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Rapid structure elucidation of drug degradation products using mechanism-based stress studies in conjunction with LC–MS^n and NMR spectroscopy: identification of a photodegradation product of betamethasone dipropionate

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ABSTRACT

Betamethasone dipropionate is an active pharmaceutical ingredient (API) that is used in various dosage forms of finished products for the treatment of inflammatory disorders. An unknown degradant was observed during a solution stability study of betamethasone dipropionate. An approach that combines LC–MS^n, mechanism-based stress studies, semi-preparative HPLC purification and structure elucidation by NMR spectroscopy was used to identify the unknown species. The key step of this approach is the design of relevant stress studies based on the plausible degradation mechanism that is revealed by the informative LC–MS^n analysis. The appropriately designed mechanism-based stress studies not only verify the degradation mechanism but also produce enough quantities of the unknown species for further structure elucidation/confirmation by NMR spectroscopy. With this strategy, the unknown degradant was rapidly identified as lumibetametasone dipropionate, a photodegradation product of betamethasone dipropionate.

1. Introduction

Pharmaceutical impurities observed from a drug substance or a drug product need to be identified when their levels exceed certain regulatory thresholds (e.g., ~0.1–0.2% for majority of drug substances and drug products according to their maximum daily doses), such as the international conference on harmonization (ICH) guidelines for new drug substances and new drug products [1–2]. Identification of drug impurities at these low levels can be very challenging due to a number of factors. One of the frequently encountered challenges is the limited availability of appropriate product samples for analysis, in particular in the case of commercial products on stability programs. Even in cases where the supply of the samples is abundant, isolation of impurities at such minute amounts can be extremely tedious, time-consuming, and difficult. To overcome these challenges, we have developed a strategy that combines LC–MS^n, mechanism-based stress studies, semi-preparative HPLC purification and structure elucidation/confirmation by NMR spectroscopy to rapidly elucidate the structures of drug impurities, in particular drug degradation products [3–5]. The key step of this approach is the design of relevant stress studies based on the plausible degradation mechanism that is revealed by the informative LC–MS^n analysis. A typical practice of this approach is to first analyze the impurity by LC-tandem mass spectrometry and high resolution mass spectrometry in conjunction with photo-diode array (PDA) detection to examine the plausible degradation mechanism by which the impurity would be formed, based on the difference and/or similarity between the impurity and the drug substance in their MS^n (n is typically 1–3) and UV spectra. A stress study is then designed and conducted according to the plausible mechanism to generate the impurity from the drug substance under relevant conditions. Typically, a stress study (or forced degradation) is carried out using acid, base, heat, oxidation or photo-irradiation, etc. In cases of degradation products in finished drug products, use of relevant excipients is often required. In addition, consideration needs to be given as to whether the stress study should be done in solution state or solid state. Once the stress study is appropriately designed and performed, the degradation products can be generated and enriched in relatively large quantities within a short period of time for isolation by semi-preparative HPLC, followed by structure elucidation by NMR spectroscopy. Frequently, the very fact that a degradant can be generated from a stress study would verify the degradation mechanism from which the structure of the unknown degradant may be inferred with high confidence level. In such cases, NMR spectroscopy is used to confirm the struc-

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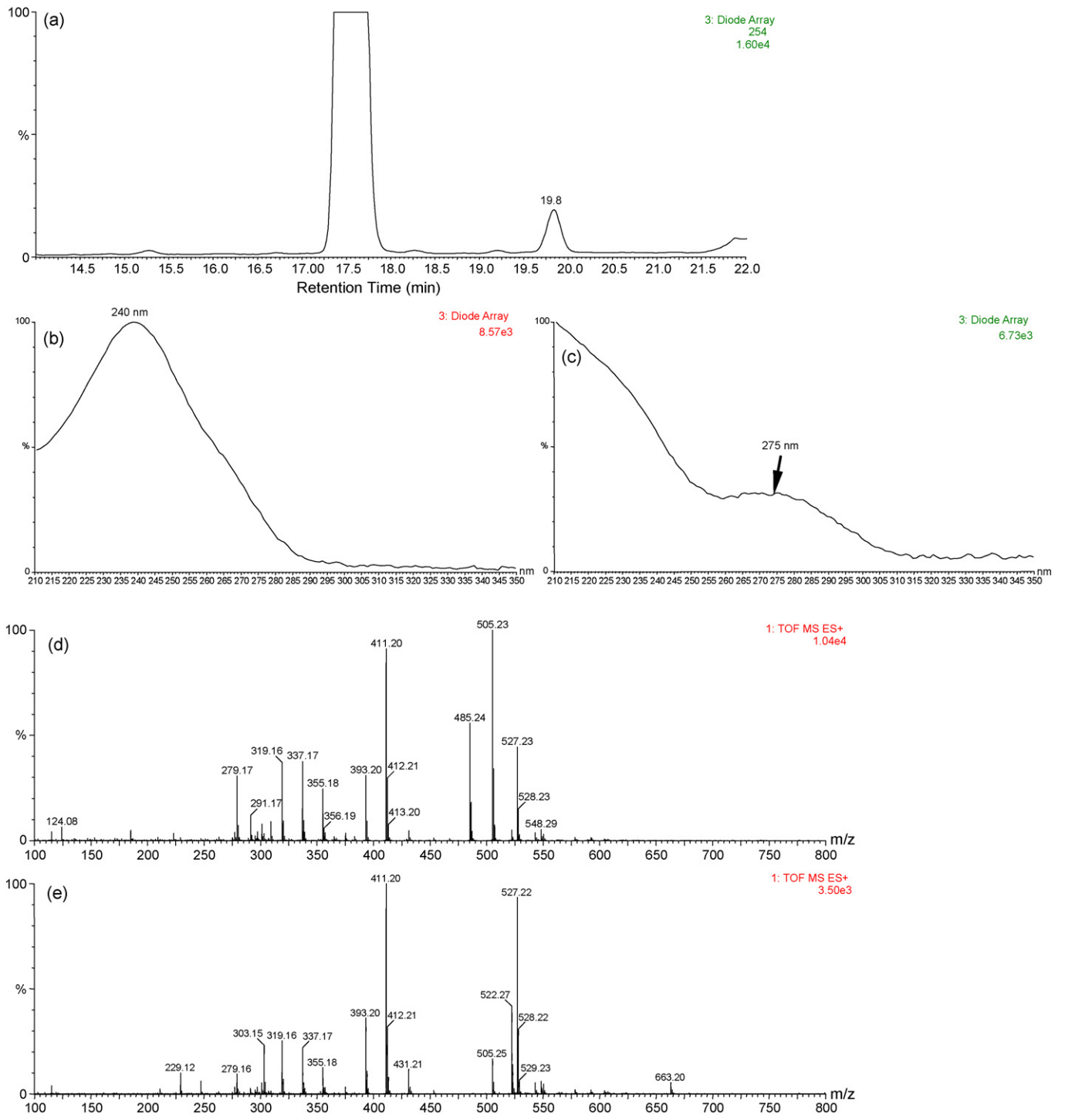


Fig. 1. (a) UV 254 nm chromatogram of the betamethasone dipropionate solution stability sample. The RRT 1.14 unknown peak was observed at retention time 19.8 min. UV absorbance spectra of betamethasone dipropionate and the RRT 1.14 unknown species are shown in (b) and (c), respectively; TOF MS spectra of betamethasone dipropionate and the RRT 1.14 unknown species are shown in (d) and (e), respectively.

ture deduced from the results of the LC–MS^n analysis and stress study.

In the current study, a degradation product was observed at a level above the identification threshold during a solution stability study of betamethasone dipropionate, a synthetic glucocorticosteroid which has been mainly used to treat certain inflammatory dermatological disorders [6]. A UV photo-stress study was designed to produce the unknown degradant based on preliminary data obtained from LC–PDA–MS analysis. The photo-degradant of betamethasone dipropionate generated from the photo stress study was purified by semi-preparative HPLC and then characterized by NMR.

2. Experimental

2.1. Materials

Betamethasone dipropionate was manufactured by Schering-Plough Corporation. All reagents used were of HPLC grade or better and obtained from Fisher Scientific (Pittsburgh, PA, USA).

Table 1
Accurate mass measurement results of the three molecular ions observed from the RRT 1.14 unknown species.

m/z	Measured accurate mass	Chemical formula	Theoretical mass	Error in ppm	Assignment
505	505.2600	$C_{28}H_{38}FO_7$	505.2602	−0.4	$(M+H)^+$
522	522.2855	$C_{28}H_{41}FO_7N$	522.2867	−2.3	$(M+NH_4)^+$
527	527.2402	$C_{28}H_{37}FO_7Na$	527.2421	−3.6	$(M+Na)^+$

2.2. LC–PDA–MS analysis

LC–PDA–MS analysis was performed on a Waters Q-Tof Premier mass spectrometer interfaced to a Waters 2695 separation module equipped with a PDA detector. The HPLC separation was carried out on a Waters YMC Basic 150 × 4.6 mm, 5 μm column at ambient temperature using a mobile phase system consisting of A, acetonitrile:water (35:65, v/v), and B, acetonitrile:water (90:10, v/v). The analyses were performed at ambient temperature with a flow rate of 1.5 mL/min and a gradient program varied according to the following program: 0 min (0% B), 5 min (0% B), 20 min (41% B), 20.1 min (100% B), 25 min (100% B), 25.1 min (0% B) and 35.0 min (0% B). The HPLC flow for the mass spectrometer was split at a ∼10:1 ratio after the PDA detector; about 150 μL/min of the LC flow was directed into the MS detector. UV spectrum was collected from 200 nm to 400 nm from the PDA detector. The Q-Tof mass spectrometer was operated at positive electrospray mode with the following source parameters: cone gas 60 L/hr, desolvation gas 600 L/hr, source temperature 100 °C, desolvation temperature 250 °C and capillary voltage 3 kV. The time-of-flight (TOF) MS analyzer was operated at positive V mode with ∼8000 full width half maximum resolution and was calibrated externally with a sodium cesium iodide solution. Betamethasone dipropionate was used as the LockSpray reference compound for accurate mass measurement. The intensity of the reference compound was adjusted to 100–200 counts/s by tuning the reference spray parameters. The LockSpray interval was set to 5 s, and TOF MS spectra were acquired at 1 scan/s scan rate and 0.1 s inter-scan time.

2.3. UV stress study and purification of the unknown degradant generated

The unknown species was generated by preparing a solution of 0.13 g betamethasone dipropionate in a mixture of 2 mL chloroform and 4 mL methanol. The solution was irradiated under a TLC UV lamp at 254 nm for 4 h. The UV degradation product was isolated using a Waters 2695 HPLC system with a Supelco Supelcosil ABZ plus, 250 × 10 mm, 5 μm semi-preparative HPLC column. The separation was performed at ambient temperature, using an isocratic program of 60% water and 40% acetonitrile at 6 mL/min. Fractions were collected and dried in a RotaVap to remove the organic solvent, followed by complete evaporation of the remaining aqueous solvent in a freeze dryer (Thermo ModulyoD).

2.4. NMR characterization

^1H and ^{13}C NMR spectra were acquired on a Varian 600 MHz spectrometer at 25 °C in $CDCl_3$. 2D NMR spectra of the degradant were acquired on a Varian 500 MHz spectrometer at 25 °C in DMSO-d_6. ^1H and ^{13}C resonances were assigned by using 2D NMR experiments: gCOSY, NOESY, gHSQC, gHSQC-TOCSY and gHMBC. Samples were prepared in DMSO-d_6 and $CDCl_3$ in concentration of ∼1–2 mg/mL. 2D gCOSY experiment was performed in a magnitude mode with gradient selection method. Eight hundred eighty points in t1 and 1024 complex points in t2 were acquired. One scan per t1 point with 1 s delay between scans was used. The spectral width was 4400 Hz in both dimensions. 2D NOESY experiment was performed in a phase sensitive mode. Three hundred complex points in t1 and 2048 complex points in t2 were acquired. Eight scans per t1 point with 0.5 s mixing time and 3 s delay between scans were used. The spectral width was 5092 Hz in both dimensions. 2D ^1H–^{13}C gHSQC experiment was performed in a phase sensitive mode with gradient selection method. Four hundred sixty complex points in t1 (^{13}C) and 1024 complex points in t2 (^1H) were acquired. Four scans per t1 point with 1.1 s delay between scans were used. The spectral widths were 18072 Hz in t1 and 4400 Hz in t2 dimension. 2D ^1H–^{13}C gHSQC-TOCSY experiment was performed in a phase sensitive mode with gradient selection method. Three hundred twenty complex points in t1 (^{13}C) and 1024 complex points in t2 (^1H) were acquired. Sixteen scans per t1 point

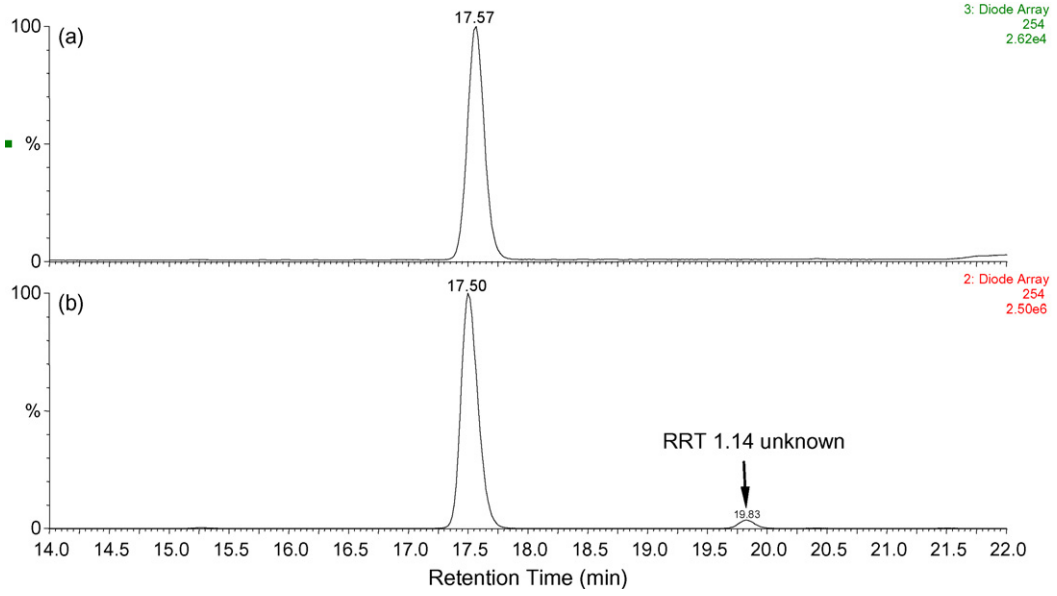
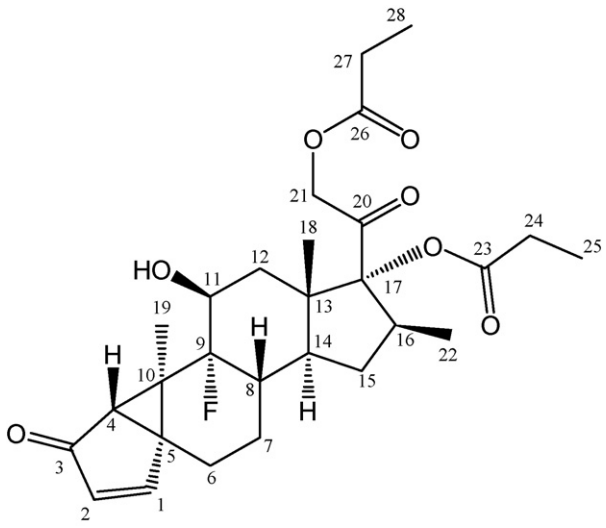


Fig. 2. UV 254 nm chromatograms of betamethasone dipropionate (a) before UV irradiation and (b) after 4 h UV irradiation at 254 nm. The RRT 1.14 unknown peak was observed at 19.8 min in the UV irradiated sample.



Scheme 1. Lumibetamethasone dipropionate.

with 0.02 s mixing time and 1.1 s delay between scans were used. The spectral widths were 18072 Hz in t1 and 4400 Hz in t2 dimension. 2D ^{1}H–^{13}C gHMBC experiment was performed in a magnitude mode with gradient selection method. Six hundred points in t1 (^{13}C) and 2000 complex points in t2 (^{1}H) were acquired. Forty eight scans per t1 point with 1.5 s delay between scans were used. The spectral width was 27128 Hz in t1 and 4400 Hz in t2 dimension. For all 2D experiments, zero-filling and a sine-bell window function were applied prior to Fourier transformation. Stereochemical analysis of the degradant was performed based on proton-proton NOE's and 3D molecular modeling by molecular mechanics algorithms (PCModel v.8.5 [7]).

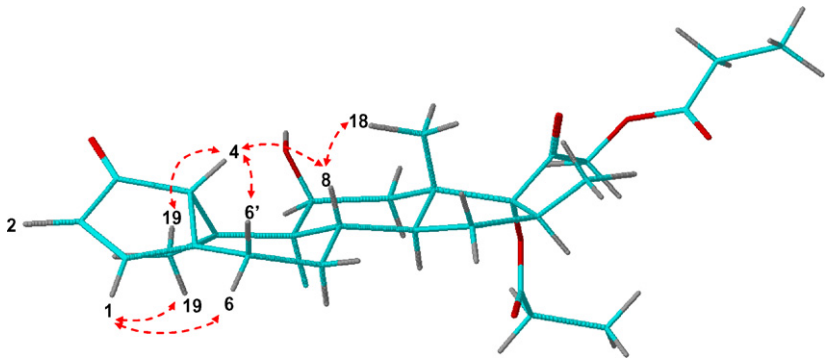
3. Results and discussion

3.1. LC–PDA–MS analysis

An unknown peak was observed in the LC–PDA–MS analysis of a stability sample of the betamethasone dipropionate solution at a relative retention time (RRT) of 1.14 to betamethasone dipropionate (Fig. 1a). The UV spectrum of betamethasone dipropionate showed a single maximum absorbance band at 240 nm (Fig. 1b), due to the conjugated double bonds in the steroid A-ring moiety. In contrast, the RRT 1.14 unknown species displayed a distinctly different UV spectrum: two absorbance bands at ~210 nm and ~275 nm, respectively (Fig. 1c). In the MS spectrum of betamethasone dipropionate (Fig. 1d), protonated and sodiated molecular ions (m/z 505 and m/z 527) were observed along with several fragments ions produced from the in-source fragmentation of the protonated molecular ion, including m/z 485 (loss of HF), m/z 411 (loss of HF and $CH_3CH_2CO_2H$), m/z 393 (loss of HF, $CH_3CH_2CO_2H$ and H_2O), and other smaller ions m/z 355, m/z 337, m/z 319, m/z 301 and m/z 279. Certain common ions were observed in the MS spectra of both betamethasone dipropionate and the RRT 1.14 unknown species (Fig. 1d and e). Accurate mass measurement using protonated betamethasone dipropionate molecular ion as the Lockmass revealed that the unknown species has a chemical formula of $C_{28}H_{37}FO_7$, which is the same as betamethasone dipropionate; its protonated, ammoniated and sodiated molecular ions (m/z 505, 522, and 527, respectively) were also observed, as summarized in Table 1. The m/z 663 ion observed in Fig. 1e was assigned as a gas phase adduct formed between the sodiated molecule ion and CF_3CO_2Na (theoretical mass 135.9748) based on the measured accurate mass difference of 135.9760 between m/z 527 and m/z 663. The MS/MS fragmentation of the m/z 663 ion produced m/z 527 as the sole product ion (data not shown). When a slightly high nose cone voltage was applied in a separate experiment, where the extent of in-source fragmentation was expected to increase, the m/z 663 peak

Table 2
^{13}C chemical shifts and ^{13}C–^{19}F J-couplings of the photo degradant of betamethasone reported in ref. [8] and lumibetamethasone dipropionate generated in the current study ($CDCl_3$, 25 °C).

Carbon number	Photo degradant of betamethasone reported in ref. [8]		Lumibetamethasone dipropionate (RRT 1.14 species)	
	δ (ppm)	J (C, F) (Hz)	δ (ppm)	J (C, F) (Hz)
20	211.7		198.51	
3	206.38		205.48	
1	165.67		164.86	
2	132.16		132.24	
9	95.93	176.2	95.47	174.5
17	89.32		94.67	
11	68.78	35.5	69.45	35.4
21	68.58		67.67	
10	53.67	27.9	52.67	28.0
13	49.12		47.65	
16	49.03		47.08	
14	42.55	2.9	42.39	3.5
4	39.42	6.4	39.20	6.3
5	38.49		38.20	
12	34.89		35.06	
15	34.94		34.77	
8	33.04	19	32.98	18.8
6	23.15		23.11	
7	20.12	2.2	20.06	2.6
22	19.71		19.42	
18	17.01		16.34	
19	8.24	5.3	8.31	5.4
23	–		174.8	
26	–		174	
24	–		28.29	
27	–		27.11	
25	–		8.99	
28	–		8.84	

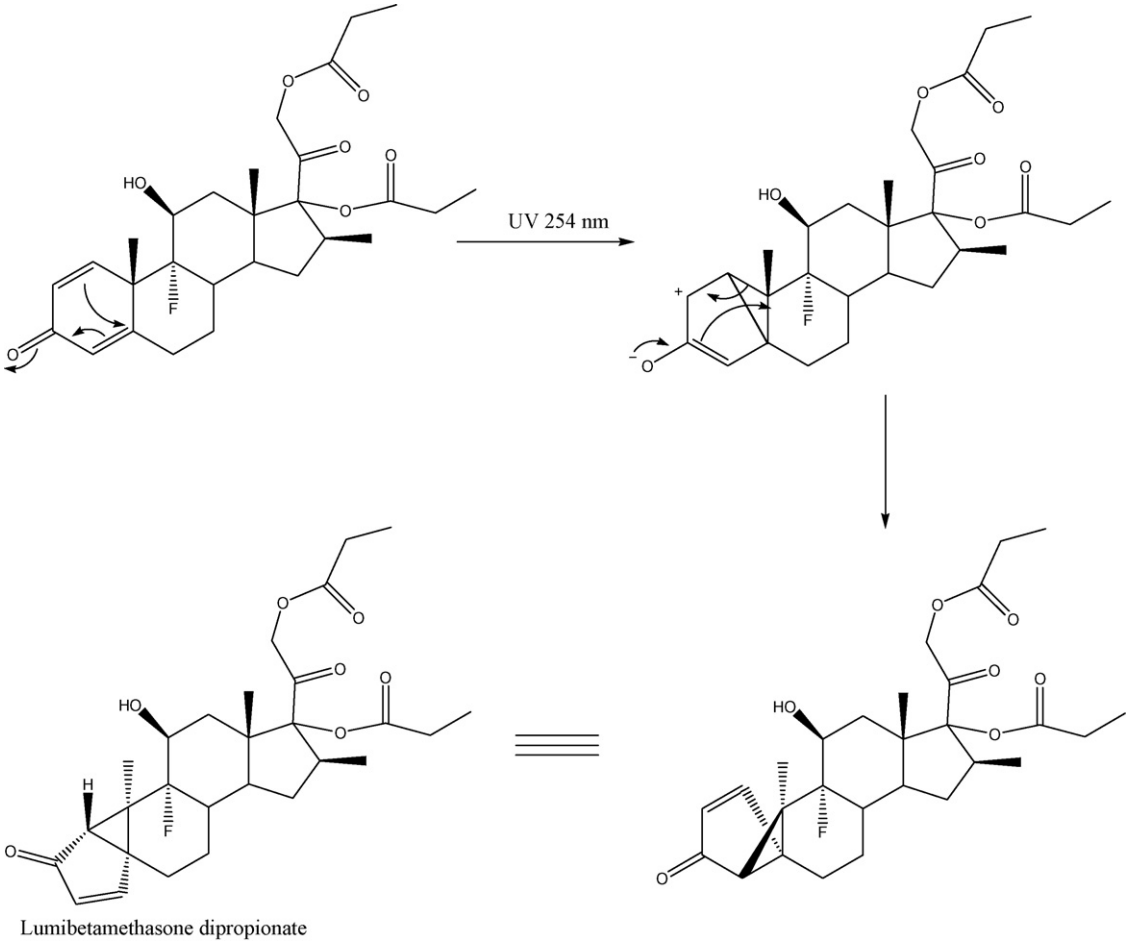


Scheme 2. Stereochemical analysis of lumibetamethasone dipropionate by nuclear Overhauser effects (NOE's). Red arrows indicate the pairs of protons which showed strong NOE's. Only the NOE's that were used to assign stereochemistry of the C10 center are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

was not observed suggesting that it was indeed a weakly bound gas phase adduct between CF_3CO_2Na and the soidated molecular ion of the unknown molecule. The source of CF_3CO_2Na was likely to be the residual trifluoroacetic acid that had been used as a mobile phase additive in the same instrument previously, but the exact source was difficult to identify. These MS data clearly suggested that the RRT 1.14 unknown species is an isomer of betamethasone dipropionate. Since the 240 nm maximum absorbance of betamethasone dipropionate is due to the characteristic conjugated double bonds in the A-ring, the A-ring structure of betamethasone dipropionate must have been altered during the isomerization process leading to the formation of the RRT 1.14 unknown species.

3.2. Plausible degradation mechanism

In an effort to identify the possible formation mechanism of the RRT 1.14 unknown species, it was found that the unknown peak was observed only when the sample solution was exposed to laboratory lighting. Since the unknown species has the same molecular formula as the drug substance, betamethasone dipropionate, but with a completely different UV absorbance spectrum, it is likely that the unknown species is a degradant of betamethasone dipropionate through photo-isomerization. Hidaka et al. [8] reported a photo-inducted, isomeric degradant of betamethasone with an altered A-ring structure, which had a maximum UV absorbance



Scheme 3. Proposed mechanism of UV photo-induced isomerization from betamethasone dipropionate to lumibetamethasone dipropionate.

band at ~270 nm. Considering the facts that the two propionate groups in betamethasone dipropionate do not contribute significantly to its UV absorbance and that the RRT 1.14 unknown species has a maximum UV absorbance at ~275 nm, it was suspected that the unknown species might be the dipropionate derivative of the photo-degradant of betamethasone reported by Hidaka et al.

3.3. UV photo-stress study

Therefore, a UV stress study outlined in Section 2.3 was performed, which was specifically designed to generate the RRT 1.14 unknown species under UV irradiation condition. The UV chromatograms obtained from LC–PDA–MS analysis of the betamethasone dipropionate sample before and after the UV irradiation are shown in Fig. 2. A peak at RRT 1.14 relative to betamethasone dipropionate clearly appeared in ~5% yield after the 4 h UV irradiation at 254 nm. The UV absorbance spectrum and MS spectrum of the photo-stress generated species also matched those of the RRT 1.14 unknown species (data not shown). Finally, spiking of the UV stressed betamethasone dipropionate sample to the betamethasone dipropionate solution, in which the unknown species was observed originally, produced a completely co-eluting peak at RRT 1.14 (data not shown), confirming that the unknown species was indeed generated from betamethasone dipropionate via a UV photo-induced isomerization process.

3.4. NMR characterization

The RRT 1.14 unknown species from the 4 h UV irradiation of betamethasone dipropionate was purified by semi-preparative HPLC. NMR characterization of the isolated RRT 1.14 unknown species confirmed that it is indeed lumibetamethasone dipropionate (Scheme 1), the photo-induced, isomeric degradant of betamethasone dipropionate. The ^{13}C chemical shifts and ^{13}C–^{19}F J-couplings of the RRT 1.14 species are shown in Table 2 along with those of the photo-degradant of betamethasone, i.e., lumibetamethasone, as reported by Hidaka et al. [8]. Overall, the steroid core structure of the RRT 1.14 species is identical to that of lumibetamethasone as the values of the ^{13}C chemical shifts and ^{13}C–^{19}F coupling are highly similar. It is noted that the chemical shifts of C13, C16, C17 and C20 carbons are different between the two compounds, which is due to the presence of the two additional propionate groups in the RRT 1.14 species (lumibetamethasone dipropionate). The structure of lumibetamethasone proposed by Hidaka et al. [8] was confirmed by 2D gHSQC and gHMBC analysis of the RRT 1.14 species, except that the S stereochemistry of the C10 carbon in the structure of lumibetamethasone reported by Hidaka et al. [8] was incorrectly assumed to be the same as in the parent compound (betamethasone). The current study showed that the stereochemistry of this carbon center had been reversed during the rearrangement of the A-ring. Thus, in the NOESY spectra of the RRT 1.14 species, strong NOE cross-peaks were detected between H4 and H6′, H8, H19 protons and between the H1 and H6, H19 protons. Three dimensional structure modeling of the lumibetamethasone dipropionate conformation indicated that the only possible structure that satisfies close proximity of these protons has the R stereochemistry at the C10 center (Scheme 2).

In a separate study, Fahmy [9] reported dexamethsone, a C16 epimer of betamethasone that has an α-oriented C16 methyl group as opposed to the β-orientation for betamethasone, can undergo a similar UV photo-induced isomerization producing lumidexamethasone. Similar to the finding in the current study, the stereochemistry of the C10 carbon center in dexamethasone is reversed as the result of the photo-induced isomerization. In addition, the same reversal of the C10 carbon center is also observed in the photo-induced isomeric degradant of prednisone, lumiprednisone [10]; the A-ring of prednisone in its steroid core is identical to that of betamethasone and dexamethasone. A plausible mechanism for the formation of lumibetamethasone dipropionate is thus proposed in Scheme 3, based on the photo-isomerization mechanism of dexamethasone [9] and prednisone [10]. During this photo-isomerization process, a new bond is formed between C1 and C5 under UV irradiation, followed by the formation of the C4–C10 bond and concomitant cleavage of the C1–C10 bond, leading to the formation of lumibetamethasone dipropionate.

4. Conclusion

A degradant observed in a solution stability study of betamethasone dipropionate was rapidly identified as lumibetamethasone dipropionate, an isomeric, photo-induced degradant of betamethasone dipropionate. The identification used a proven strategy, involving LC–MS analysis, mechanism-based stress study, semi-preparative HPLC purification and NMR characterization. This strategy can be utilized effectively to identify drug degradants in general from drug substances and drug products.

Acknowledgements

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References

[1] ICH Harmonised Tripartitie Guideline, Impurities in New Drug Substances, Step 4 version, dated 25 October 2006.
[2] ICH Harmonised Tripartitie Guideline, Impurities in New Drug Products, Step 4 version, dated 2 June 2006.
[3] M. Li, B. Chen, M. Lin, A. Rustum, Amer. Pharm. Rev. 11 (1–2) (2008) 98–103.
[4] M. Li, M. Lin, A. Rustum, J. Pharm. Biomed. Anal. 48 (2008) 1451–1456.
[5] M. Li, X. Wang, B. Chen, T.-Z. Chan, A. Rustum, J. Pharm. Sci. 98 (2009) 894–904.
[6] M. Avery, G. Detre, D. Yasuda, W.R. Chao, M. Tanabe, D. Crowe, R. Peter, W. Chong, J. Med. Chem. 22 (1990) 1852–1858.
[7] PCModel v. 8.5, Serena Software, Bloomington IN 47402-3076, USA.
[8] T. Hidaka, S. Huruumi, S. Tamaki, M. Shiraishi, H. Minato, Yakugaku Zasshi 100 (1980) 72–80.
[9] O.T.Y. Fahmy, 1997. Generation, isolation, characterization and analysis of some photolytic products of dexamethasone and related steroids. University of Mississippi doctoral thesis.
[10] J.R. Williams, R.H. Moore, R. Li, J.F. Blount, J. Am. Chem. Soc. 101 (1979) 5019–5025.