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Impurity profile tracking for active pharmaceutical ingredients: Case reports

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Abstract

Tracking the impurity profile of an active pharmaceutical ingredient (API) is a very important task for all stages of drug development. A systematic approach for tracking impurity profile of API is described. Various real pharmaceutical applications are presented through successful examples of impurity profile tracking for three different novel APIs. These include MK-0969, an M3 antagonist; MK-0677, an oral-active growth hormone secretagogue and API-A, a cathepsin K inhibitor. A general strategy including selection of a reversed phase high performance liquid chromatographic (RP-HPLC) impurity profile method based on screening various stationary phases and changing the pH of the mobile phase and elucidation of impurity structures through the utilization of LC–MS, preparative-LC and NMR is demonstrated. A series of studies were conducted on the peak purity check by using the LC-UV diode-array and LC–MS detections. The advantages and disadvantages of each technique in the evaluation of peak purity are discussed.

Keywords: Active pharmaceutical ingredient (API); Impurity profile tracking; Reversed phase high performance liquid chromatographic (RP-HPLC); Elucidation of impurity structures; Peak purity check

1. Introduction

Most active pharmaceutical ingredients (API) are produced by organic chemical synthesis. Various components, including residual solvents, trace amounts of inorganic, and organic components can be generated during such a process. Those components remaining in the final API are considered as impurities. Profile tracking for such impurities, especially for the organic impurities is a very important task for the development of an API from early to late stage in order to ensure its safety, efficacy, purity, stability and quality.

The organic impurities present in the API could be process-related impurities such as starting materials, intermediates, by-products, reagents, ligands, and process degradation products. They could also be formed as a result of degradation experienced under storage conditions. Prior to clinical studies, safety toxicology studies on animals are required for organic impurity qualification. Qualified impurities will have levels consistent with those used in safety and clinical batches. The International Conference on Harmonization (ICH) guidelines for the API indicate that new impurities at levels >0.15% for a ≤2 g/day daily dose or those >0.05% for a >2 g/day daily dose should be qualified or reduced by purification of the batch prior to use in clinical studies. If there are known human relevant risks of an identified impurity, the impurity level will need to be reduced to lower safe levels [1].

A chromatographic method, especially a reversed phase high performance liquid chromatographic (RP-HPLC) method with UV detection is commonly developed for controlling trace amount of organic impurities. This is done through a systematic approach that screens different stationary phases, changes the pH of the mobile phase, varies the organic modifiers and column temperature [2]. Each individual unqualified impurity exceeding the 0.15% level is identified by different techniques, such as authentic sample spiking, HPLC with diode-array detection (LC-diode-array), HPLC with mass spectroscopic detection (LC–MS), HPLC with NMR (LC-NMR), and isolation by preparative-LC followed by NMR. In addition, to prevent possible impurity peak co-elution with the main compound peak, a peak purity of the main compound is often assessed by using

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LC-UV diode-array or LC–MS detections. Although all these techniques are very useful for the structural identification of the impurity, the most important aspect is still to have a clear understanding of the chemistry used for the synthetic route and for the chemical degradation process to elucidate a reliable impurity structure with a clear mechanism.

In the past two decades, there are several publications on the topic of API impurity profiles [3–6], but in this paper, we focused on modernized and streamlined practices of pharmaceutical industry for impurity tracking process based on state-of-art techniques through various successful examples of real pharmaceutical cases. Three novel and distinct APIs and their related impurities are tracked based on the synthetic chemistry, the selected RPLC method, data collected by utilizing LC–MS and preparative-LC followed by NMR. These include MK-0969, an M3 antagonist [7]; MK-0677, an oral-active growth hormone secretagogue [8] and an API-A, a cathepsin K inhibitor [9]. A series of studies were conducted on peak purity check by using LC-UV diode-array and LC–MS. The advantages and disadvantages of each technique for the evaluation of peak purity are discussed.

2. Experimental

2.1. Instrumentation

In the RPLC mode, an Agilent LC 1100 (Hewlett Packard Co., Wilmington, DE, USA) system was used. Data collection and analysis were performed with a PE Nelson data system equipped with Turbochrom software (PE Nelson, Cupertino, CA, USA).

LC–MS experiments were performed using an HP 1100 LC and a quadrupole ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionization (ESI) or an atmospheric pressure chemical ionization (APCI) interface. LC conditions were the same as used for regular LC except that mobile phase A was 0.1% (v/v) aqueous formic acid. Parameters for ESI or APCI ion source were selected based on automatic tune with studied compound. The acquired mass range for full scan experiment was from *m/z* 50 to 1500 at 3 s per scan. MS data acquisition and analysis were performed with Xcalibur® software Version 1.2.

Preparative-LC was carried out using a Waters® LC system (Milford, MA, USA) equipped with a Waters Symmetry C18 column (5 cm × 25 cm). The mobile phases were 0.1% (v/v) aqueous formic acid (A) and acetonitrile (B). A 500 mL MK-0677 API solution was injected into the LC system and isocratically (75/25 v/v acetonitrile/0.1% formic acid) eluted at 50 mL/min. UV detection was set at 220 nm wavelength and desired fractions were collected and analyzed by analytical LC to check identity and purity. A rotary evaporator (Büchi, Meierseggstrasse, Switzerland) with a 40 °C water bath was used to remove acetonitrile in the LC fraction under a 15 Torr vacuum. Then, the fraction was lyophilized. Afterwards, a small aliquot of the collected solid was reconstituted in 50/50 (v/v) acetonitrile/water and reanalyzed by analytical LC to confirm the retention time.

In NMR mode, an AVANCE DRX-400 system (Bruker, Boston, MA, USA) was used. The frequency was set 399.9 MHz for ^{1}H and 100 MHZ for ^{13}C.

2.2. Chromatographic columns

The columns used in RPLC mode were Zorbax XDB-C8, Zorbax Rx C8, Zorbax CN, Zorbax phenyl (Agilent Technologies, Wilmington, DE, USA); SymmetryShield™ RP18, SymmetryShield™ RP8, Symmetry C18, Symmetry C8, Xterra-RPC18, Xterra-RPC8, YMC-AQ ODS, YMC basic, YMC pro-C18 (Waters, Bedford, MA, USA). All columns were 25 cm long and 4.6 mm in ID with a particle size of 5 μm.

2.3. Chromatographic conditions

All LC separations, except where specified, were performed at a temperature of 25 °C. Each mobile phase gradient profile was varied. The flow rate was 1.0 mL/min, the injection volume was 10 μL; UV detection wavelength was at 210–230 nm.

2.4. Reagents

MK-0969, MK-0677, API-A and all related authentic samples were synthesized by Process Research chemists at Merck & Co., Inc. (Rahway, NJ). HPLC grade potassium di-phosphate, potassium mono-phosphate and phosphoric acid were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC grade acetonitrile (MeCN) was obtained from EMS Scientific (Springfield, NJ, USA). Deionized (D.I.) water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

3. Results and discussions

3.1. Selection of impurity profile methods

The most common impurity profile method for organic impurities is RP-HPLC with UV detection. Several parameters such as screening various stationary phases, changing the pH of the mobile phase, varying organic modifiers and column temperature are important for the development of an accurate, precise, reproducible, and rugged RP-HPLC impurity method. However, in this paper, we only focus on column and mobile phase pH, two most critical parameters.

3.1.1. Column selections

Numerous RP-HPLC columns are commercially available now. Each individual stationary phase would provide different levels of selectivity for different impurities due to various modifications, such as end capping, mixed-modes, amide bone-linkages, and phenyl and cyano bonding. The goal of an RP-HPLC impurity profile method is to separate different impurities from the main API peak and from each other as well. Thus, it is recommended that different stationary phases that possess large differences in polarity, wettability, and retention ability should be screened. This increases selectivity since the properties of the APIs and each impurity will differ greatly. Such

properties include ionic status, molecular size, and whether or not the chemical is hydrophobic or hydrophilic. Some impurities may possess structural features and properties very similar to API, such as diastereomers, positional isomer and oxidative degradation products from API. Therefore, they easily co-elute with the API peak. In those cases, the screening of different columns becomes even more important. The accurate number of columns for a screen is case dependent. In general, two sets of commercial available column switches each consistent of six to nine columns with low, and high pH mobile phases are recommended for an initial screen. Further screen with more columns, different organic modifies and temperatures can be conducted as necessary. Fig. 1 shows an example of how a column screen separated a diastereomeric impurity from the main peak. MK-0969 contains two chiral centers resulting in four stereoisomers. Three of the four are considered as impurities. The enantiomer cannot be separated by an achiral column since it has the same physical–chemical properties as the API, but the diastereomers may be separated by an achiral column. One of diastereomers of the MK-0969 has been controlled in the precursor, but other as shown in Fig. 1a could remain in the API based on the synthetic chemistry. Nine columns, including Zorbax XDB-C8, Zorbax phenyl, SymmetryShield™ RP8, SymmetryShield™ RP18, Xterra-RPC8, Xterra-RPC18, YMC-AQ ODS, YMC basic and YMC Pro-C18 were screened with 0.1% phosphoric acid/MeCN and pH 7.5 phosphate buffer/MeCN mobile phases gradient. The SymmetryShield™ RP8 bonded with amide linkage gave an adequate separation between the MK-0969 and its diastereomeric impurities (see Fig. 1b).

3.1.2. Mobile phase pH selection

Many pharmaceutical APIs are ionic compounds. The molecules of these compounds contain one or more acidic or basic functional groups and some contain both. In an RP-HPLC mode, sample retention increases for more hydrophobic compounds. When an acidic or basic functional group undergoes ionization, it becomes much less hydrophobic [10]. As a result, its retention k in RP-HPLC will be reduced significantly. In general, it is suggested that the mobile phase pH should be controlled to about 2 units away from the compound pK_a in order to get good peak shape and reproducible retention time. But in some cases, narrower pH band may be needed for the desired separations. Adjusting the mobile phase pH can be crucial to achieve the separations of ionic compounds that are structurally similar. We have successfully separated six positional isomers by utilizing the small pK_a differences of each isomer [11]. In general, if

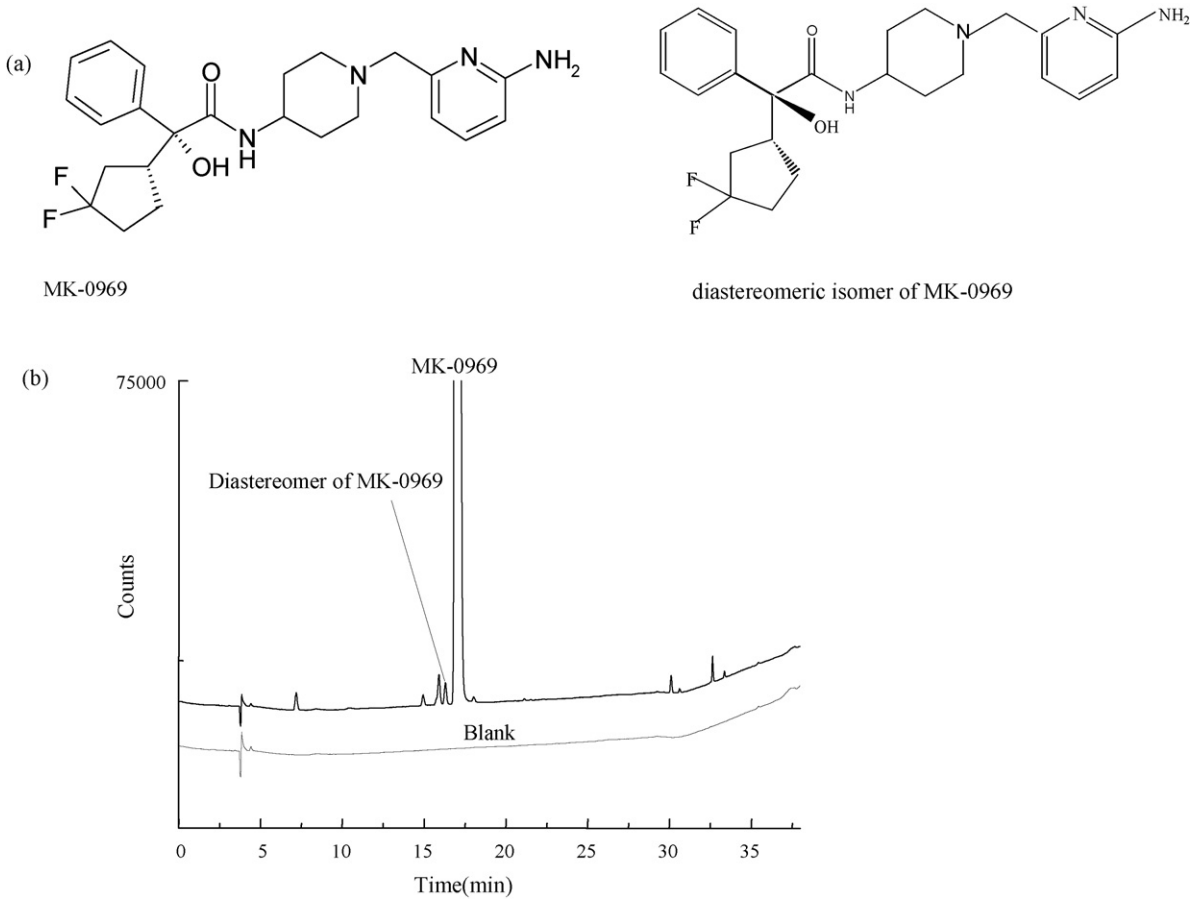


Fig. 1. (a) The structures of MK-0969 and its diastereomer. (b) The chromatogram of impurity profile of MK-0969. Conditions: column, SymmetryShield™ RP8, 250 mm × 4.6 mm, 5 μm particle diameter. Mobile phase—A: 0.1% phosphoric acid; B: acetonitrile. Mobile phase gradient profile: start with 95%A/5%B, gradient to 70%A/30%B in 25 min, then gradient to 10%A/90%B in 20 min. Flow rate: 1.0 mL/min. UV detection: 220 nm. Column temperature: 25 °C. Injection volume: 10 μL. Sample solution: 0.5 mg/mL in 10/90 MeCN/H_2O. Equilibration time: 10 min.

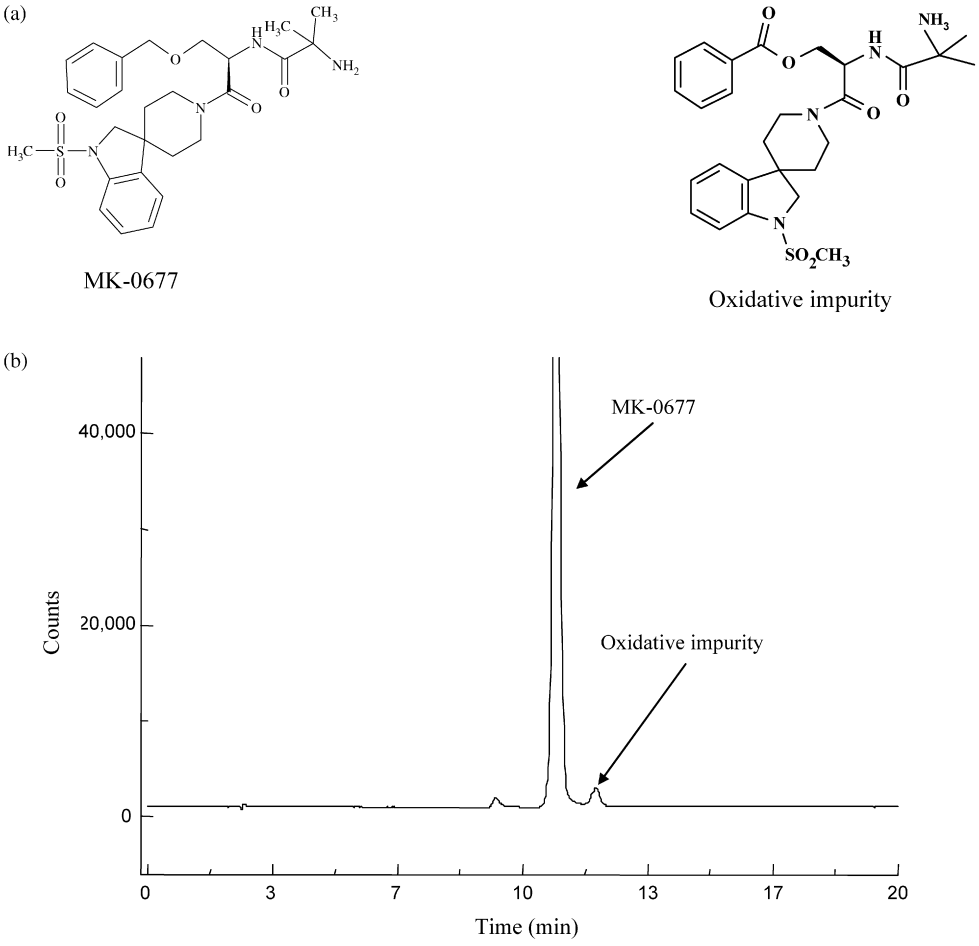


Fig. 2. (a) The structures of MK-0677 and its oxidative product. (b) The chromatogram of separation MK-0677 and its oxidative product. Conditions: column, Zorbax XDB C8, 250 mm × 4.6 mm, 5 μm particle diameter. Mobile phase—A: 15 mM potassium phosphate buffer, pH was adjusted to 6.5; B: acetonitrile. Mobile phase gradient profile: start with 70%A/30%B, gradient to 65%A/35%B in 5 min, then gradient to 60%A/40%B in 5 min, hold for 10 min, then further gradient to 30%A/70%B in 20 min. Flow rate: 1.0 mL/min. UV detection: 230 nm. Column temperature: 40 °C. Injection volume: 10 μL. Sample solution: 0.5 mg/mL in 10/90 MeCN/H_2O. Equilibration time: 10 min.

the structural differences are on hydrophobic functional groups, suppressing the ionization is helpful. Separation of MK-0677 from its oxidative degradation product is a good example. MK-0677 molecule contains a primary amine; therefore it is a base with a pK_a of 7.8. Its oxidative degradation product has very small structural differences compared to MK-0677 as shown in Fig. 2a. The pK_a of the two compounds are similar too. In such a case, suppression of ionization would help in the separation process. Investigating results of the pH screen indicated that both compounds were co-eluted with a low pH of mobile phase, the separations occurred when the pH of mobile phase increased to 6.0. The resolution increased as the pH of the mobile phase further increased. As a compromise, the pH of the mobile phase was set at 6.5 for the separation of the other impurities as shown in Fig. 2b. The detailed experimental conditions are listed in the figure caption.

3.1.3. Other parameters selection

Other parameter selection, such as column temperature, organic modifiers, and sample diluent strength are also very important. In general, higher column temperatures produce better peak shapes. However, both the column and sample can be degraded if the column temperature is too high. The sample dissolved into the mobile phase with the composition of the initial gradient point is preferred. But the solubility of the compound and the stability of the solution should also be carefully considered.

Organic modifiers can alter separation selectivity especially for compounds with closely related structures. However, from a practical perspective, it is best to use acentonitrile as an organic modifier. The main reason is a consideration of the method detection limit. In most cases impurity profile methods are carried out with gradient mobile phase profile and UV detection at wavelength below 230 nm in order to detect the most impurities. The most useful organic modifiers for the RPLC method include methanol, tetrahydrofuran (THF), isopropanol (IPA) and acetonitrile. Acetonitrile has the lowest UV cut-off while THF has the highest. Methanol and IPA are in between. In addition, the viscosities of methanol and IPA are relatively high, resulting in high column back-pressure and slowing down the mass transfer of the solutes. This broadens the peak. Therefore, the best choice of mobile phase for a RPLC impurity profile method is

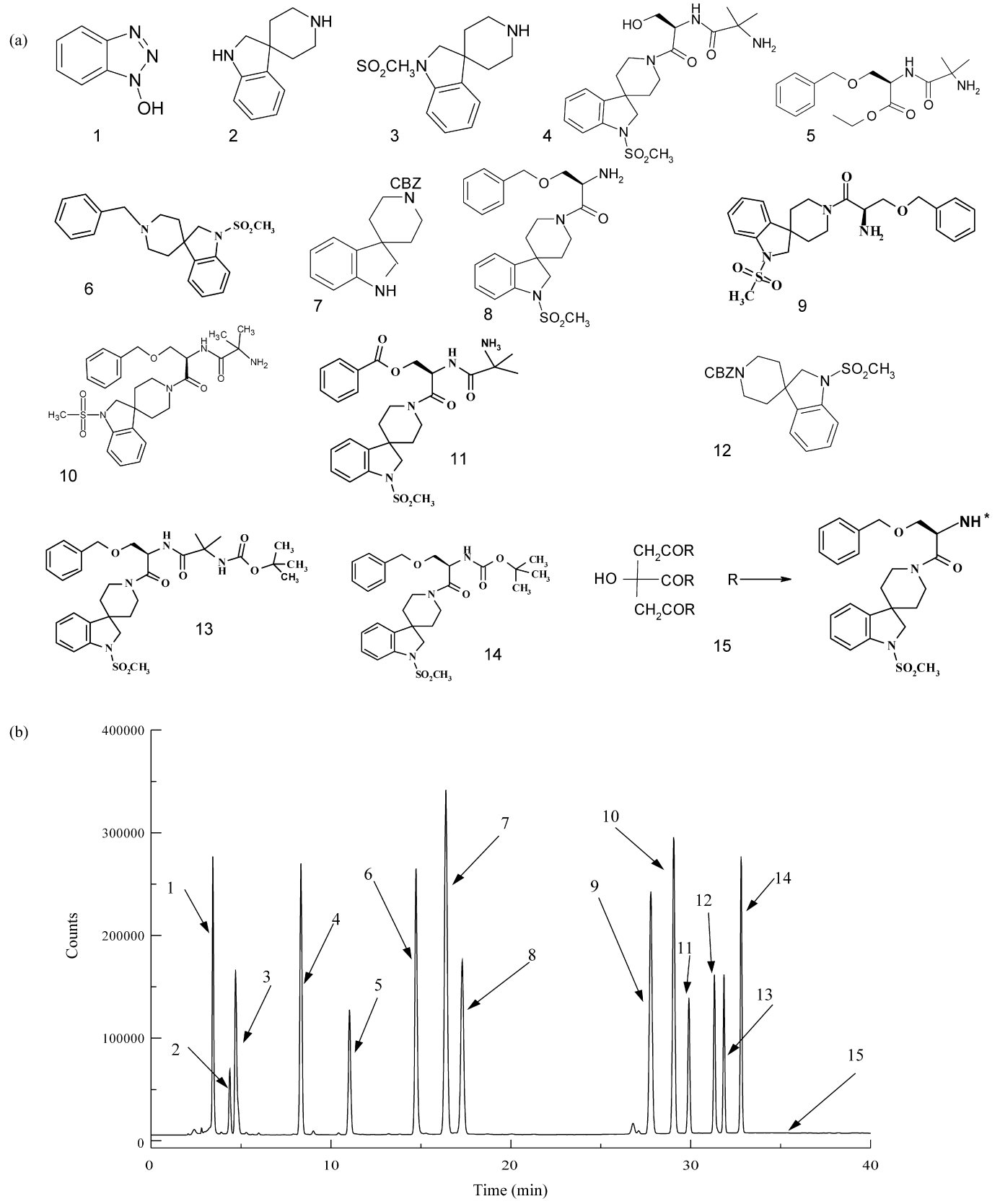


Fig. 3. (a) Structures of all MK-0677-related impurities. (b) Chromatogram of separation of all MK-0677-related impurities. Experimental conditions are the same as in Fig. 2b.

combination of phosphate buffer with acetonitrile. The methods conditions listed in the figure captions for both MK-0969 and MK-0677 reflect the above considerations.

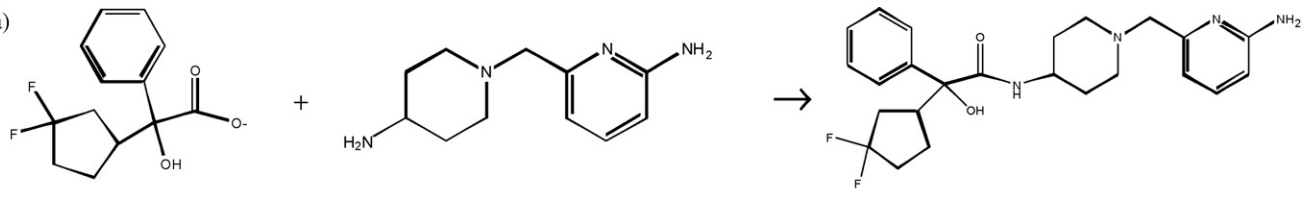
3.2. Impurity structural elucidation

Once all of the impurities are separated, identification becomes a very important next step for further control. Various analytical tools can be used for the elucidation of impurity structure.

3.2.1. Spiking known authentic impurities into API solution

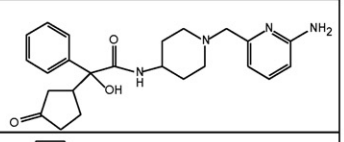
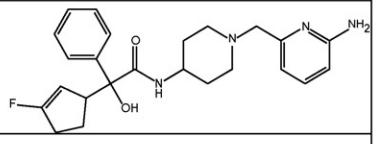
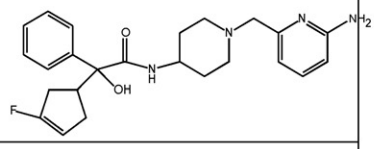
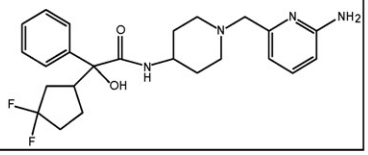
Some API-related impurities may be as simple as starting materials or intermediates carried over from previous reaction steps. In those cases, the easiest way to identify an unknown impurity in the RPLC chromatography is to spike a known authentic impurity into API solution. Then, inject the API solution and determine the relative retention time of the impurity. Fig. 3b shows a chromatogram of MK-0677 spiked with 13 other known impurities. The structure of each spiked impurity is listed in Fig. 3a. Those impurities can be divided as starting materials

(a)



Acid **Base** **MK-0969**

(b)

Impurities	RRT	MW	Structure
Ketone	0.42	422.2	
Vinylfluoro(A)	0.92	424.2	
Vinylfluoro(B)	0.94	424.2	
Diastereomer of API-A	0.96	444.3	

(c)

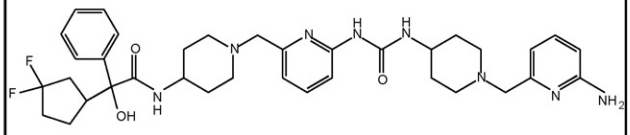
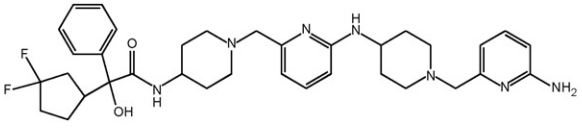
Impurities	RRT	MW	Structure
Urea	0.86	676.4	
Bis-Amine	0.88	633.4	

Fig. 4. (a) Reaction scheme of the final step of MK-0969. (b) Proposed structures for difluorohydroxy acid-related impurities. Experimental conditions: HPLC conditions are the same as in Fig. 1b. MS conditions are the same as listed in Section 2. (c) Proposed structures for 2-amino-6-(4-aminopiperdin-1-ylmethyl) pyridine base-related impurities. Experimental conditions are the same as in (b).

(1 and 2), process intermediates (3, 4, 9 and 12–14), process side products (6 and 15), process degradation products (4, 5 and 8) or storage degradation products (11). Their relative retention time versus the MK-0677 peak (10) can be determined based on the reliable chromatographic method.

3.2.2. LC–MS determination

In the past decade we have seen the rapid development of LC–MS systems, especially open access on-line LC–MS systems such as Finnigan's LCQ and Agilent's LC-MSD. These systems have gained more and more popularity allowing chemists to identify structures of impurities more rapidly. However, when using LC–MS for the determination of impurity structures, it is critical to understand the chemistry of synthetic process and product. Fig. 4a is the process scheme of a final reaction step for MK-0969. It shows that MK-0969 was synthesized through coupling of difluorohydroxy acid and 2-amino-6-(4-aminopiperdin-1-ylmethyl) pyridine base. The chemistry suggests that these acid and base-related impurities will go through a similar process as MK-0969 as long as the acidic and basic functional groups on those impurities remain the same as those of MK-0969. Fig. 4b and c summarizes proposed structures for those impurities in the final API of MK-0969 based on their molecular weights and chemistry.

In some cases, the LC–MS spectra provide certain isotopic patterns which help to determine the impurity structure. Fig. 5a

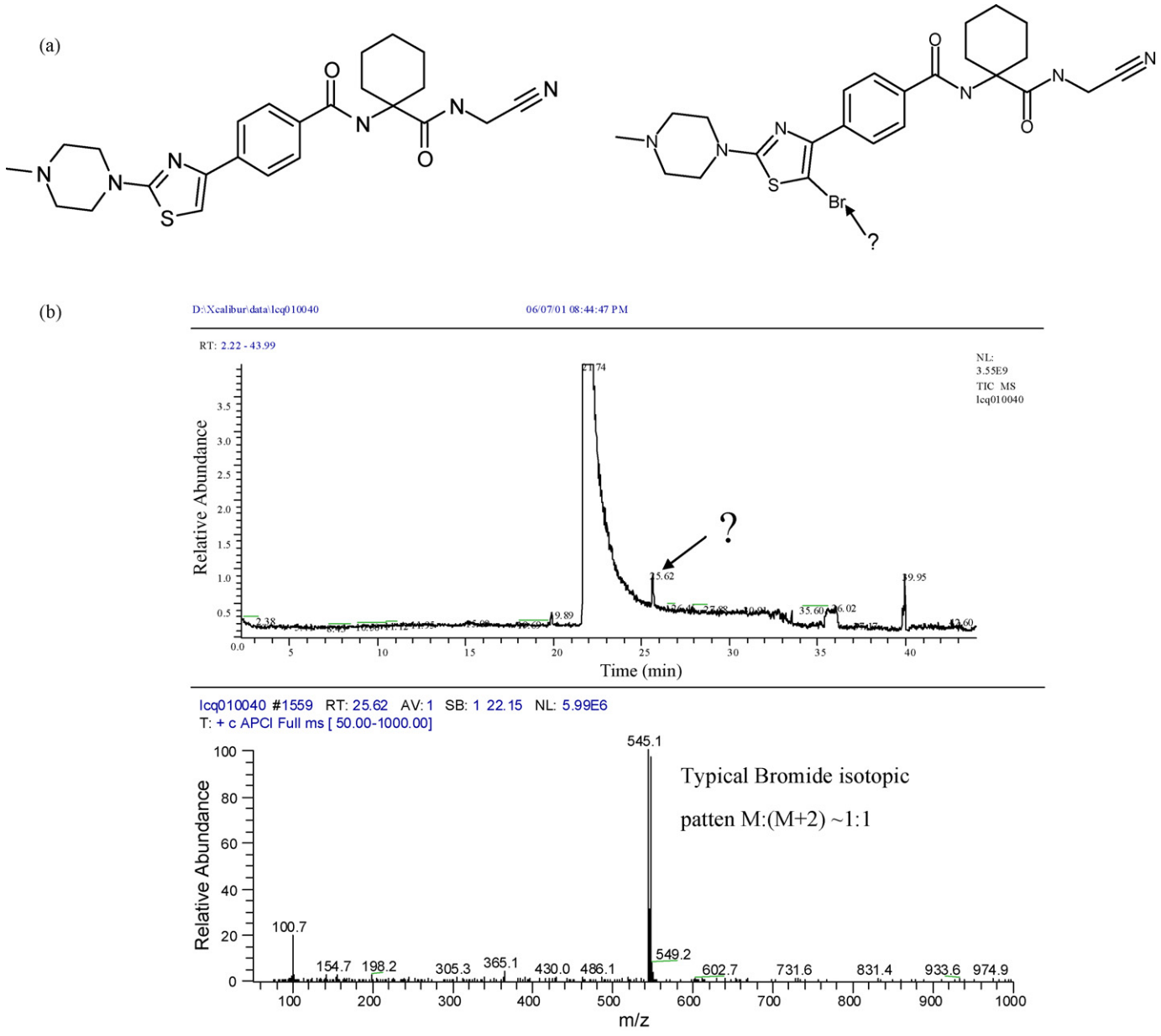


Fig. 5. (a) The structures of API-A and its bromide impurity. (b) LC–MS spectrum of bromide impurity of API-A. Experimental conditions: YMC ODS-AQ, 250 mm × 4.6 mm, 5 μm particle diameter. Mobile phase—A: 0.1% phosphoric acid; B: acetonitrile. Mobile phase gradient profile: start with 95%A/5%B, gradient to 70%A/30%B in 25 min, then gradient to 10%A/90%B in 20 min. Flow rate: 1.0 mL/min. UV detection: 215 nm. Column temperature: 25 °C. Injection volume: 10 μL. Sample solution: 0.25 mg/mL in 30/70 MeCN/H_2O. Equilibration time between run: 10 min. MS conditions are the same as listed in Section 2.

shows structures of API-A and its possible bromine-containing impurity. During a pilot plant campaign, an unknown impurity in a crude API-A front run sample was recognized and evaluated by LC-APCI-MS. The MS spectrum as shown in Fig. 5b indicated that the impurity has a typical bromide isotopic pattern M:($M+2$) ~ 1:1 and its molecular weight also confirmed that it is API-A plus a bromine atom as shown in Fig. 5a. Although the location of the bromine atom in the molecule was not clear since the MS–MS did not show clear fragments, a bromide source in the process was recognized after further reviewing the process chemistry. Hence, the impurity was well controlled.

3.2.3. NMR determination

In many cases, the on-line LC–MS and LC-NMR may not be able to provide a clear lead of the impurity structure. Subsequently, preparative-LC can be used to isolate the respective impurity and the relatively pure authentic impurity compound can be evaluated by NMR. In the early development of MK-0677, one of the pilot plant batches showed a 0.15 area % of the new impurity. This impurity had a relative retention time to MK-0677 of about 2.2, indicating that the compound was either large or very hydrophobic. Further LC–MS showed that the compound had a molecular weight of 1468.7. All these results indicated that the impurity could be a dimmer or a trimmer. But the puzzle could not be completely solved. The impurity level is too low to be determined by LC-NMR. Therefore, the impurity was isolated by preparative-LC. A few millgrams of 99.9% pure material were collected; then the pure compound was evaluated by ^{1}H NMR, ^{13}C NMR and 2-D NMR. The NMR results suggested that the impurity was formed by a citrate linkage with three mono-peptides of MK-0677 intermediates through amide bonds as shown in Fig. 3a compound 15. To confirm the suggestion, an authentic compound with proposed structure was synthesized. This authentic sample was evaluated by high-resolution MS, NMR and spiked back to MK-0677 API to confirm the prediction. All results indicated that the suggestion was correct. However, the question of the location of the citrate source remained. Further screens of every raw material and reagent indicated that the citrate came from one of raw materials. Further contact with the supplier confirmed that citric acid was used to adjust the pH during the reaction of raw material. Once the source was identified, the impurity was well controlled during the later pilot plant process.

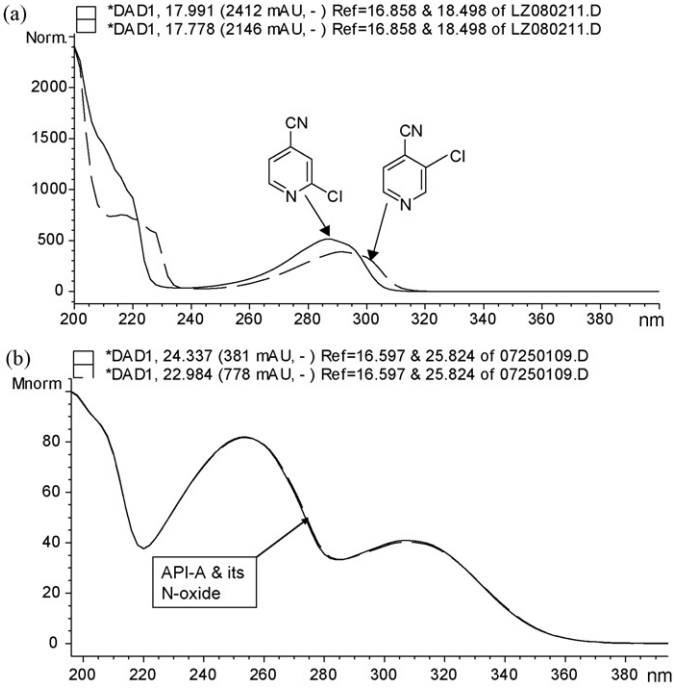


Fig. 6. (a) Examples of dissimilar UV spectra from two structural closely related compounds and (b) examples of similar UV spectra from two structural closely related compounds.

3.3. Peak purity evaluation

Sometimes, even after all of the column and mobile phase pH screens, temperature optimizations and mobile phase alterations have been conducted; an unknown impurity may still co-elute with the main API peak. The common way to control

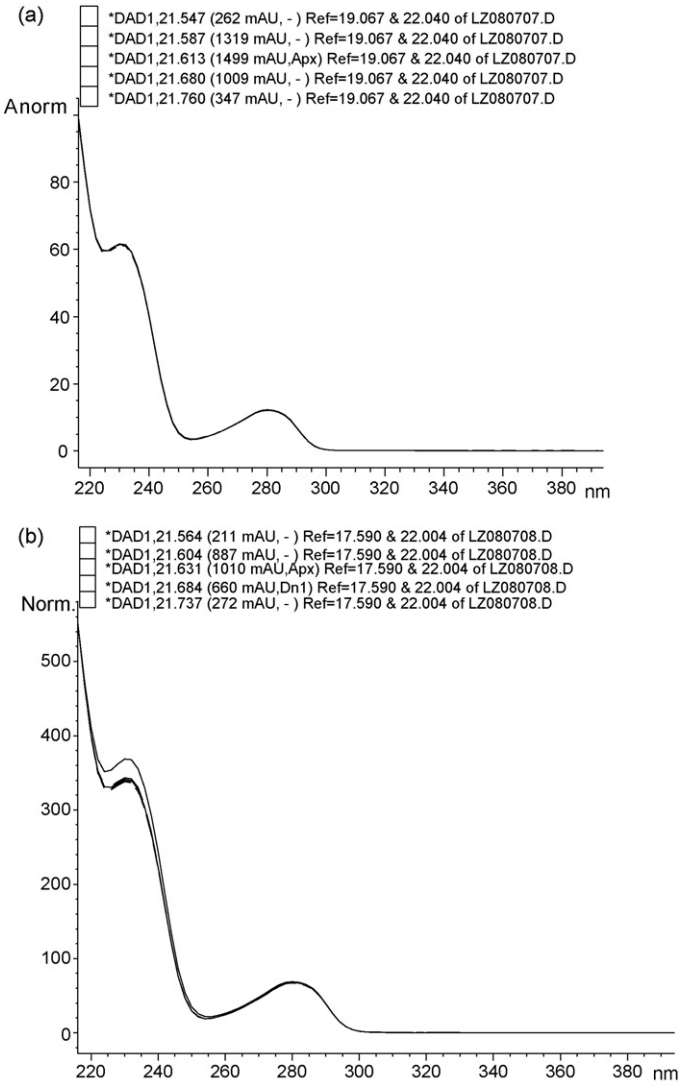


Fig. 7. (a) Peak purity check for the MK-0677 at 0.1% spiking level of OX-MK-0677 and (b) peak purity check for the MK-0677 at 0.5% spiking level of OX-MK-0677.

these co-eluting impurities is to check the peak purity by way of UV diode-array detection. However, such evaluations are based on the assumption that "Peak purity may be observed by looking for changes in spectra measured over a peak" [12]. In other words, only those compound which posses dissimilar UV spectra as the main peak can be recognized. Most co-eluted peaks have structural features similar to the main peak. These include positional isomers, diastereomers, and oxidative degradation products. In most cases, the positional isomer due to different positions on the aromatic or other un-saturated rings and the oxidative of un-saturated rings would result in different UV spectra from the main peak. But a diastereomer or an N-oxidative on a saturated ring will have very similar UV spectra compared to the parent compound in most cases. Fig. 6a and b is two examples. Therefore, the peak purity check has limitations for those impurities which have similar UV spectra as the main peak. Another limitation of the peak purity check by diode-array comes from detection sensitivity. One possibility to enhance detection sensitivity is to utilize the LC–MS. However, LC–MS has limitations too. LC–MS cannot recognize the co-eluted peak if it is an isomeric impurity. LC–MS may also provide misleading results as a result of its narrow linearity range and interference from the main component. Fig. 7a and b summarizes a series of studies on peak purity check by LC-diode-array and LC–MS detections. MK-0677 and its oxidative product (see Fig. 2a for structures) were selected as molecular probes. A chromatographic system in which both MK-0677 and its oxidative product co-eluted together was selected for the studies. The oxidative product of MK-0677 (OX-MK-0677) was spiked to a pure MK-0677 solution at 0.01%, 0.05%, 0.1% and 0.5% levels, respectively. As can be seen from Fig. 7a and b, the dissimilar spectra of the OX-MK-0677 compound were not detected below the 0.5% level. The second derivative UV spectra showed the same trends. As expected, when a LC–MS system was utilized to evaluate the spiked solution, sensitivity increased significantly. LC–MS can recognize the spiked OX-MK-0677 at levels as low as 0.01%. However, using the area % of the LC–MS-SIM spectrum of OX-MK-0677 versus MK-0677 over-estimated the concentration at the 0.05% spiked level. Overall, it can be concluded that both techniques have advantages and disadvantages and should be used as complimentary techniques.

4. Conclusion

The impurity profiles of three different classes of APIs were tracked by systematic approaches. The structures of the impurities in each case were elucidated by using multiple complimentary techniques. The most important aspect is a thorough knowledge of the process and product chemistry when conducting the elucidation of the impurity profiles.

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