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Development and Validation of a Stability-Indicating RP-HPLC Method for Assay of Alphamethylepoxide and Estimation of its Related Compounds

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Abstract

Alphamethylepoxide (16α-methyl-$\Delta_{1,4}$-pregnadiene-9β-11β-oxide-17α,21-diol-3,20-dione) is a key intermediate for the synthesis of various active pharmaceutical ingredients of steroid compounds. A stability-indicating reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the assay of alphamethylepoxide and estimation of its related compounds has been developed and validated. It can accurately quantitate alphamethylepoxide in the presence of numerous structurally related compounds (including the β-epimer, known as betamethylepoxide). This method can also adequately separate most of the impurities from each other and estimate their quantities in alphamethylepoxide samples. The stability-indicating capability of this method has been demonstrated by adequate separation of the degradation products from alphamethylepoxide in stress degraded and aged stability samples. A 15 cm × 4.6 mm i.d. ACE C_{18} HPLC column is the primary column used in this method, and a 15 cm × 4.6 mm i.d. A Develosil ODS UG column serves as the alternative column. The mobile phase consisted of 10mM sodium sulfate, 0.05% (v/v) phosphoric acid, and acetonitrile. This method can accurately assay the content of alphamethylepoxide (in a given lot) with a % relative standard deviation of less than one. It can also estimate individual impurities down to 0.05% level compared with the alphamethylepoxide peak in the sample.

Introduction

In the pharmaceutical industry, the control of the purity of key intermediates is critical to ensure the quality of active pharmaceutical ingredients (APIs) and the final drug products. The impurities in the key intermediates can potentially be carried over throughout the subsequent synthetic steps, or they can undergo similar reactions to form "new impurities" in the APIs or final products. The time and effort spent on the identification of the impurities and their sources, or on the toxicity study of the "new impurities" can be saved if the type and amount of impurities in the key intermediates are tightly controlled. Therefore, it is desirable to have an analytical method that can not only separate the key intermediate from all the potential related compounds (including process-related impurities and degradation products), but also separate all the related compounds from each other.

Alphamethylepoxide (16α-methyl-$\Delta^{1,4}$-pregnadiene-9β-11β-oxide-17α,21-diol-3,20-dione, see Figure 1 for structure) is the key intermediate for synthesizing various APIs of steroid compounds, such as dexamethasone, dexamethasone-21-acetate, dexamethasone-21-phosphate, and dexamethasone-17,21-dipropionate. As indicated in the literature, development of a reversed-phase high-performance liquid chromatographic (RP-HPLC) stability-indicating method for analysis of typical steroid compounds has always been a challenging task (1,2,3). One of the major causes of the challenge is the presence of a great number of structurally similar compounds in the samples. In the case of alphamethylepoxide, we needed to develop a method that not only can separate 11 impurities with known structures (listed in Figure 1), but also separate impurities with unknown identities as well any new degradation products that might form during storage of the samples. The HPLC separation of steroid compounds becomes extremely challenging when separation of the epimers of the APIs or key intermediates is necessary for accurate quantitation of the major peak and each individual epimer impurity peak in the samples (4–7). In the case of alphamethylepoxide, the most challenging part of an RP-HPLC analysis of this material is the separation of alphamethylepoxide from its β-epimer [i.e., the betamethylepoxide (16α-methyl-$\Delta^{1,4}$-pregnadiene-9β-11β-oxide-17α,21-diol-3,20-dione, see Figure 1 for structure)]. Betamethylepoxide is the key intermediate for the synthesis of other steroid APIs, such as betamethasone and the esterification compounds of betamethasone. The β- and α- forms (i.e., 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 an opposite direction from the plane. Physicochemical characteristics of these two forms of the compounds are very similar but not identical as is the case for enantiomers (8,9). Therefore, it would be a challenging task to obtain a mobile phase and a stationary phase that would provide adequate differences in thermodynamic parameters (entropy,

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enthalpy, etc.) between these epimers for a true baseline separation. However, because different isomeric forms of an API may have vastly different physiological effects (10–12), it is preferred that the API of a pharmaceutical product is in one pure form instead of mixed isomers. In our case, accurate quantitation of betamethylepoxide is deemed necessary, even when it is present in trace quantity (e.g., ~ 0.1% or lower) in the alphamethylepoxide samples. The epimeric purity of the APIs has to be controlled at the intermediate stage as it can be expected from the similarity in the structures that the epimers will go through the synthesis in a highly similar manner and would result in epimeric impurities in the final APIs.

To our knowledge, there is no literature report available on the HPLC analysis of alphamethylepoxide and its related compounds. In fact, a stability-indicating HPLC method for the analysis of betamethylepoxide and its related compounds has been developed and validated, and the results will be published elsewhere.

In this paper, we describe an RP-HPLC method for the assay of alphamethylepoxide and estimation of its related compounds. The method has been demonstrated to be accurate, linear, precise, reproducible, repeatable, specific, and robust, and therefore suitable for routine analysis of alphamethylepoxide. This method has also been demonstrated to be stability indicating because it can separate degradation peaks from the alphamethylepoxide peak that were present in stress degraded samples or in aged stability samples.

In addition, we have identified an alternative column (Develosil ODS UG) to the primary column (ACE C_{18}). The prescribed method has also been validated on the alternative column.

Experimental

Chemical and reagents

Alphamethylepoxide and related compounds were provided by the Global Quality Services– Analytical Sciences Group in Schering-Plough (Union, New Jersey). All HPLC-grade solvents were obtained from Fisher Scientific (Fisher Scientific International Inc. Liberty Lane Hampton, NH). Water (18.2 MΩ.cm) was obtained using a Milli-Q system (Millipore, Billerica, MA).

Apparatus

A Hitachi LaChrom Elite HPLC system (Hitachi High Technologies America, Inc. San Jose, CA) equipped with ChromSword method development software (Merck KGaA, Darmstadt, Germany); an Agilent Technologies 1100 Series HPLC system (Santa Clara, CA) equipped with a LC Spiderling column switching system (Chiralizer Services, L.L.C., Newtown, PA); and a Waters 2695 Alliance HPLC systems (Milford, MA) 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. During the validation, Waters HPLC systems equipped with 2695 separation modules and 2996 photodiode array detector or 2487 dual wavelength UV detectors were used. The primary HPLC column was an ACE C_{18} (15 cm × 4.6 mm i.d., 3 µm particle size) purchased from Mac-Mod Analytical, Inc. (Chadds Ford, PA). The alternative column was a Develosil ODS UG (15 cm × 4.6 mm i.d., 3 µm particle size) purchased from Pheno-menex (Torrance, CA).

Chromatographic conditions

The mobile phase consisted of (A) a 1:4 (v/v) mixture of acetonitrile and an aqueous solution consisting of 10mM sodium sulfate and 0.05% (v/v) phosphoric acid and (B) a 6:4 (v/v) mixture of acetonitrile and the aqueous solution consisting of 10mM sodium sulfate and 0.05% (v/v) phosphoric acid. The gradient program is listed in Table I. The total run time was 58 min including an approximately 15-min re-equilibration time. The flow rate was 1.5 mL/min and the column temperature was 40°C. The detection wavelength was 254 nm. The stress degraded samples and the solution stability samples were analyzed using a PDA detector covering the range of 200 nm to 400 nm. The sample injection volume was 15 µL.

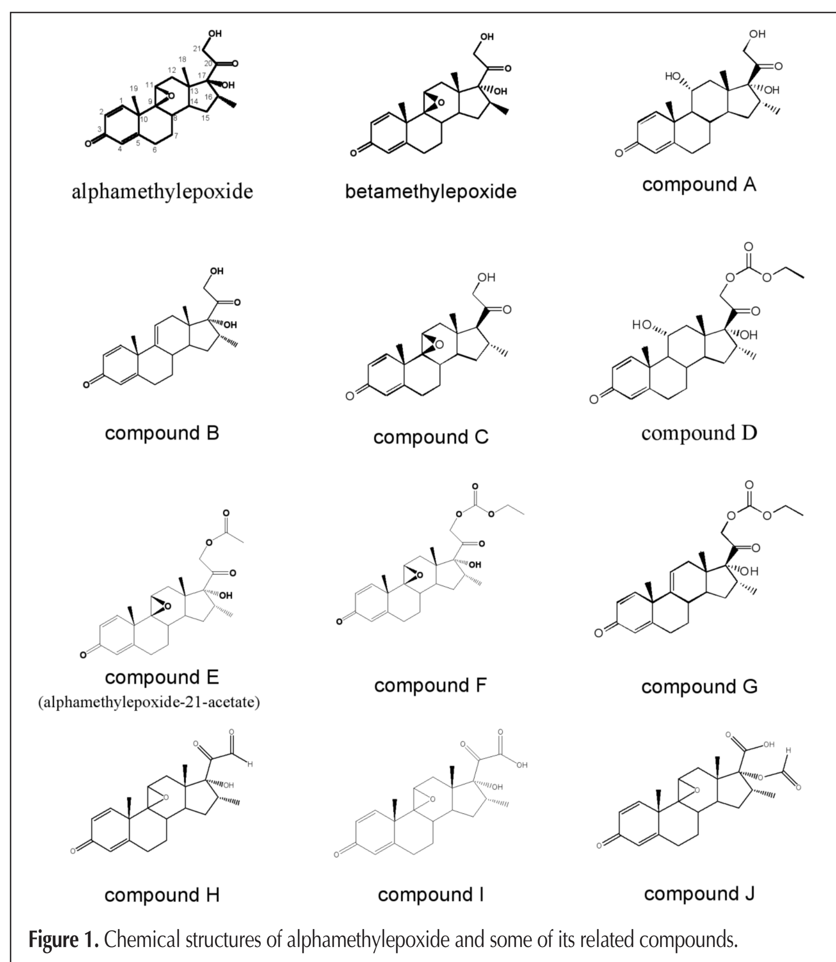

Figure 1. Chemical structures of alphamethylepoxide and some of its related compounds.

Table I. Gradient Program of the Method*

Time (min)	Mobile phase A	Mobile phase B	Gradient curve
0	97%	3%	Linear
25	65%	35%	Linear
40	0%	100%	Linear
43	0%	100%	Linear
44	97%	3%	Linear
58	97%	3%	Linear

* Flow rate was 1.5 mL/min.

Solution preparations

Sample solutions were prepared by dissolving appropriate amounts of alphamethylepoxide or its related compounds into methanol. The analytical concentration of alphamethylepoxide was 0.25 mg/mL. To determine the linearity of alphamethylepoxide, triplicate preparation of alphamethylepoxide in methanol 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 alphamethylepoxide analytical concentration (which was 0.25 mg/mL). The linearity of alphamethylepoxide related compounds were also determined using triplicate preparations of alphamethylepoxide related compounds in methanol at each of the six concentration levels of 0.05%, 0.1%, 0.25%, 0.5%, 1%, and 2% of the alphamethylepoxide analytical concentration.

Stress degradation of alphamethylepoxide samples

The solid powder of alphamethylepoxide was subjected to light and heat exposures. Heat stress was conducted by heating the alphamethylepoxide at 105°C in an oven for 7 days. Photo stress was performed by exposing the alphamethylepoxide to CWF (Cool White Fluorescent) for 2.6 million lux-h followed by exposing to UVA (Ultraviolet A Radiation) for 400 watt-hours/m^2 at 25°C ± 2°C.

For solution stability studies, solutions containing alphamethylepoxide at a concentration of ~ 0.25 mg/mL were prepared. The solutions were stored in volumetric flasks (either amber glass or transparent glass flasks wrapped with or without aluminum foil) at ambient laboratory temperatures and stored under refrigeration (2–8°C).

Calculation

The quantitation of alphamethylepoxide or its related compounds were carried out using an external alphamethylepoxide reference standard prepared at 0.25 mg/mL in methanol. The sample solutions were bracketed between two alphamethylepoxide reference standard solutions and the experimental concentration was obtained from the following equation:

$$\text{Experimental Conc.} = \frac{P2}{RRF \times P1} \times C1$$

where: P1 = Average peak area of alphamethylepoxide in the adjacent alphamethylepoxide bracketing standards; P2 = Peak area of alphamethylepoxide or each individual related compound in linearity sample solution; C1 = Concentration of alphamethylepoxide in alphamethylepoxide bracketing standard; RRF = relative response factor (RRF is the ratio between the response factor of each individual related compound and the response factor of alphamethylepoxide). The RRF was the quotient obtained by dividing the slope of the linear regression curve of alphamethylepoxide 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:

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

Results and Discussion

HPLC method development

In the beginning we believed that it was clear from the molecular structures of alphamethylepoxide and the known related compounds (Figure 1) that there were no functional groups, which could be easily ionized. Therefore, mobile phase pH or ionic strength should not affect the retention/separation of these compounds under the conditions of reversed-phase chromatography. Hence, the method development was focused on the selection of a suitable 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 final elution profile. However, experimental data showed that, potentially, a degradation could occur once the alphamethylepoxide samples were dissolved in a mixture of acetonitrile and water, but it was stable when dissolved in neat methanol or in a mixture of methanol and water. The actual cause of instability of alphamethylepoxide in neat acetonitrile is not clear. A literature search also revealed no report on this topic. The degradation products were identified to be acids (compound I and J, which were degraded from compound H, see Figure 1) that appeared as broad peaks with irregular shapes. A 10mM sodium sulfate together with 0.05% (v/v) phosphoric acid were used to control the ionic strength and to lower the pH of the mobile phase. The peak shapes of the acidic degradants became symmetrical after the addition of salt and acid. Although because we changed the sample diluent to neat methanol or a 1:1 (v/v) mixture of water–methanol, the degradation was never observed, we still kept the salt and acid in the mobile phase in case there might be any acidic degradants observed in the future. In addition, although the injection precision was found to be better when alphamethylepoxide was dissolved in the 1:1 (v/v) mixture of water–methanol, some of the impurities were difficult to be dissolved in this mixture. Therefore, the samples were dissolved in neat methanol throughout the method validation study.

During the method development activities, advanced HPLC technologies such as ChromSword, a computer-aided chromatographic method development tool, and a LC Spiderling automated 9-port column switching system were used in combination with the knowledge and experiences of the bench analytical scientists. The use of the advanced method development tools largely improved the efficiency of method development activities and also enhanced the probability of finding an

optimum separation condition in a short time period (13–18). The details and specifics of the peak identification of compound H, I, and J, and our strategy for efficient method development will be reported elsewhere.

Initial strategy for any new method development activities should be to identify the pairs of known peaks that would pose the greatest challenge to achieve baseline separation. Although there were a number of related compounds that needed to be separated, however, the initial focus of the method development activities was to separate alphamethylepoxide peak from trace level (~0.1% of the alphamethylepoxide peak) of betamethylepoxide peak. The final method was then built based on the conditions that were suitable for separating this critical pair and meanwhile separating all other alphamethylepoxide-related compounds with known and unknown identities. During the method development work, ~ 50 different HPLC columns were screened and a large number of combinations of different organic modifiers, including acetonitrile, methanol, isopropanol, and tetrahydrofuran, were investigated. Table II lists some representative columns that were tested and their ability to separate alphamethylepoxide and betamethylepoxide is briefly described. Many columns showed good separation between betamethylepoxide and alphamethylepoxide (i.e., resolution greater than 1.5). Furthermore, we found that the separation between betamethylepoxide and alphamethylepoxide could be achieved within 10 min under certain conditions. However, the separation became much more challenging and complicated due to the presence of many other peaks that eluted in the close vicinity of the betamethylepoxide peak. Many columns in Table II, which showed adequate separation between alphamethylepoxide and betamethylepoxide failed to show adequate separation for some other impurities. Based on the assessment of the overall separation including all the betamethylepoxide related compounds, the 15 cm ACE C_{18} column was selected as the primary column for the method development. The final mobile phase and gradient conditions of this method was selected after testing the separation of all the potential impurities using a large number of alphamethylepoxide samples of various ages and from different sources.

Table II. Columns Screened During Method Development

Column name	Column description (4.6 mm i.d.)	Comments*
YMC-Pack Pro C_{18}	5 cm (3 µm, 5 µm), 15 cm	1
YMC Basic	5 cm	1
YMC J'sphere ODS-H80	15 cm	1
YMC J'sphere ODS-M80	15 cm	1
YMC J'sphere ODS-L80	15 cm	1
YMC Hydrosphere C_{18}	5 cm, 15 cm	1
YMC ODS AQ	5 cm, 15 cm	1
YMC ODS AQ	5 cm (200 Å pores)	2
YMC ODS A	5 cm (300 Å pores)	2
YMC-Pack CN	5 cm	2
YMC-Pack Phenyl	5 cm	1
TSK Super ODS	5 cm	1
TSK Super Octyl	5 cm	1
Ace C_4	5 cm	2
Ace C_8	5 cm	1
Ace C_{18}	100 Å or 300 Å pores for 5 cm or 15 cm	15 cm (100 Å) is the primary column
Waters Sunfire C_{18}	5 cm, 15 cm	1
Waters Atlantis dC_{18}	5 cm	1
Waters XTerra MS C_{18}	15 cm	1
Waters XTerra RP C_{18}	15 cm	1
Waters XBridge C_{18}	15 cm	1
Waters Symmetry Shield RP	15 cm	1
Novapak C_{18}	15 cm	1
µBondapak C_{18}	15 cm	2
Phenomenex Luna C_{18} (2)	15 cm	1
Phenomenex Gemini C_{18}	5 cm, 15 cm	1
Phenomenex Inertsil ODS-3	15 cm	1
Phenomenex Prodigy ODS (3)	15 cm	1
Zorbax Eclipse XDB-C_{18}	5 cm	1
Zorbax RX-C_{18}	15 cm	1
Supelco ABZ	5 cm	2
Develosil ODS UG	15 cm	Alternative column
Macherey-Nagel Nucleosil 100 C_{18}	15 cm	1
Alltech Platinum 100 C_{18}	15 cm	1
Shiseido Capecell-Pak C_{18} UG,	15 cm	1
Beckman Ultrasphere C_{18}	15 cm	1

* 1 = Good separation can be achieved between betamethylepoxide and alphamethylepoxide. 2 = Separation not adequate for betamethylepoxide and alphamethylepoxide.

In addition to the primary ACE C_{18} column, we also identified an alternative column, the Develosil ODS UG column. It is beyond the scope of this manuscript to present the detailed work carried out for the search of alternative columns. The following only provides the rationale for identification of an alternative column. For any HPLC method that is meant for routine analysis in pharmaceutical quality control (QC) laboratories, it is important to identify an alternative column to the primary column. An optimized separation can be jeopardized even if there is only a little change taking place in the physicochemical characteristics of the primary column. This variation of the primary column does not mean the batch-to-batch variation that can occur in the routine manufacturing of the HPLC columns. The variation refers to some permanent changes in the column manufacturing process, such as using silica from different sources or if surface modification techniques are modified. This can have negative impact in a pharmaceutical QC laboratory because typically all the method details, including the column information, are approved by regulatory agencies. One way to avoid this situation is to identify and qualify one or more alternative columns using the same HPLC method conditions that are in the original validation package. For this purpose, an alternative column is a column that provides resolution, selectivity, and retention time of all the peaks of interests that are similar to the primary column. The true equivalency can be demonstrated by the fact that the alternative column meets the requirements of system suitability, peak identification, and other critical elements that are set for the primary column. In this study, the alternative column search was conducted mainly by looking for columns that had similar stationary phase surface properties, such as carbon loading, endcapping, and surface functionality. The selected columns were then screened on the LC Spiderling system using the conditions of the developed method.

Analytical method validation

Method validation was performed with respect to parameters such as response linearity, assay accuracy, limit of detection (LOQ), and quantitation (LOD), ruggedness and precision, specificity, robustness, sample stability, and equivalency between the primary and alternative columns (all results described in this section were obtained using the ACE C_{18} column).

Method specificity

Method specificity was demonstrated during the linearity and accuracy/recovery studies of the tested alphamethylepoxide-related compounds. The method specificity was further demonstrated by the separation of alphamethylepoxide from its related compounds, with known or unknown identities, in different material sources. Representative chromatograms of alphamethylepoxide samples from different sources and an alphamethylepoxide standard solution spiked with available related compounds are shown in Figure 2. The chromatograms illustrate that the alphamethylepoxide peak is free from the interferences of methanol blank solvent peaks; alphamethylepoxide and betamethylepoxide are adequately separated; and alphamethylepoxide and its related compounds are adequately separated from each other.

Linearity

The structures of the related compounds listed in Figure 1 were confirmed by NMR and/or LC–MS. Due to the limited availability of some of the alphamethylepoxide-related compounds, eight related compounds (i.e., betamethylepoxide and compound A to G listed in Figure 1) were selected to conduct experiments to determine the linearity, accuracy/recovery, precision, LOQ, LOD, and column equivalency.

The analytical (assay) concentration of alphamethylepoxide was ~ 0.25 mg/mL. For alphamethylepoxide, the investigated linearity range covered the concentrations from 0.000125 to 0.4 mg/mL in methanol, which corresponded to 0.05% to 160% of the alphamethylepoxide analytical concentration. For alphamethylepoxide-related compounds, the investigated linearity range covered the concentrations from 0.000125 to 0.005 mg/mL of each tested related compounds, which corresponded to 0.05% to 2% of the alphamethylepoxide analytical concentration. Although the typical concentrations of the related compounds were not greater than 0.5%, we tested a wider range to cover any future potential increase in concentrations of the related compounds in alphamethylepoxide samples. The related compounds were dissolved together in a diluent which contained approximately 0.25 mg/mL alphamethylepoxide. 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 regression coefficient r^2 greater than 0.999 for alphamethylepoxide (n = 24) and for all the tested related compounds (n = 18) were obtained from both analysts. Y-intercepts obtained from the alphamethylepoxide linearity curves were very insignificant, which were almost 0% of the corresponding alphamethylepoxide responses obtained at the analytical concentration. The Y-intercepts obtained for each individual alphamethylepoxide-related compounds linearity curves were less than the 50% responses of the corresponding related compound obtained at the 0.05% level.

LOD and LOQ

The LOQ 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 a S/N = 3 is regarded as the LOD. Practically, however, different compounds can possess different LOQ and LOD concentrations at the selected UV wavelength for the method detection. Moreover, depending on the different sensitivity of the detectors used, either a PDA or a dual wavelength UV detector, the S/N ratios can be very different at the selected LOQ and LOD levels. To be consistent, in this paper we set the

Compound names	RT* in combined compounds (min)	RT in AP† from source II	RT in AP† from source I
Compound A	10.802	10.908	ND‡
Alphamethylepoxide	17.030	17.146	17.095
Betamethylepoxide	17.852	17.970	17.919
Compound B	19.352	19.473	ND‡
Compound C	22.211	22.345	22.272
Compound D	29.918	ND‡	ND‡
Compound E (alphamethylepoxide-21-acetate)	30.451	ND‡	30.498
Compound F	35.210	35.257	ND‡
Compound G	36.615	ND‡	ND‡

* RT = Retention time.
† AP = Alphamethylepoxide.
‡ ND = Not detected.

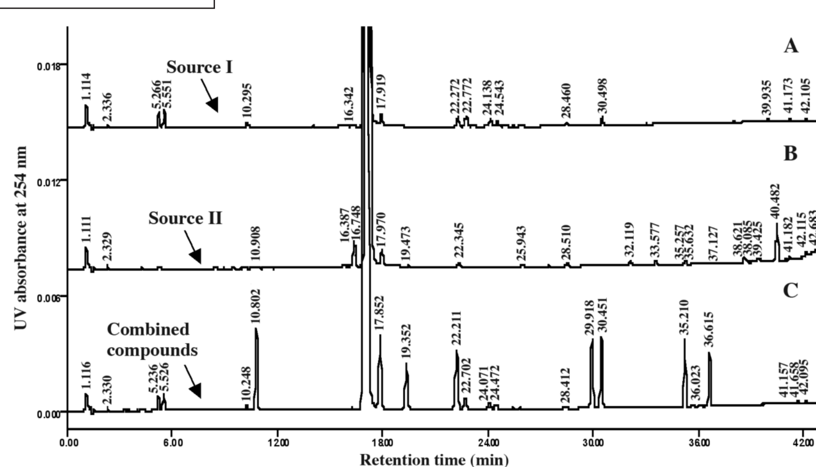

Figure 2. Typical chromatograms of alphamethylepoxide from source I (A), alphamethylepoxide from source II (B), alphamethylepoxide from source I that was spiked with approximately 0.5% of the related compounds (C) obtained on the ACE C_{18} column.

LOQ at 0.05% and the LOD at 0.02% of the alphamethylepoxide analytical concentration level (i.e., LOQ at 0.000125 mg/mL and LOD at 0.00005 mg/mL). At the selected LOQ and LOD concentrations, all S/N for LOQ were larger than 10 and all the S/N for LOD were larger than 3.

Accuracy

The solutions used for the response linearity studies were also used to generate the recovery data to evaluate the assay accuracy. The quantitation, which was determined as weight/weight%, was carried out by using an external bracketing alphamethylepoxide standard prepared at 0.25 mg/mL. Relative response factors (RRFs, see Table III) of the alphamethylepoxide-related compounds were used to calculate the weight percentages of the alphamethylepoxide-related compounds. The RRF of the related compounds that were either not quantitatively tested in the method validation (e.g., compounds H–J in Figure 1) or with unknown identities could be used as 1 in routine analysis. The experimental results showed ~ 98% to 101% recoveries were obtained for alphamethylepoxide from 1% to 160% levels. At the 0.05% concentration level (i.e., the LOQ level), the recoveries ranged from 98% to 127%. The typical recoveries of alphamethylepoxide related compounds were ~ 87% to 109% for the tested compounds. In this study, the quantitation was based on the external standard that was prepared at the analytical concentration level, regardless whether the quantitation was for alphamethylepoxide, which was at the same analytical concentration level or the related compounds, which were at typically less than 0.5% level. Therefore, based on the recovery data, we believe the assay of alphamethylepoxide and estimation of its related compounds has been demonstrated to be accurate enough for routine analysis.

Method precision and ruggedness

International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use considers ruggedness as the method reproducibility and intermediate precision. Again, the data obtained from the linearity study was used for the evaluation. The method reproducibility was evaluated by the %RSD of the recoveries obtained from nine samples prepared as triplicates at the low (40%), middle (100%), and high (160%) alphamethylepoxide concentration levels. For alphamethylepoxide-related compounds, the method reproducibility was evaluated by 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 evaluated based on the difference in the average recoveries and the difference in the % RSD of recoveries between analyst 1 and analyst 2. The results for all the tested compounds are listed in Table IV, which reveal that the method has a good reproducibility and intermediate precision.

Stress degradation

A newly developed analytical method is stability-indicating if this method can separate all the process-related impurities and all the degradation products from the major peak of the sample. Although stress studies can be conducted under conditions such as heat, light, acid, base, and oxidation, those conditions are in fact recommended for testing new compounds that do not have long-term stability data under various storage conditions. During method development work, we tested aged samples that were obtained from stability studies of alphamethylepoxide. These aged stability samples represented the true degradation chemistry under the real life scenario. The results obtained from the aged samples clearly demonstrated that the method was capable of resolving the alphamethylepoxide peak from all the

Table III. Typical values of RRF and RRT Obtained on ACE C_{18} and on Develosil Column

Compound name	RRF		RRT	
	ACE C_{18}	Develosil	ACE C_{18}	Develosil
Compound A	1.11	1.16	0.63	0.65
Alphamethylepoxide	1.00	1.00	1.00	1.00
Betamethylepoxide	0.98	1.02	1.05	1.05
Compound B	0.81	0.81	1.14	1.12
Compound C	1.01	0.99	1.31	1.29
Compound D	0.89	0.92	0.98*	0.99*
Compound E (Alphamethylepoxide-21-acetate)	0.86	0.87	1.00	1.00
Compound F	0.76	0.80	1.16*	1.15*
Compound G	0.61	0.61	1.21*	1.19*

* The RRTs of compounds D, F, and G are obtained against the RT of compound E.

Table IV. Intermediate Precision of Assay of Alphamethylepoxide and Its Related Compounds

Compound name	Average recovery%		Difference from analyst
	Analyst 1	Analyst 2	Analyst 2
Compound A	99.47	92.56	7 (6.91)
Alphamethylepoxide	99.69	100.18	0 (0.49)
Betamethylepoxide	100.57	99.72	1 (0.85)
Compound B	98.55	92.33	6 (6.22)
Compound C	98.68	N/A*	N/A*
Compound D	99.89	93.42	6 (6.47)
Compound E (alphamethylepoxide-21-acetate)	98.70	91.63	7 (7.07)
Compound F	97.90	92.19	6 (5.71)
Compound G	98.02	92.56	5 (5.46)
Compound name	**% RSD of recovery**		**Difference from analyst 1**
	Analyst 1	**Analyst 2**	**Analyst 2**
Compound A	0.78	0.78	0 (0.00)
Alphamethylepoxide	0.38	0.76	0 (0.38)
Betamethylepoxide	0.99	1.41	0 (0.42)
Compound B	0.58	1.84	1 (1.26)
Compound C	0.25	N/A*	N/A*
Compound D	1.26	0.52	1 (0.74)
Compound E (alphamethylepoxide-21-acetate)	0.84	3.60	3 (2.76)
Compound F	0.86	2.24	1 (1.38)
Compound G	1.09	3.09	2 (2.00)

* Due to limited availability of compound C, only analyst 1 performed the validation study of this compound.

peaks of the impurities in the samples. Therefore, in this validation, alphamethylepoxide stress studies were conducted only under heat and light exposures based on the precautionary consideration that during storage or transportation, the alphamethylepoxide sample might be exposed to excessive light or heat.

Figure 3 contains representative chromatograms of the heat- and photo-stressed alphamethylepoxide. By comparing the chromatograms before (refer to the middle chromatogram in Figure 2) and after stress experiments (Figure 3), it can be seen that no major degradation peaks appeared in the chromatogram even after 7 days of heat stress; while there was one major degradation peak appeared at ~ 13.9 min after photo stress. The homogeneity of the alpha-methylepoxide peak in the heat- or photo-stressed samples was estimated based on photo diode array (PDA) scans from 200 nm to 400 nm using Waters Millennium32 software. Peak purity was obtained by comparing the purity angle and purity threshold. The results showed that in every stressed sample, the alpha-methylepoxide peak had purity angles much less than the purity thresholds, indicating identical UV spectra across the peak. Quantita-tion of heat- or photo-stressed alphamethylepoxide revealed that the absolute difference in the amounts of alphamethylepoxide obtained before and after the heat stress studies was less than 1 wt%, while the difference was larger than 6 wt% before and after the photo stress. Therefore, it was concluded that the alphamethylepoxide was stable under high temperatures while it is unstable under light exposures.

The quantitation of the solution stability samples was performed against freshly prepared alphamethylepoxide bracketing standard at each testing point. The differences in the amounts of alphamethylepoxide were determined for the solutions stored at room temperature and at 2°C~8°C between days 1, 3, 7, vs. day 0, respectively. It was found that the sample solutions were stable when the solutions were stored in amber glass flasks or in transparent glass flasks that were wrapped with aluminum foil or in a refrigerator. Under those three conditions, the differences in the assay of alphamethylepoxide were within 2% of the initial amount after 7 days. The sample solutions were not stable when stored in transparent glass flasks that were left on lab bench, exposed to the lab lights. Two major degradation peaks appear at ~ 13.8 min and ~ 16.1 min (Figure 3). The difference in the amounts of alphamethylepoxide in solutions was around 4% even just for one day and around 27% after 7 days. From the solution stability study, it was concluded that the alphamethylepoxide solution was not stable when exposed to light.

Based on the solid-state stress and liquid solution stability studies, we have demonstrated that the proposed method is a stability-indicating method. The method adequately separates the degradation peaks from the alphamethylepoxide peak and accurately quantitates alphamethylepoxide in the stressed and stability samples.

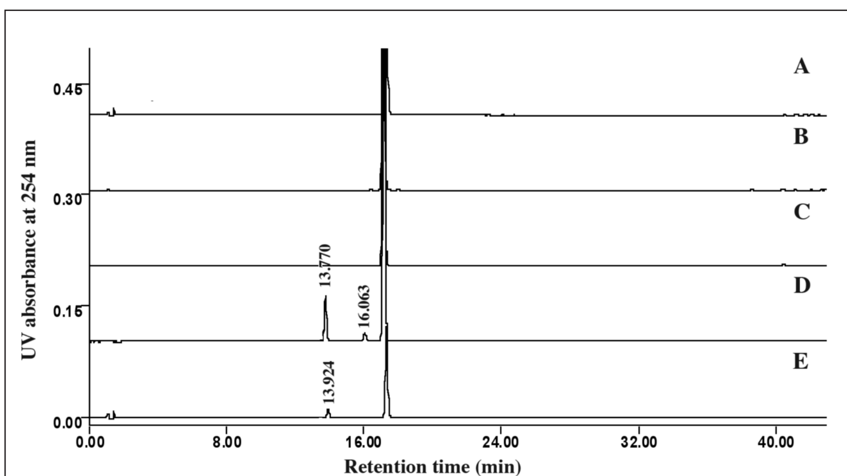

Figure 3. Typical chromatograms obtained from stability studies of alphamethylepoxide from source II on the ACE C_{18} column. Solid powder after heat stress for 7 days (A), solution stored in refrigerator for 7 days (B), solution in flask wrapped with aluminum foil for 7 days (C), solution exposed to lab lights for 7 days (D), and solid powder after photo stress (E).

Table V. RRTs Obtained Under Conditions Studied for Method Robustness

Compound name	Method condition		Different lots	Column Temp.	
	Waters HPLC	Agilent HPLC	ACE C_{18} column	35°C	40°C
Compound A	0.63	0.63	0.63	0.63	0.64
Alphamethylepoxide	1.00	1.00	1.00	1.00	1.00
Betamethylepoxide	1.05	1.05	1.05	1.05	1.05
Compound B	1.14	1.14	1.14	1.13	1.14
Compound C	1.31	1.31	1.30	1.30	1.31
Compound D*	0.98	0.98	0.98	0.98	0.99
Compound E (alphamethylepoxide-21-acetate)	1.00	1.00	1.00	1.00	1.00
Compound F*	1.16	1.16	1.16	1.15	1.17
Compound G*	1.21	1.21	1.20	1.20	1.22

Compound name	No salt, no acid in mobile phase	Gradient		Flow rate	
		10% faster	10% slower	1.35 mL/min	1.65 mL/min
Compound A	0.63	0.64	0.63	0.64	0.63
alphamethylepoxide	1.00	1.00	1.00	1.00	1.00
betamethylepoxide	1.05	1.05	1.05	1.05	1.05
Compound B	1.14	1.13	1.14	1.13	1.14
Compound C	1.31	1.30	1.31	1.30	1.31
Compound D*	0.99	0.98	0.98	0.98	0.98
Compound E (alphamethylepoxide-21-acetate)	1.00	1.00	1.00	1.00	1.00
Compound F*	1.16	1.15	1.16	1.15	1.17
Compound G*	1.21	1.20	1.21	1.19	1.21

* The RRTs of compound D, F, and G are obtained against the retention time of compound E.

Method robustness

Deliberate variations in HPLC parameters were made to demonstrate the robustness of the method. We evaluated the method robustness based on the changes in relative retention times (RRTs) under the tested conditions. For RRT calculation, we used two RRT markers, the first one was the peak of alphamethylepoxide and the second one was the peak of alphamethylepoxide-21-acetate. The RRTs of the peaks eluting within 0 to 25 min were calculated against the alphamethylepoxide peak and the RRTs of the peaks eluting after 25 min were calculated against the alphamethylepoxide-21-acetate peak. Because the method has multiple elution steps, using a second RRT marker makes the RRT determination much more reliable. The RRTs of the tested compounds obtained under some representative HPLC conditions are summarized in Table V. It can be seen that the RRTs obtained under various chromatographic conditions maintained almost unchanged. Therefore, the proposed method has been demonstrated to be robust enough for quantitation analysis.

In addition, the resolution factors (R_s) between alphamethylepoxide and betamethylepoxide obtained under various HPLC conditions were found to be larger than 2.2, which demonstrated a robust separation between alphamethylepoxide and betamethylepoxide.

Validation performed on the alternative column

The column equivalency was first demonstrated from the method specificity study. Figure 4 shows overlay chromatograms of alphamethylepoxide sample spiked with related compounds obtained on the ACE C_{18} column and on the Develosil ODS-UG column. It can be seen that the overall separation is highly similar on these two columns.

Compound names	RT* on ACE C18 column (min)	RT* on Develosil ODS UG column (min)
Compound A	10.795	11.531
Alphamethylepoxide	17.036	17.843
Betamethylepoxide	17.856	18.711
Compound B	19.336	20.031
Compound C	22.217	22.982
Compound D	29.919	30.585
Compound E (alphamethylepoxide-21-acetate)	30.450	31.029
Compound F	35.195	35.676
Compound G	36.583	36.949

* RT = Retention time.
† ND = Not detected.

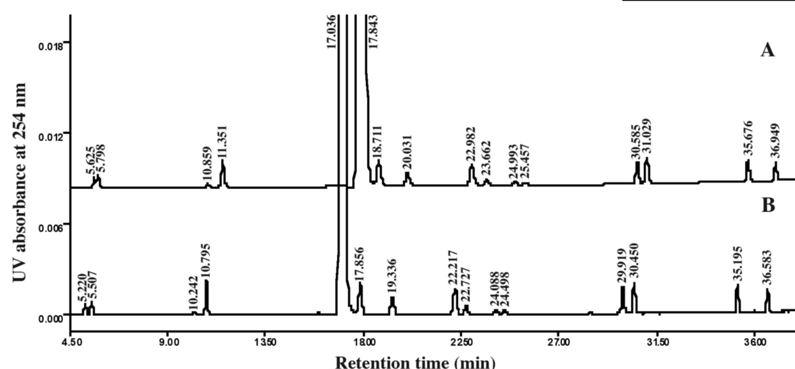

Figure 4. Overlay chromatograms of alphamethylepoxide and related compounds obtained on the Develosil ODS UG column (A) and on the ACE C_{18} column (B).

The same linearity solutions used for the validation study performed on the ACE C_{18} column for assay of alphamethylepoxide and estimation of its related compounds were used for the validation study (by analyst 1) on the Develosil ODS UG column. All the system suitability acceptance criteria set for the ACE C_{18} column had been met on the Develosil column in all corresponding experiments. Linear regression analysis showed that a regression coefficient $r^2 = 1.000$ for alphamethylepoxide ($n = 24$) and for all the tested related compounds ($n = 18$) were obtained on the Develosil column. Y-intercepts obtained from the alphamethylepoxide linearity curves were very insignificant, which were almost 0% of the corresponding alphamethylepoxide responses obtained at the analytical concentration. The Y-intercepts obtained for each individual alphamethylepoxide-related compounds' linearity curves were less than the 50% responses of the corresponding related compound obtained at the 0.05% level. At the selected LOQ and LOD concentrations, all S/N for LOQ were larger than 10 and all the S/N for LOD were larger than 3. The experimental results showed that 99.7% to 100.6% recoveries were obtained for alphamethylepoxide from 1% to 160% levels. At the 0.05% concentration level (i.e., the LOQ level) the recoveries ranged from 104.2% to 105.7%. The typical recoveries of alphamethylepoxide-related compounds were approximately 92.0% to 107.4% for the tested compounds. The method reproducibility was evaluated by the %RSD of the recoveries obtained from nine samples prepared as triplicates at the low (40%), middle (100%), and high (160%) alphamethylepoxide concentration levels. The obtained %RSD was 0.2. For alpha-methylepoxide-related compounds, the method reproducibility was evaluated by 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 obtained % RSD ranged from 0.5 to 1.5. The resolution factors between alphamethylepoxide and betamethylepoxide were found all larger than 2.3. The RRTs and RRFs obtained under the method conditions are listed in Table III. It can be seen that the RRTs and RRFs obtained on the Develosil ODS-UG column are highly similar to those of obtained on the ACE C_{18} column. Finally, the photo stressed sample was injected on the Develosil column and the major degradation peak eluted at 14.4 min, which was again very similar to that on the ACE C_{18} column.

Therefore, the equivalency of the Develosil ODS-UG column to the ACE C_{18} column in the prescribed HPLC method for alphamethylepoxide analysis has been demonstrated based on its successfully meeting the system suitability, the acceptance criteria set for the assay and related compounds' linearity, recovery and accuracy, based on its successfully identifying the alphamethylepoxide and its related compounds via retention times and RRTs, and based on its capability of separating stress-induced degradation peaks.

Conclusions

The analytical method described in this paper is suitable for assay of alphamethylepoxide and estimation of its related compounds. The method has been demonstrated to be accurate, linear, precise, reproducible, repeatable, specific, and robust, and therefore suitable for routine analysis of alphamethylepoxide. This method is demonstrated to be stability-indicating because it can separate degradation peaks from the alphamethylepoxide peak and accurately quantitates the content of alphamethylepoxide in stability samples. An alternative column has also been identified and validated which will enhance the method endurance.

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