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Short communication

# Application of LC–MS$^n$ in conjunction with mechanism-based stress studies in the elucidation of drug impurity structure: Rapid identification of a process impurity in betamethasone 17-valerate drug substance

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ABSTRACT

Through a case study, the use of LC–MS$^n$ technique in conjunction with a mechanism-based stress study is shown to be a very effective way in the rapid elucidation of unknown drug impurities. In this case, the drug substance sample was first analyzed using LC–MS$^n$ through which the unknown species was found to be a valeryl-containing, isomeric impurity of the active pharmaceutical ingredient (API), betamethasone 17-valerate, based on its molecular ion and major fragments. Since a substantial knowledge regarding a large number of isomeric impurities of betamethasone has been accumulated in the literature as well as in our laboratory, a hydrolytic stress study (forced degradation) of the isolated unknown species was then designed and carried out accordingly in order to remove the valeryl group from the unknown species. During the stress study, a betamethasone isomer was generated as expected. However, a new unknown species isomeric to betamethasone 17-valerate was also formed unexpectedly. By comparing the UV spectra and more importantly MS$^n$ fragmentation patterns of the two newly formed species with those of betamethasone, dexamethasone, betamethasone 17-valerate, and betamethasone 21-valerate, these two unknown species generated in the stress study were identified as dexamethasone and dexamethasone 21-valerate, respectively. Based on the plausible reaction mechanism of the forced degradation, the original impurity present in betamethasone 17-valerate drug substance was then identified as dexamethasone 17-valerate; the structure assignment was later confirmed by various 1D and 2D NMR experiments. The efficient conversion from dexamethasone 17-valerate to dexamethasone 21-valerate was also observed during a 2D NMR acquisition of the isolated dexamethasone 17-valerate sample.



## 1. Introduction

Pharmaceutical impurities are components found in a drug substance or drug product that are neither the drug substance nor excipients according to the definition by the International Conference on Harmonization (ICH) [1]. For practical purpose, pharmaceutical impurities can be divided into the following categories: process impurities of drug substances (process impurities), degradation products of drug substances (degradants), packaging/label related impurities (leachables/extractables), contaminant peaks, solution degradants (degradation due to inadequate sample preparation and storage or intrinsic instability of drug substances), and artifact peaks. Of these six categories, the first four are due to real impurities present in the drug product analyzed and thus, the structures and/or sources of the unknown impurities need to be identified once they exceed certain specified levels as required by regulatory guidelines. For instance, ICH guideline stipulates that the identification threshold of impurities in a drug substance is 0.1% if the daily dose is $\leq$2 g/day or 0.05% if the daily dose is >2 g/day [2]. LC–MS$^n$ (typically, $n$ = 1–3) is most often employed as the first line of analytical tool for identification of these impurities/degradants [3,4]. Nevertheless, complementary analytical techniques such as NMR spectroscopy are often required for structure identification of unknown impurity peaks with a high level of confidence. This exercise can be quite time-consuming due to the fact that a very limited amount of these unknown species would usually be available from the drug substance or formulated product for isolation and subsequent structure elucidation via NMR spectroscopy.

We have utilized a strategy that combines LC–MS$^n$ with a mechanism-based approach for design of effective stress studies (forced degradation), which has enabled us to quickly identify structures of drug impurities with a high level of confidence without going through comprehensive impurity purification and NMR

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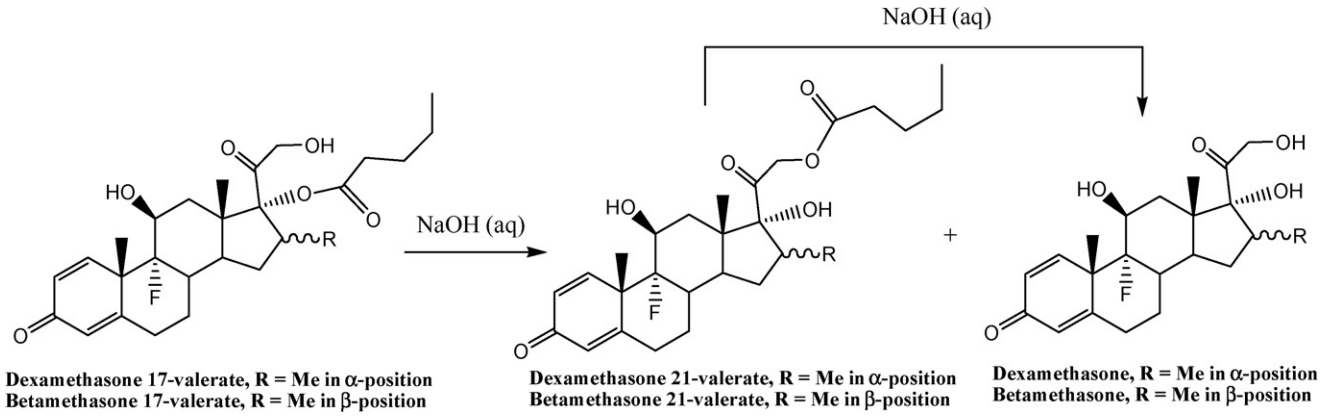



**Scheme 1.** Stress study of dexamethasone 17-valerate and betamethasone 17-valerate under alkaline condition. Approximately 1 mg of dexamethasone 17-valerate or betamethasone 17-valerate was dissolved in 1 mL of acetonitrile and one drop of 1N NaOH solution was added. The reaction solution was then mixed well and monitored by LC–MS for up to 1 h at room temperature.

structure elucidation [5]. This strategy is particularly effective for the elucidation of drug degradant structures and degradation pathways. However, this strategy can also be very useful in the structure identification of certain process impurities. In this paper, we demonstrate the use of this strategy in the identification of an unknown peak observed at a relative retention time (RRT) of 0.90 during the analysis of a drug substance, betamethasone 17-valerate, an anti-inflammatory agent used in a number of drug products. The unknown peak was observed at a level slightly above 0.1% relative to the drug substance, which exceeded the 0.1% ICH threshold for impurity identification in a drug substance whose daily dose is below 2 g/day. The unknown species was rapidly identified (within a few days) as dexamethasone 17-valerate, a process impurity, through the use of LC–$MS^n$ followed by stress studies which were designed based on the LC–$MS^n$ results.

## 2. Experimental

### 2.1. Materials

All corticosteroids used in this study are in-house materials. Solvents and reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA) and are of HPLC grade or better for all HPLC and LC–MS experiments.

### 2.2. LC-PDA/UV-$MS^n$ experiments

LC–MS and LC–MS/MS experiments were performed on a Waters Q-TOF Premier mass spectrometer interfaced to a Waters 2695 separation module and a PDA detector. The HPLC analysis of betamethasone 17-valerate and its impurities was performed

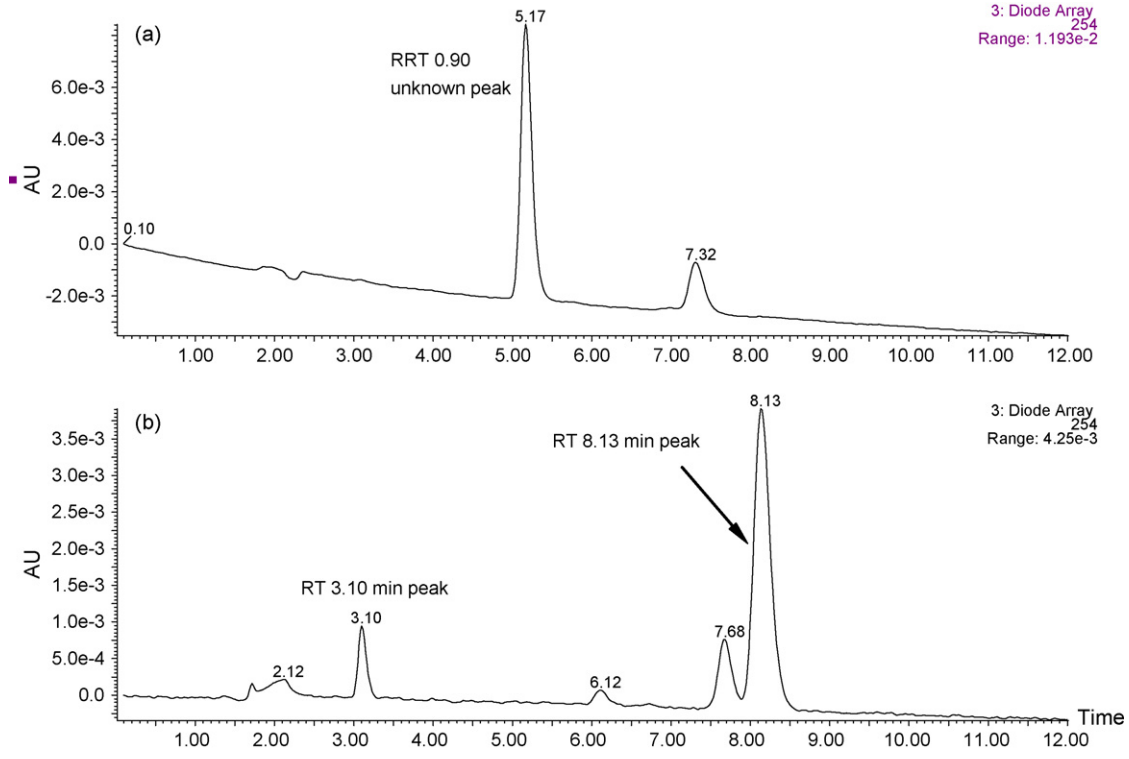



**Fig. 1.** UV 254 nm chromatograms of the isolated RRT 0.90 unknown species: (a) before treatment with NaOH and (b) immediately after treatment with NaOH.

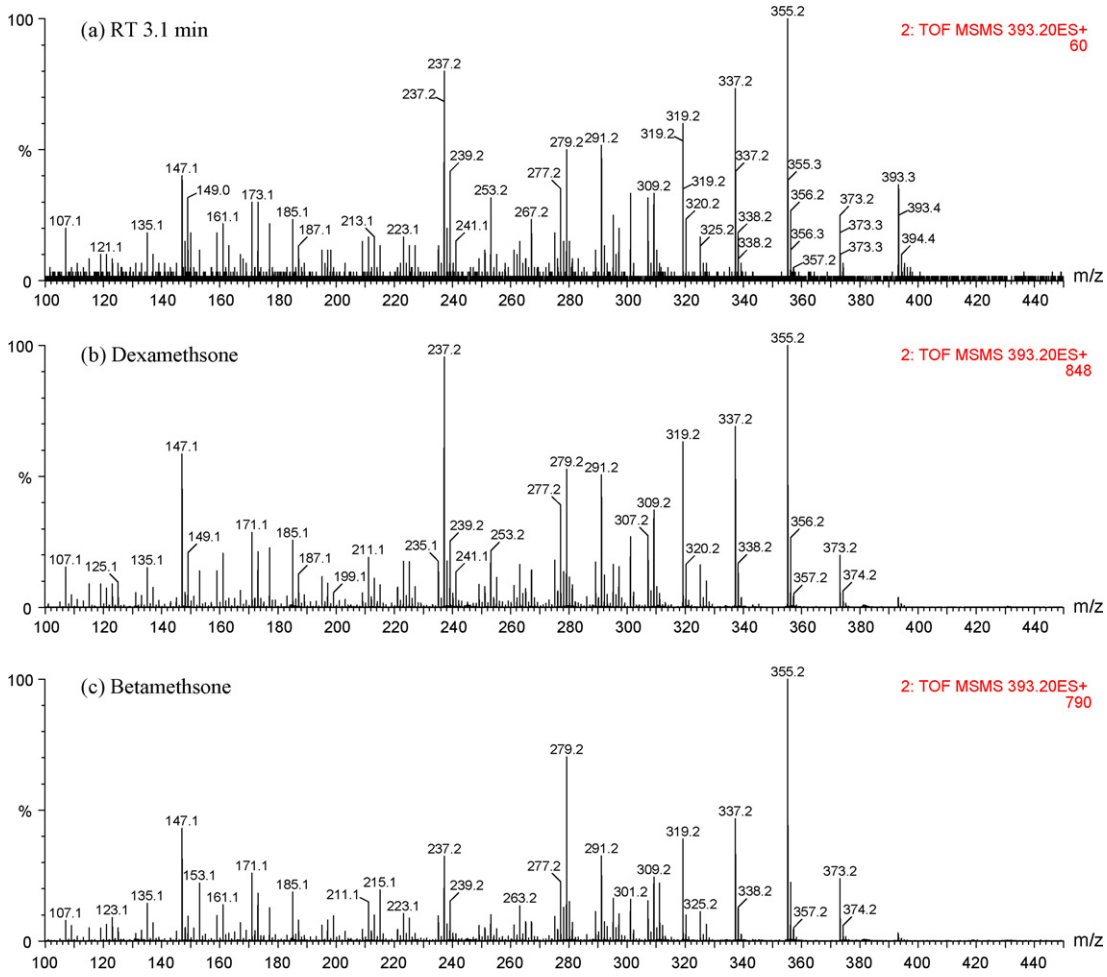



**Fig. 2.** MS/MS product ion spectra of the molecular ions *m*/*z* 393 from: (a) the RT 3.10 min species, (b) dexamethasone, and (c) betamethasone.

on a Beckman Ultrasphere ODS, 250 mm × 4.6 mm, 5 μm column under isocratic elution using a mobile phase consisting of 60% acetonitrile and 40% water. The analysis was carried out at ambient temperature with a flow rate of 1.0 mL/min. The HPLC flow for the mass spectrometer was split at a ratio of approximately 10:1 after the PDA detector; about 100 μL/min of the LC flow was directed into the MS detector. The mass spectrometer was operated under positive electrospray ionization (ESI) mode and the source was operated under the following condition: spray voltage 4.0 kV, cone voltage 30 V, cone gas 60 L/h, desolvation gas 600 L/h, source temperature 100 °C and desolvation temperature 150 °C. The Time-of-Flight (TOF) mass analyzer was operated at V mode and TOF MS spectra were collected from 50 to 1000 daltons, with 1 s/scan rate and 0.1 s inter scan delay. TOF MS/MS spectra were collected with a mass selection window set as LM 4.7 and HM 15.0. The collision energy was set to 10–15 V. For all LC–MS analyses, the betamethasone 17-valerate solution was prepared in a mixture of acetonitrile:water:acetic acid (60:40:0.1, v/v/v) at a concentration of ~2.5 mg/mL.

### 2.3. Isolation of RRT 0.90 unknown species for stress study

The isolation of RRT 0.90 was conducted on a Waters semi-preparative HPLC system using an ACE 5 C18, 250 mm × 10 mm, 5 μm column. The separation was effected under isocratic elution using a mobile phase consisting of 60% acetonititrile and 40% water with a flow rate of 6 mL/min. A betamethasone 17-valerate solution of ~8 mg/mL was prepared for isolation. Fractions collected from several repeated runs were combined and evaporated to dryness in a SpeedVac. For the hydrolytic stress study of the RRT 0.90 unknown species with NaOH, a small amount of the isolated sample was dissolved in 1 mL acetonitrile and one drop of 1N NaOH solution was added. The solution was analyzed by LC–MS immediately and then at an interval of 15 min, 30 min and 1 h, respectively.

### 2.4. 1D and 2D NMR experiments

The conditions used for these experiments are similar to those reported previously by our group [6].

## 3. Results and discussion

### 3.1. Preliminary LC–MS analysis

An unknown peak at RRT 0.90 was observed in betamethasone 17-valerate (structure shown in Scheme 1) drug substance at levels above the ICH identification threshold when analyzed by a compendial method. It was clear from the LC–MS results that the RRT 0.90 peak is an isomer of betamethasone 17-valerate (since they both have the same molecular ions at *m*/*z* 477) and appeared very likely to be another valeryl derivative of betamethasone or one of its isomers.

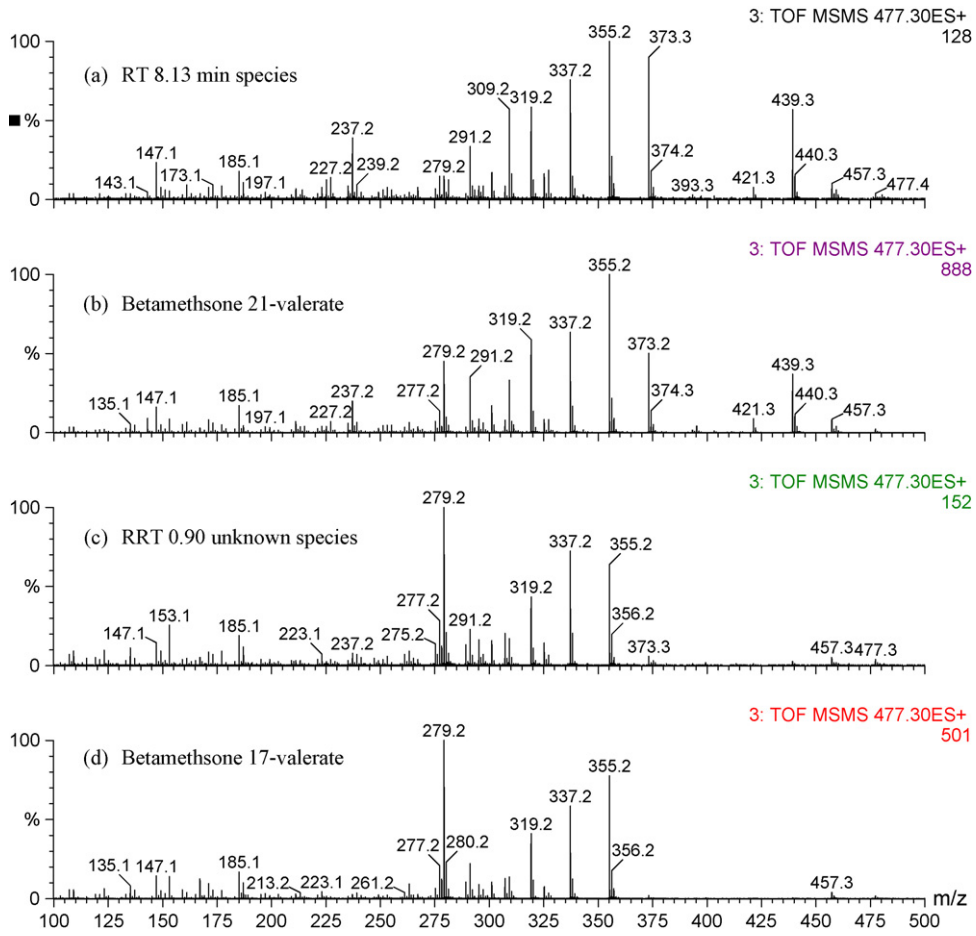



**Fig. 3.** MS/MS product ion spectra of the ions at *m/z* 477 from: (a) RT 8.13 min, the isomer from NaOH treatment of the RRT 0.90 species, (b) the RRT 0.90 unknown species, (c) betamethasone 21-valerate, and (d) betamethasone 17-valerate. Spectra were obtained under the sample experimental conditions with collision energy set to 15 V.

### 3.2. Stress studies based on LC–MS results

To test the above hypothesis, the RRT 0.90 peak was isolated by semi-preparative HPLC and then treated with a small aliquot of 1N NaOH in acetonitrile in an attempt to remove the valeryl group via hydrolysis. Since a fair amount of knowledge had been accumulated in our laboratory regarding the chemistry of betamethasone and related compounds, the identity of the RRT 0.90 species might be deduced based on the structure of the product(s) from the hydrolysis of the RRT 0.90 species. It was found that, upon the treatment with NaOH, the majority of the RRT 0.90 peak disappeared instantaneously (Fig. 1), while a major product peak occurred which eluted at 8.13 min (Fig. 1b) and also had a molecular ion of *m/z* 477. At the meantime, a smaller peak with a molecular ion of *m/z* 393 was observed at 3.10 min, where dexamethasone and betamethasone should elute under this LC condition. When the alkaline solution was examined 15 min, 30 min and 1 h after NaOH was added, it was found that the 8.13 min peak decreased, while several new peaks appeared.

### 3.3. Structure assignment based on MS/MS molecular fingerprinting

It has been known that the MS/MS fragmentation patterns of betamethasone and dexamethasone showed some subtle but distinguishable differences according to the literature [7] as well as the observation made in our own laboratory. We have taken advantage of these distinguishable but characteristic differences to determine structures of unknown species in a speedy and reliable way [8]. In this particular case, this strategy was used again to determine whether the 3.10 min peak resulting from the hydrolysis of the RRT 0.90 unknown peak was betamethasone or dexamethasone. Hence, the MS/MS fragmentation of the *m/z* 393 ion from the RT 3.10 min peak was obtained and compared with those of betamethasone and dexamethasone, respectively, obtained under the same experimental conditions. As shown in Fig. 2b and c, most of the fragment ions from dexamethasone are the same as those from betamethasone. The common fragment ions observed are *m/z* 373 (loss of HF), *m/z* 355 (loss of HF and $H_2O$), *m/z* 337 (loss of HF and two $H_2O$), *m/z* 319 (loss of HF and three $H_2O$), and two smaller fragments (*m/z* 279 and *m/z* 237) that were proposed by Arthur et al. [7] as fragments containing only the steroid core structure. Nevertheless, there are two characteristic differences between the two spectra: (1) the relative abundance of *m/z* 237 ion to other fragments is higher in dexamethasone than in betamethasone and (2) the intensity ratio of *m/z* 277 to *m/z* 279 is higher in dexamethasone than in betamethasone. These observations were highly reproducible and have been reported by Arthur et al. [7] as a reliable way to differentiate dexamethasone and betamethasone. The comparison of MS/MS product ion spectrum of the RT 3.10 min species (Fig. 2a) with those from dexamethasone and betamethsone led to the assignment of the RT 3.10 species as dexamethasone. This assignment suggested that the RRT 0.90 was a valeryl derivative

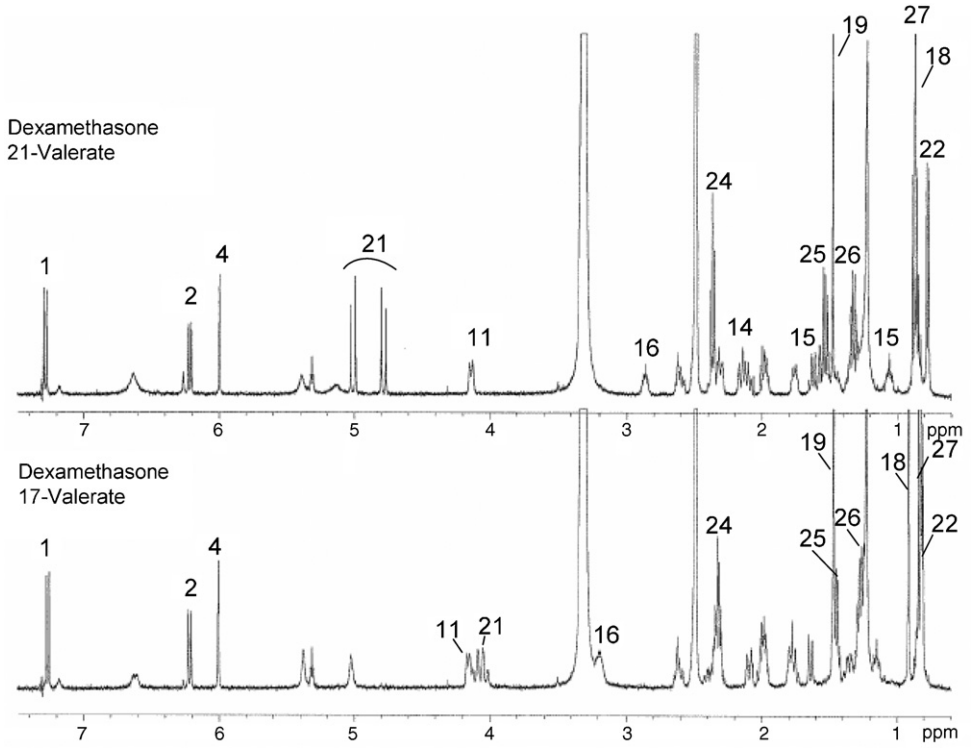
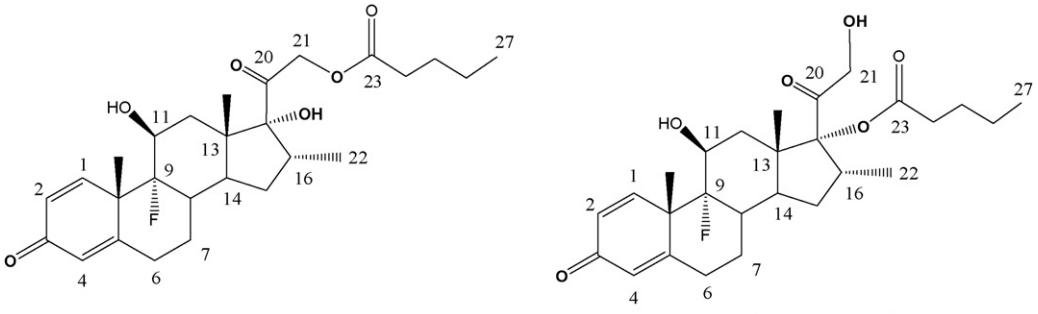



**Fig. 4.** $^1$H NMR spectrum of dexamethasone 17-valerate (bottom) which was obtained on the DMSO-$d_6$ solution of the isolated RRT 0.90 peak on a Varian Inova 500 spectrometer operating at a proton frequency of 500 MHz with a 3-mm carbon-proton dual probe at 25 °C. Various 2D experiments such as COSY, NOESY, and HMBC were conducted after the initial $^1$H NMR spectrum was performed in order to establish the connectivity of the proton and carbon nuclei. After all the 2D NMR experiments were completed (~24 h), it was found that dexamethasone 17-valerate had completely converted into dexamethasone 21-valerate (upper). The numbering of the two steroid rings is shown below.



Dexamethasone 21-Valerate

Dexamethasone 17-Valerate

of dexamethasone, which is consistent with the original hypothesis outlined above. During the alkaline stress study of the isolated RRT 0.90 species, it was also noted that the RT 8.13 min peak, the major product peak initially formed, was an isomerization product since it displayed the same *m*/*z* value as that of RRT 0.90 species (*m*/*z* 477). It was likely that, between the RRT 0.90 unknown peak and its isomeric degradant (the 8.13 min peak), one was dexamethasone 17-valerate and the other was dexamethasone 21-valerate based on the expected degradation chemistry (isomerization) between the two valerate isomers. By adopting the same strategy of using MS/MS molecular fingerprinting as an effective tool for structural identification/verification, the MS/MS product ion spectra of the RRT 0.90 unknown peak and the 8.13 min peak were collected and compared with the MS/MS spectra of betamethasone 17-valerate and betamethasone 21-valerate, respectively. These two compounds were used as surrogates for MS/MS molecular fingerprinting due to their availability in our laboratory; the two counterpart valerates of dexamethasone were not available at the time of the study. As shown in Fig. 3b and d, betamethasone 17-valerate and betamethasone 21-valerate share most of the common fragment ions, including *m*/*z* 457 (loss of HF) and fragments observed from betamethasone (*m*/*z* 373, *m*/*z* 355, *m*/*z* 337 and *m*/*z* 279). However, betamethasone 17-valerate distinctly differs from betamethasone 21-valerate by the following two observations: (1) the absence of *m*/*z* 439 ion (loss of HF and $H_2O$) and *m*/*z* 421 (loss of HF and two $H_2O$) and (2) the most abundant ion being *m*/*z* 279. It turned out that the MS/MS spectrum of the RRT 0.90 unknown peak (Fig. 3c) resembled that of betamethasone 17-valerate, while the 8.13 min peak (Fig. 3a) resembled that of betamethasone 21-valerate. Therefore, the RRT 0.90 species was assigned as dexamethasone 17-valerate and its isomeric product formed under the alkaline condition, the 8.13 min peak, was assigned as dexamethasone 21-valerate. The same acyl-transfer isomerization from dexamethasone 17-valerate to the corresponding 21-valerate under the alkaline condition was later observed between betamethasone 17-valerate and betamethasone 21-valerate as well (Scheme 1), which provided further support for the current peak assignments.

### 3.4. NMR confirmation

The rapid structure determination of the RRT 0.90 unknown peak as dexamethasone 17-valerate via the above MS/MS studies and accompanying hydrolytic stress studies is already of very high level of confidence and appropriate decisions were made promptly with regard to the quality of the batch of betamethasone 17-valerate tested. In order to validate this strategy of rapid structure identification, the isolated RRT 0.90 peak was analyzed by NMR. The results obtained from various 1D and 2D NMR experiments confirmed that the RRT 0.90 species was indeed dexamethasone 17-valerate (Fig. 4, bottom). Quite surprisingly, a complete conversion from dexamethasone 17-valerate to the 21-valerate isomer (Fig. 4, upper) was also observed when acquiring 2D NMR data during a 24-h period.

## 4. Conclusion

In summary, the unknown peak at RRT 0.90 was rapidly identified as dexamethasone 17-valerate, a process impurity of betamethasone 17-valerate, by using the strategy of combining LC–$MS^n$ with the mechanism-based stress studies; the structure identification was later confirmed by full NMR characterization. The keys for success in this strategy are the effective use of LC–$MS^n$ (including $MS^n$ molecular fingerprinting) and design of informative stress studies based on LC–$MS^n$ results. This overall approach can be adopted as a very effective general strategy for the rapid identification of pharmaceutical impurities at low levels (∼0.1%).

## Acknowledgment

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