into a diluent (1:1 volume ratio of acetonitrile and water). The analytical concentration of betamethasone was 1.0 mg/mL.

To determine the linearity of betamethasone, triplicate preparations of betamethasone in the diluent at each of the eight concentration levels were carried out. The eight levels of sample concentrations were 0.05, 1, 10, 40, 80, 100, 120 and 160% of the betamethasone analytical concentration. The linearity of betamethasone related compounds was also determined using triplicate preparations of betamethasone related compounds in the diluent at each of the six concentration levels of 0.05, 0.1, 0.25, 0.5, 1 and 2% of the betamethasone analytical concentration. The limit of quantitation (LOQ) and the limit of detection (LOD) of all the tested compounds were 0.05 and 0.02%, respectively, of the betamethasone analytical concentration.

2.5. Calculation

The quantitation of betamethasone or its related compounds was carried out based on an external standard method using betamethasone reference standard. The sample solutions were bracketed between two betamethasone reference standard solutions and the experimental concentration was obtained from the following equation:

$$\text{Experimental Concentration} = \frac{P_2}{RRF \times P_1} \times C_1 \quad (1)$$

where P_1 = average peak area of betamethasone in the adjacent betamethasone bracketing standards, P_2 = peak area of betamethasone or each individual related compound in linearity sample solution, C_1 = concentration of betamethasone in betamethasone bracketing standard, RRF = relative response factor

RRF (relative response factor) is the ratio between the response factor of each individual related compound and the response factor of betamethasone. The RRF was the quotient obtained by dividing the slope of the linear regression curve of betamethasone by the slope of the linear regression curve of the individual related compound. The recovery of each concentration level was then determined by the following equation:

$$\%\,\text{Recovery} = \frac{\text{Experimental Concentration}}{\text{Prepared Concentration}} \times 100 \quad (2)$$

3. Results and discussion

3.1. HPLC method development

The molecular structures of betamethasone and the known related compounds (Fig. 1) clearly show that there are no functional groups available in BM and in any known compounds of BM which can be easily ionized. Therefore, the retention and separation

Table 1
HPLC gradient program for sample analysis.

Time (minute)	Flow rate (mL/minute)	Mobile phase A (water/acetonitrile 90:10, v/v)	Mobile phase B (acetonitrile/isopropanol 80:20, v/v)	Gradient curve
0	2.00	92.0	8.0	linear
25	2.00	87.0	13.0	linear
35	2.00	65.0	35.0	linear
40	2.00	63.0	37.0	linear
*41	2.00	92.0	8.0	linear
*50	2.00	92.0	8.0	linear

* Column re-equilibration step.

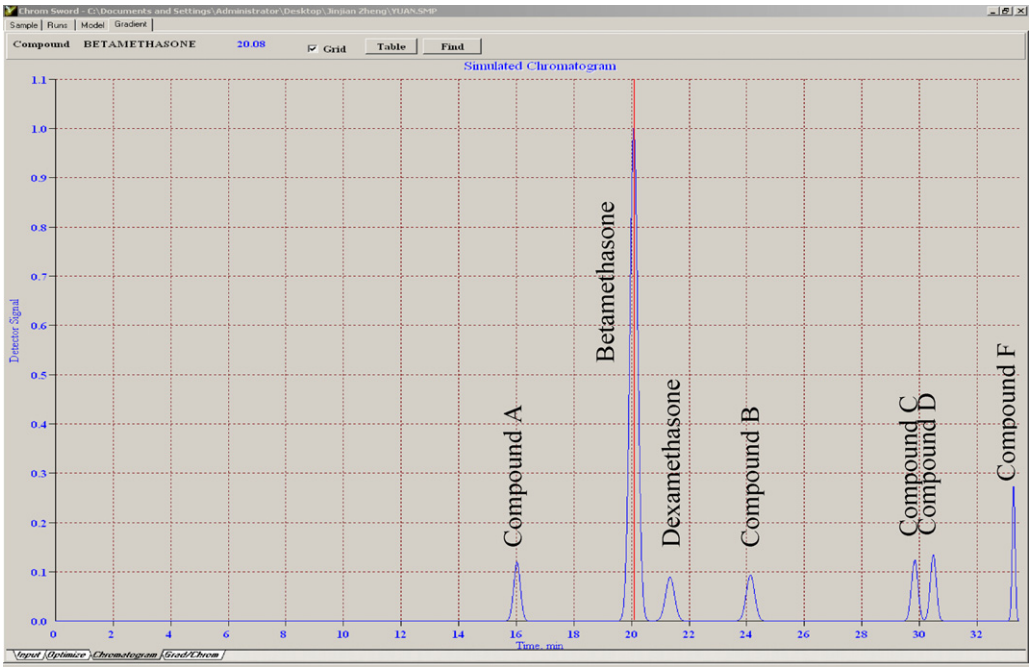
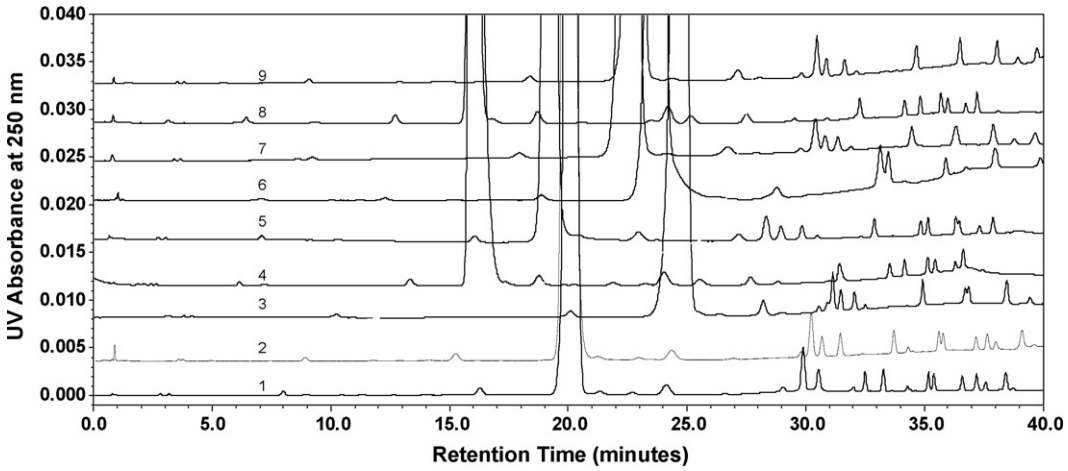


Fig. 2. The chromatogram simulated by Chromsword-Auto software for the separation of some key impurities. (Betamethasone solution spiked with Compounds A–D and F was used in the simulation).

of these compounds under the mobile phase and stationary phase conditions of reversed-phase chromatography should not have any significant impact with the pH and ionic strength changes of the mobile phase. Hence, the method development was focused on the selection of the most suitable stationary phase (i.e., the HPLC column), optimization of the compositions of organic modifiers in the mobile phases, investigating the impact of flow rates and temperatures, and fine-tuning the conditions of the gradient profile to obtain the final and optimum elution profile of the method. During the method development activities, state-of-the-art HPLC method development technologies such as ChromSword-Auto (which is a smart computer-aided chromatographic method development tool) and a LC Spiderling (which is an automated 9-port column switching system), were heavily used in combination of the knowl-

Table 2
HPLC columns screened during method development.

HPLC column	Column description	Comments
ACE C_{18}	5 cm × 4.6 mm ID and 10 cm × 4.6 mm ID, 3 μm particles, 100 Å pores Carbon loading: 15.5%	Base deactivated stationary phase, good peak shape.
ACE C_{18} 300 Å	5 cm × 4.6 mm ID and 10 cm × 4.6 mm ID, 3 μm particles, 300 Å pores Carbon loading: 9%	Wide pore silica particles which may enhance the access of steroid molecules to the intra-particle surfaces.
ACE C_8	5 cm × 4.6 mm ID and 10 cm × 4.6 mm ID, 3 μm particles, 100 Å pores Carbon loading: 9%	Shorter chain stationary phases require less diffusion for the steroid molecules to reach to the surface under the carbon chains, where the secondary interaction takes place.
ACE Phenyl	5 cm × 4.6 mm ID and 10 cm × 4.6 mm ID, 3 μm particles, 100 Å pores Carbon loading: 9.5%	Hydrophobicity between C4 and C8 phases and also with increased polar selectivity
TSK-Gel Super-ODS	5 cm × 4.6 mm ID, 2 μm particles, 110 Å pores Carbon loading: 8%	Packed with 2 μm particles, which may increase the column efficiency.
TSK-Gel Super-Octyl	5 cm × 4.6 mm ID, 2 μm particles, 110 Å pores Carbon loading: 6%	Packed with 2 μm particles and also shorter chain stationary phase.
TSK-Gel Super-Phenyl	5 cm × 4.6 mm ID, 2 μm particles, 140 Å pores Carbon loading: 3%	Packed with 2 μm particles and potential $\pi - \pi$ interactions between the stationary phase and the steroid molecules.
YMC-Pack Pro C_{18}	5 cm × 4.6 mm ID, 3 μm particles, 80 Å pores Carbon loading: 22%	Stationary phase modified with Lewis acid–Lewis base chemistry that may possess unique interactions with steroid molecules.
YMC Hydrosphere C_{18}	5 cm × 4.6 mm ID, 3 μm particles, 120 Å pores Carbon loading: 12%	Can be used under 100% aqueous conditions, which suggests a strong hydrogen bonding interaction with the steroid molecules.
Thermo fluophase PFP	5 cm × 4.6 mm ID, 5 μm particles, 100 Å pores Carbon loading: 12%	Greater dipole of carbon–fluorine bond versus the carbon–hydrogen bond makes the perfluorinated stationary phase unique in the retention of polar and halogenated compounds.
Thermo fluophase RP C_{18}	5 cm × 4.6 mm ID, 5 μm particles, 100 Å pores Carbon loading: 10%	Perfluorinated C6 phase provides extra interactions with polar samples and greater retention of polar and halogentated compounds



1. ACE Phenyl column (Mac-Mod Analytical, Inc.)
2. ProntoSIL Phenyl column (Mac-Mod Analytical, Inc.)
3. Ultra Phenyl column (Restek)
4. BetaBasic Phenyl column (Thermo Scientific)
5. Pinnacle II Phenyl column (Restek)
6. Nucleosil Phenyl column (Macherey-Nagel)
7. Clipeus Phenyl column (Higgins Analytical Inc.)
8. Alltima Phenyl column (Alltech)
9. YMC pack Phenyl column (YMC)

Fig. 3. Comparisons of chromatograms of betamethasone and its related compounds (impurity profile solution) on different phenyl columns.

edge and experiences of the bench analytical scientists. The use of the advanced method development tools immensely improved the efficiency of method development activities by significantly decreasing the time-consuming manual trial and error screening and optimization experiments. The method development tools also enhanced the probability of finding an optimum separation condition in a much shorter period of time. As an illustration, a simulated chromatogram for the separation of some key impurities was shown in Fig. 2. This simulation was obtained with only a few scout runs and the peak retentions are almost identical with the corresponding peaks in the real chromatogram (refer to the middle chromatogram in Fig. 4), which was obtained based on the chromatographic conditions predicted from the simulation.

During the method development work, more than 10 different HPLC columns (50 mm × 4.6 mm I.D. or 100 mm × 4.6 mm I.D.) were screened (Table 2). The columns for screening studies were selected to cover a wide range of stationary phase surface properties, such as carbon chain length, carbon loading, separation mechanism and surface functionality. The highlight of the scientific rationale for the selection of the most appropriate column for the intended purpose of this method and the column information are provided in Table 2. Each of the selected columns was screened with mobile phases containing various types and percentages of organic modifier such as acetonitrile, methanol, isopropanol, and tetrahydrofuran (THF). The initial results of column and mobile phase screening studies provided a few conditions that could separate betamethasone and dexamethasone with resolution greater than 2.7 when the level of DM is approximately 0.1% (or lower) compared to the BM peak. However, the separation became much more challenging and complicated due to the presence of many other impurity peaks with similar chemical structures. Certain columns such as the ACE C_8 column provided better separation between betamethasone and dexamethasone peaks but failed to provide good separation between all other impurity peaks. On the other hand, the ACE 3 Phenyl column was able to provide adequate separation of DM and BM and it also separated all the other impurities in a satisfactory manner. Therefore, the 10 cm ACE 3 Phenyl column was selected as the primary column for the method development. Numerous experiments were conducted using various combinations of a wide range of mobile phases to obtain the one that is optimum to achieve the overall goal and purpose of this method. Although the use of Acetonitrile as the mobile phase B provided an acceptable separation on the ACE Phenyl column, the separation between BM and DM was not acceptable on the ProntoSIL Phenyl column, the back-up column. The acceptable separation was achieved on both columns (Refer to Fig. 3), after adding isopropanol into the mobile phase B. The final mobile phase conditions and the gradient program are presented in Table 1. Analysis of a large number of BM aged samples clearly demonstrated that the final conditions of this method can adequately separate all the impurities (including dexamethasone) that are present in the samples of typical commercial lots of betamethasone.

One of the important elements of an analytical method that is intended for routine use in a pharmaceutical quality control (QC) laboratory is that the method should have capabilities of generating similar results (i.e., meeting all the requirements of system suitability) with columns packed with same type or class of stationary phase but manufactured by different vendors. On the basis of column chemistry and known physicochemical characteristics of the ACE Phenyl column, we selected eight different phenyl columns from different vendors to identify a back-up/alternate column for this method. Selective samples of betamethasone API were analyzed using the mobile phase conditions of this method. The results of the column screening studies are presented in Fig. 3. The chro-

matograms in Fig. 3 demonstrate that the ProntoSIL-Phenyl column gave results that were very similar to the results obtained from ACE Phenyl column. Therefore, the ProntoSIL-Phenyl column can be used as a back-up column for the primary column (the ACE Phenyl column) in case the ACE Phenyl column becomes unavailable in the future.

3.2. Analytical method validation

Two laboratory analysts performed the method validation work (using two different HPLC systems) with respect to parameters such as linearity, assay accuracy, limit of quantitation (LOQ), limits of detection (LOD), ruggedness, precision, specificity, robustness, and sample stability in solution.

3.2.1. linearity

The structures of the related compounds listed in Fig. 1 were verified by NMR and/or LC-MS. Due to limited availability of the reference materials of certain impurities, compounds A–D and F (Fig. 1) and dexamethasone were selected to conduct experiments to determine the linearity, accuracy/recovery, precision, LOQ and LOD.

The six related compounds selected for this study were dissolved together in the diluent which already contained approximately 1.0 mg/mL betamethasone. The slope, *Y*-intercept, and coefficient of determination (r^2) were obtained from linear regression analysis performed by the SAS system JMP version 4. The peak areas of each individual compound were plotted against corresponding concentrations, which were corrected for purity. Linear regression analysis showed that a coefficient of determination $r^2 = 1.000$ was obtained for betamethasone and also for all the other related compounds that were tested in this study. *Y*-intercepts obtained from the betamethasone linearity curves were insignificant, which were almost 0% compared to the betamethasone responses obtained at the analytical concentration. The *Y*-intercepts obtained for each individual betamethasone related compounds linearity curves were also insignificant compared to the response of a typical related compound obtained at the 0.05% level.

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

The limit of quantitation refers to the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. There are different approaches to determine the LOQ and LOD. Typically the concentration level that generates a signal-to-noise (S/N) of 10 is regarded as the LOQ and the concentration level that generates S/N = 3 is regarded as the LOD. The LOD and LOQ of betamethasone standard solutions are approximately 0.0002 and 0.0005 mg/mL, respectively, which is equivalent to 0.02 and 0.05% of the analytical concentration (1.0 mg/mL) of betamethasone. At the selected LOQ and LOD concentrations, all the S/N for LOQ standard solutions were larger than 10 and all the S/N for LOD standard solutions were larger than 3. These results suggest that the proposed HPLC method has good sensitivity for the estimation of BM related compounds that are typically present in commercial lots of betamethasone.

3.2.3. Accuracy of the method

The solutions used for the linearity studies were also used to determine the recovery and hence the accuracy of the assay. The quantitation (weight/weight %) was carried out by using an external betamethasone standard prepared at the betamethasone analytical concentration. Relative response factors (RRFs) of the betamethasone related compounds were used to calculate the weight percentages of the betamethasone related compounds. The RRF of any individual related compounds that were either not tested in the method validation or with unknown identities is assumed as 1 and used for all calculations. The experimental results showed approximately 99–102% recoveries obtained for betamethasone from 1 to 160% levels. The typical recoveries of betamethasone related compounds were approximately 90–118% for the tested compounds from 0.1 to 2% levels. Therefore, based on the recovery data, the assay of betamethasone and estimation of its related compounds that are prescribed in this report has been demonstrated to be accurate for intended purpose and is adequate for routine analysis in a quality control (QC) laboratory.

3.2.4. Method precision

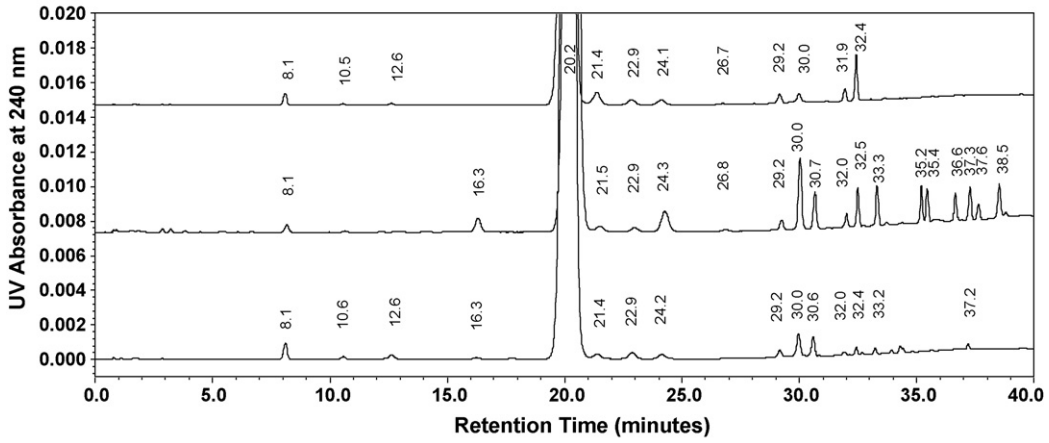
ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) states that method precision may be considered at three levels: repeatability, intermediate precision and reproducibility [11]. Only repeatability and intermediate precision were evaluated in this validation. The data obtained from the linearity study was used for the evaluation of method precision. The repeatability was investigated by calculating the %RSD of the recoveries obtained from nine samples [triplicates at the low (40%), middle (100%), and high (160%) concentration levels] and the intermediate precision was evaluated by calculating the differences in %RSD of recoveries and the average percent recovery obtained from nine samples [triplicates at the low (40%), middle (100%), and high (160%) concentration levels] between analyst 1 and analyst 2. For betamethasone related compounds, the method repeatability was determined from the %RSD of the recoveries obtained from nine samples prepared as triplicates at the low (0.25%), middle (0.5%), and high (1%) concentration levels of the corresponding related compounds. The intermediate precision was determined from the difference in the %RSD of recoveries between both analysts. The results for all the tested compounds are listed in Table 3, which reveals that this method has good repeatability and intermediate precision.

3.2.5. Method specificity

Method specificity was demonstrated during the linearity and accuracy/recovery studies of the six tested betamethasone related compounds because the separation and quantitation were performed in the presence of 1.0 mg/mL betamethasone. The method specificity was also demonstrated by the separation of the remaining betamethasone related compounds listed in Fig. 1. Representative chromatograms of betamethasone samples from different sources and a betamethasone solution spiked with avail-

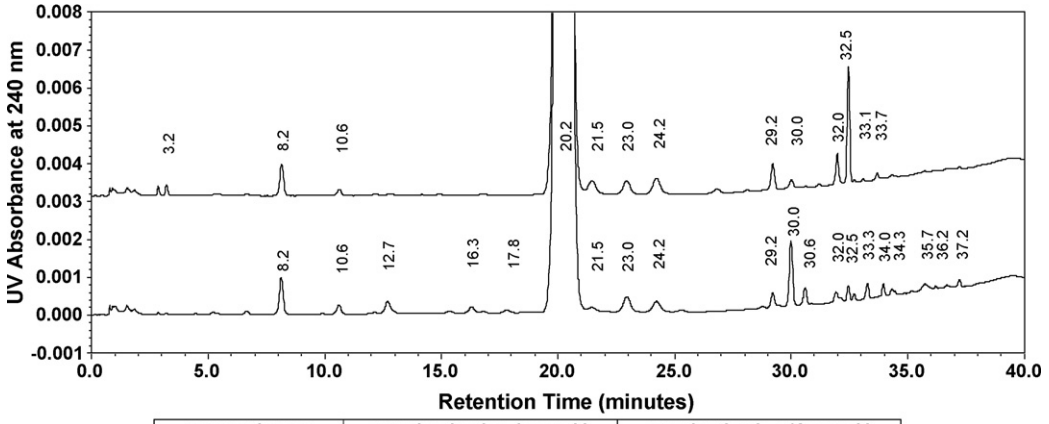
Table 3
Intermediate precision of assay of betamethasone and its related compounds.

Compound name	Average recovery%		Absolute difference from analyst 1
	analyst 1	analyst 2	analyst 2
Compound A	98.34	97.98	0.4%
Betamethasone	100.26	99.37	0.9%
Dexamethasone	100.84	103.33	2.5%
Compound B	104.61	100.57	4.0%
Compound C	104.62	98.37	6.3%
Compound D	101.79	100.85	0.9%
Compound F	103.55	102.41	1.1%
Compound name	**%RSD of recovery**		**Absolute difference from analyst 1**
	analyst 1	analyst 2	analyst 2
Compound A	1.8%	1.3%	1%
Betamethasone	0.6%	0.3%	0%
Dexamethasone	2.8%	2.7%	0%
Compound B	5.2%	4.0%	1%
Compound C	4.0%	1.8%	2%
Compound D	2.5%	1.3%	1%
Compound F	3.0%	1.1%	2%



compound names	retention time in sample from source I (minutes)	retention time in impurity profile sample (minutes)	retention time in sample from source II (minutes)
compound A	Not Detected	16.3	16.2
betamethasone	20.1	20.2	20.1
dexamethasone	21.4	21.5	21.4
compound B	24.1	24.3	24.1
compound C	30.0	30.0	30.0
compound D	Not Detected	30.7	30.6
compound E	32.4	32.5	32.4
compound F	Not Detected	33.3	33.2
compound G	Not Detected	35.2	Not Detected
compound H	Not Detected	35.4	Not Detected
compound I	Not Detected	36.6	Not Detected
compound J	Not Detected	37.3	37.2
compound K	Not Detected	37.6	Not Detected
compound L	Not Detected	38.5	Not Detected

Fig. 4. Typical chromatograms of (a) betamethasone from source I (top chromatogram), (b) betamethasone and its related compounds (impurity profile solution, middle chromatogram), and (c) betamethasone from source II (bottom chromatogram).



compound names	retention time in a 4-year old sample from source I (minutes)	retention time in a 10-year old sample from source II (minutes)
compound A	Not Detected	16.3
betamethasone	20.2	20.2
dexamethasone	21.5	21.5
compound B	24.2	24.2
compound C	30.0	30.0
compound D	Not Detected	30.6
compound E	32.5	32.5
compound F	Not Detected	33.3
compound G	Not Detected	Not Detected
compound H	Not Detected	Not Detected
compound I	Not Detected	Not Detected
compound J	Not Detected	37.2
compound K	Not Detected	Not Detected
compound L	Not Detected	Not Detected

Fig. 5. Chromatograms of a 4-year old betamethasone sample from source I (top chromatogram) and a 10-year old betamethasone sample from source II (bottom chromatogram).

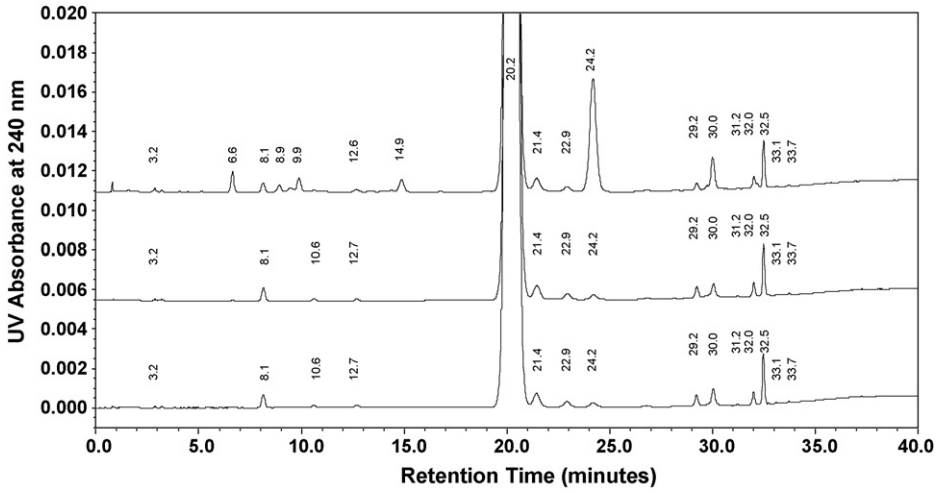


Fig. 6. Typical chromatograms obtained from stability studies of betamethasone from source I. Sample solution exposed to lab lights for 7 days (top chromatogram); solution stored in refrigerator for 7 days (middle chromatogram), solution in flask wrapped with aluminum foil at room temperature for 7 days (bottom chromatogram).

able related compounds are shown in Fig. 4. The chromatograms illustrate that the betamethasone peak is free from any interferences of blank solvent peaks, betamethasone and dexamethasone is adequately separated, and betamethasone and its related compounds are separated from each other.

An analytical method is stability indicating if the method can separate all the process related impurities and all the degradation products from the major peak (i.e., the API peak) of the sample. Stress degradation studies are typically conducted under conditions such as heat, light, acid, base, and oxidation, for compounds that do not have aged samples. Aged BM samples from more than 30 different batches (stored under various stability and storage conditions) were analyzed using the new method that is prescribed in this report. These aged samples represented the true degradation chemistry containing actual degradation products that are generated under real stability and or bulk storage conditions. The results obtained from the aged samples clearly demonstrated that this method is capable of resolving betamethasone peak from all the impurities peaks in the samples. For presentation purpose, the overlay chromatograms of two representative expired betamethasone stability samples, one was 10-year old sample and the other one was 4-year old sample, are shown in Fig. 5. The homogeneity of the betamethasone peak in Fig. 3 was determined by a PDA scanning the wavelength range from 200 to 400 nm using Waters Millennium software. Peak purity results were obtained by comparing the purity angle and purity threshold. In every stability sample, betamethasone peak had purity angles at least 10 times less than the purity thresholds, indicating identical UV spectra across the peak. Therefore, the betamethasone peak is homogeneous and is free of any interferences by other peaks,

3.2.6. Solution stability

Solutions with betamethasone (standard and sample) at a concentration of approximately 1.0 mg/mL and the LOQ level were prepared in duplicate. Standard and sample solutions were stored at ambient laboratory temperatures (clear volumetric flasks with or without aluminum foil wrapping) and under refrigeration (2–8 °C, clear glassware without aluminum foil wrapping). The relative percent difference was determined for the solutions stored at room temperature and at 2–8 °C in day 1, 3, and 7 vs. day 0, respectively. It was found that the sample and/or standard solutions were stable when the solutions were stored in flasks that were wrapped with aluminum foil or in a refrigerator. Under those two conditions, the difference of the amounts obtained for betamethasone in each of the sample and standard solution is within 2% of the initial amount. Also under those two conditions, the difference of the peak area percentage obtained for betamethasone related compounds in each of the sample solution is within ±0.1% of the initial amount. All injections of QL solutions of betamethasone showed a signal-to-noise ratio >10. The sample and/or standard solutions were not stable when the solutions were stored in flasks that were left on lab bench, exposed to lab lighting. The decrease in the amounts of betamethasone in those solutions was around 3% after three days and around 7% after 7 days. Several degradation peaks appeared at ~6.6, ~8.9, ~9.4, ~9.9, ~14.9, and ~24.2 min (Fig. 6). The degradation peak at 24.2 min was determined to be compound B. Therefore, it was concluded that the betamethasone solution was not stable when stored at ambient laboratory conditions and exposed to regular lab lighting. The method has thus been demonstrated to be a stability-indicating method because it can adequately separate degradation peaks from the betamethasone peak and accurately quantitate the amount of betamethasone and its degradation products in the stability samples.

3.2.7. Method robustness

The robustness of the method demonstrated by showing the capacity of the method remained unaffected while deliberately changing HPLC parameters. Several parameters including HPLC column batch, flow rate, detector wavelength, temperature, injection volume, mobile phase ratios and gradient conditions were varied around the procedural values to assess the results under each HPLC parameter variation against those obtained under the procedural parameters. Betamethasone related compounds solution, betamethasone QL solution, betamethasone standard solution, and betamethasone sample solution were prepared to obtain the following results: (1) the relative retention times (RRTs) of the betamethasone related compounds, (2) the resolution between betamethasone and dexamethasone, (3) the estimation of the related compounds, (4) the S/N of QL, (5) the assay of betamethasone in reference standard and sample. Each sample or standard solution was injected twice to obtain average of the RRTs, the S/N of QL, the assay of betamethasone and the estimation of the related compounds.

For RRT calculation, two RRT markers were used: the first one was the peak of betamethasone and the second one was the peak of compound D. The RRTs of the peaks eluting before 25 min were calculated against the betamethasone peak and the RRTs of the peaks eluting after 25 min were calculated against the compound D peak.

Table 4
Relative retention times (RRTs) obtained under representative conditions studied for method robustness.

Compound name	Method condition (on Waters HPLC)	Method condition (on Agilent HPLC)	Another ACE 3 Phenyl column	Column temperature = 25 °C	Column temperature = 35 °C
Compound A	0.80	0.80	0.80	0.79	0.80
Betamethasone	1.00	1.00	1.00	1.00	1.00
Dexamethasone	1.06	1.06	1.06	1.07	1.06
Compound B	1.21	1.21	1.21	1.20	1.21
Compound C	0.98	0.98	0.98	0.98	0.98
Compound D	1.00	1.00	1.00	1.00	1.00
Compound F	1.09	1.09	1.09	1.09	1.09

Compound name	Injection volume = 15 μL	Gradient 10% faster	Gradient 10% slower	Flow rate = 1.8 mL/min	Flow rate = 2.2 mL/min
Compound A	0.80	0.80	0.79	0.80	0.80
Betamethasone	1.00	1.00	1.00	1.00	1.00
Dexamethasone	1.07	1.06	1.07	1.06	1.07
Compound B	1.21	1.20	1.21	1.20	1.21
Compound C	0.98	0.98	0.98	0.98	0.98
Compound D	1.00	1.00	1.00	1.00	1.00
Compound F	1.09	1.09	1.09	1.09	1.09

Because the method has multiple elution steps, using a second RRT marker makes the RRT determination of the peaks that are eluting after the major peak much more reproducible and reliable. The RRTs of the tested compounds obtained under a few representative HPLC conditions are summarized in Table 4. It can be seen that the RRTs obtained under various chromatographic conditions remained almost unchanged.

The resolution factors (R_s) between betamethasone and dexamethasone obtained under various HPLC conditions were found to be larger than 2.1 (except at 35 °C which was ~1.8) that demonstrated a robust separation between these two epimers. The average peak area percentages of betamethasone related compounds under various robustness conditions were found to be within ±0.1% of the average result obtained using the procedural parameters. The average %assay of betamethasone in standard and sample solutions under various robustness conditions was found to be within 2% of the average result obtained using the procedural parameters.

4. Conclusions

The analytical method described in this paper is the first known RP-HPLC method that can separate and accurately estimate all the known and unknown related compounds of betamethasone. Therefore, this method is suitable for assay of betamethasone and estimation of its related compounds that are typically present in commercial lots of betamethasone. This method has been demonstrated to have good accuracy, high sensitivity, linearity, precision, reproducibility, repeatability, specificity, and robustness. This method has also been demonstrated to be a stability-indicating method because it can also separate all the known and unknown degradation product peaks of betamethasone from betamethasone peak (and also from each other) and can accurately quantitate the content of betamethasone in the stability samples. Therefore this method can be used in quality control labs for routine analysis of commercial lots of betamethasone for the purpose of lot release and also for the purpose of conducting stability studies.

Acknowledgements

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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