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Identification of an unknown extraneous contaminant in pharmaceutical product analysis

Mingxiang Lin, Min Li *, Abu Rustum

Global Quality Services-Analytical Sciences, Schering-Plough Corporation, 1011 Morris Avenue, Union, NJ 07083, United States

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Abstract

During the content uniformity test of a drug product (tablet formulation), an unknown peak was observed in the HPLC chromatograms. Upon further investigation, it was determined that the unknown peak was originated from an external source and, therefore, the drug product is free of this unknown peak. The next step was to identify the structure of this unknown peak in order to determine the source of this contaminant species. In this paper, we wish to present the strategy and the results of the experiments that led to the identification of this unknown peak. LC–PDA/UV and LC–MS^n analyses were conducted to obtain the UV spectrum, molecular weight and MS^n fragmentation pathways of the unknown peak. The MS analysis revealed certain structural features of the unknown species and a number of model compounds that contain such features were then examined for their UV absorbance profiles in an attempt to establish the functional group connectivity within the unknown species. A careful examination of these results in conjunction with the determination of the high-resolution molecular weight led to a short list of potential candidates for the unknown species, among which the most likely one was 1,3-diphenylguanidine. The identification of the unknown contaminant was confirmed by spiking experiments using the authentic compound. The potential source of this contaminant was also identified as derived from the safety filler of the pipette bulb used to prepare the sample solutions during the drug analysis.

Keywords: Identification; Impurity; Extraneous contaminant; 1,3-Diphenylguanidine; LC–MS

1. Introduction

By the definition of the FDA, drug impurities are components found in a drug product that are neither its drug substances nor excipients [1]. When exceeding certain thresholds [2,3], the identification of these unknown impurities is required by regulatory guidelines and their toxicity and/or safety may also need to be evaluated dependent upon the nature and amount of the identified species. According to their origins, the commonly observed drug product impurities can be classified into several categories: degradation products of drug substance (or active pharmaceutical ingredient, API), synthetic process impurities, extractable/leachable components from packaging materials, and external contaminants. The external contaminants may be introduced during sample preparation steps of drug analysis. Since the external contaminants are usually not structurally related to the drug substance and/or the excipients, their identification can be challenging due to the lack of relevant structural information.

The current paper presents a case study for identifying an impurity that was originated from the safety filler of a laboratory pipette bulb, which is an external contamination. The unknown impurity peak was observed at greater than 1% level during the content uniformity test of a drug product (tablet formulation). However, this impurity was found only in two out of the ten final content uniformity sample solutions and none of the 10 stock sample solutions, from which the final solutions were made through dilution, showed this unknown peak. We utilized a strategy that started with LC–MS^n including its high-resolution mode, which is a widely used technique to elucidate the structures of unknown pharmaceutical impurities [4,5]. The LC–MS^n analysis revealed certain structural features of the unknown species and a number of model compounds that contain such features were then examined for their UV absorbance profiles in an attempt to establish the functional group connectivity within the unknown species. This systematic process to extract

* Corresponding author.
E-mail address: min.li@spcorp.com (M. Li).

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structural features of the unknown species by combining the two techniques, i.e., LC–MS^n and UV profiling of structurally relevant model compounds, has been proven quite effective in the determination of this impurity as 1,3-diphenylguanidine. We have performed literature search to determine if there has been any report of this impurity in the sample preparation of pharmaceutical products. It is quite surprising that there has been no previous report on this impurity in such cases, although the source of this contaminant impurity (i.e., the safety filler of a laboratory pipette bulb) could be widely present in a pharmaceutical testing laboratory.

2. Experimental

2.1. Materials

All reagents used to prepare mobile phases for the HPLC analyses were HPLC grade, obtained from Fisher Scientific (USA). Other compounds were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. HPLC analyses

A Waters Alliance 2695 HPLC system equipped with a PDA/UV detector was used for sample analysis and for the development of an LC–MS compatible method. For the drug content uniformity test, an Agilent Zorbax SB-CN 250 mm × 4.6 mm, 5 μm column was used with a mobile phase consisting of acetonitrile:water (38:62, v/v) containing 5 mM sodium dodecyl sulfate (SDS) and 0.05% phosphoric acid. The analyses were performed with isocratic elution mode at a flow rate of 2.0 mL/min and a column temperature of 40 °C. UV spectra were collected from the PDA/UV detector with a wavelength range of 210–400 nm.

An LC–MS compatible method was developed which employed a Waters YMC Pack Pro C18 150 mm × 4.6 mm, 3 μm column and a mobile phase system consisting of A, water with 5 mM ammonium acetate, and B, acetonitrile:water (90:10, v/v). The analysis was performed at ambient column temperature, a flow rate of 1.5 mL/min, and a gradient with the percentage of the mobile phase B varied according to the following program: 0 min (5% B), 5 min (5% B), 20 min (60% B), 20.1 min (100% B), 25 min (100% B), 25.1 min (5% B), 30 min (5% B). For certain analyses, a variation of this LC–MS compatible method was also used, in which the original mobile phase A was replaced by an aqueous 0.1% trifluoroacetic acid solution while other conditions remained unchanged.

2.3. LC–PDA/UV–MS^n analyses

LC–PDA/UV–MS^n experiments were performed on a Thermo Surveyor HPLC system interfaced to a PDA/UV detector and a Thermo Finnigan LTQ mass spectrometer operating under positive electrospray ionization (ESI) mode. The HPLC mobile phase flow to the mass spectrometer was split at a ratio of 10:1 prior to entering the mass spectrometer. The electrospray

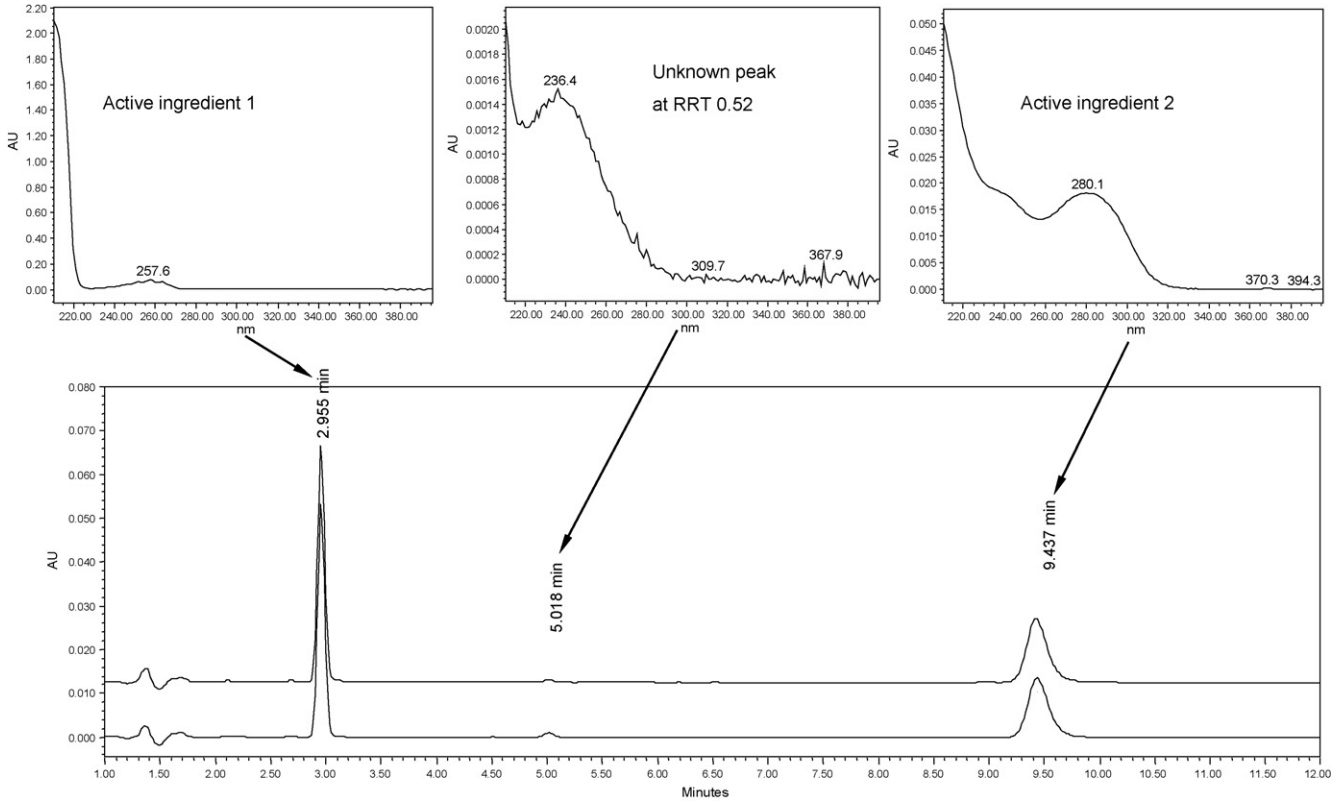


Fig. 1. Observation of the RRT 0.52 unknown peak from the two content uniformity test working solutions. The UV spectra of active ingredient 1 (RT 3.0 min), active ingredient 2 (RT 9.4 min), and the unknown peak (RT 5.0 min) are displayed as insets.

ionization voltage was set at 4.0 kV and the capillary temperature at 300 °C. The full MS scan was acquired from 50 to 500 Da. The MS^n experiments were conducted on ions of interest with normalized collision energies set between 28 and 40, depending on the intensities of product ions produced.

2.4. Unknown peak isolation

The unknown peak was isolated from multiple runs of HPLC fractionations. After all fractions were combined, the organic solvent (acetonitrile) was removed by a Rotavapor (Büchi R-200 with a V-800 vacuum controller) in a 30 °C water bath. The remaining aqueous portion was extracted by methylene chloride (2 × 5 mL each) and the organic extraction solutions were then combined and concentrated to ∼0.5 mL. The concentrated organic solution was reconstituted with a 50:50 (v:v) methanol:water mixture to a final volume of 1–2 mL.

2.5. High-resolution MS analyses

The high-resolution MS analysis of the isolated unknown species was performed on a Waters Q-Tof Premier mass spectrometer equipped with a LockSpray ionization source. The instrument was operated at positive ESI mode with an electrospray voltage 3.5 kV, cone voltage 35 V, source temperature 100 °C, desolvation temperature 250 °C, cone gas flow 60 L/h, and desolvation gas flow 600 L/h. The instrument was calibrated externally by a sodium cesium iodide solution. The sample was infused by a syringe pump at a flow rate of 5 μL/min through the analyte channel. A reference compound with the chemical formula of $C_{13}H_{21}NO_3$ was used in the reference resolution which was infused into the reference channel by a second syringe pump at a flow rate of 5 μL/min. The time-of-flight (TOF) MS spectra were acquired at 1 scan/s rate and 0.1 s inter-scan time. The intensity of the peaks of interest was adjusted to ∼200–300 counts/s.

3. Results and discussion

3.1. Preliminary investigation and development of an MS compatible HPLC method

The unknown peak was observed in two batches of the product tablets at a relative retention time (RRT) of 0.52 during the content uniformity test, as shown in Fig. 1. Preliminary investigation revealed that this unknown peak only occurred in two working solutions and was not observed in the corresponding stock solutions, indicating that this unknown peak was most likely due to an external contaminant introduced during sample preparation or analysis process. The UV spectrum of the

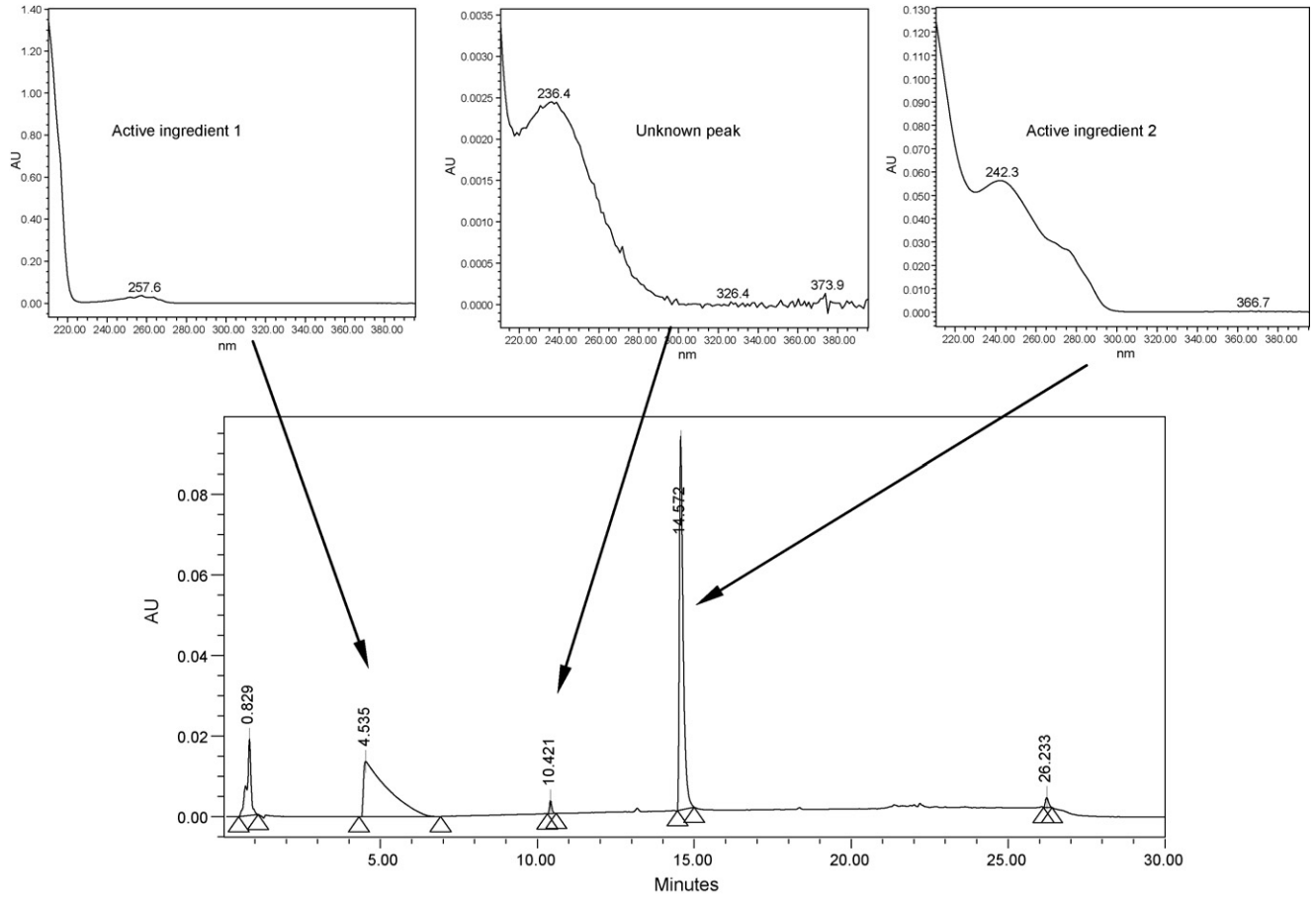


Fig. 2. HPLC chromatogram obtained using the ammonium acetate buffer method (Section 2.2). The UV spectra of the three peaks are shown as insets. RT 9.0, 11.5 and 15.2 min peaks are assigned as active ingredient 1, the RRT 0.52 unknown peak, and active ingredient 2, respectively, based on their UV spectra.

unknown species displayed a single maximum absorbance at 236 nm, which bears no similarity to either of the two APIs (Fig. 1 insets).

The mobile phase in the original content uniformity test contains phosphoric acid and 5 mM sodium dodecyl sulfate (SDS) as ion-pairing reagent. Both of these two components are non-volatile and known for suppressing electrospray ionization; thus, the original method is not MS compatible. Considering the pK_a values of the two APIs in the drug product, an MS compatible, gradient method was developed which utilized a neutral ammonium acetate buffer as mobile phase A on a C18 column. With this MS compatible method, the unknown species was observed at a retention time (RT) of 10.4 min, which was well separated from the two APIs (Fig. 2). The identity of the unknown peak under the new method was revealed by its UV profile which was identical to the one obtained under the original method.

3.2. LC–MS^n analyses (n = 1–3)

LC–MS analyses were performed using the LC–MS compatible method described in Sections 2.2 and 3.1. As shown in Fig. 3, the unknown species at RT 10.9 min clearly showed an *m/z* 212 ion in the MS spectrum after background subtraction. The extract single ion chromatogram at *m/z* 212 (Fig. 3b) gave a single chromatographic peak at the corresponding retention time, confirming that *m/z* 212 was indeed produced from the unknown species. MS/MS fragmentation of *m/z* 212 yielded three major fragments: *m/z* 195, *m/z* 119, and *m/z* 94 (Fig. 3d). The loss of 17 from *m/z* 212 to form *m/z* 195 would be most likely a neutral NH_3 molecule. To rule out the possibility that *m/z* 212 ion might be the ammoniated molecular ion $(M + NH_4)^+$ of the unknown species, the ammonium acetate buffer was replaced by a 0.1% trifluoroacetic acid aqueous solution as the mobile phase A. Under the latter condition, in which the three components were also well separated and the same elution order maintained (active ingredient 1, the unknown species, and active ingredient 2 eluted at 8.4, 11.3, and 11.7 min, respectively), the *m/z* 212 ion was still observed as the predominant ion of the unknown species (data not shown). Thus, the *m/z* 212 ion was confirmed as the protonated molecular ion of the unknown species, indicating that the molecule weight of the unknown species should be 211. This odd number of the molecular weight suggested that the molecule should contain an odd number of nitrogen atom(s).

To obtain further structural information, MS^3 fragmentations of the three MS^2 product ions were subsequently performed individually. As shown in Fig. 4, the main product ions of *m/z* 195 were *m/z* 168, 117 and 92; the main product ions of *m/z* 119 were *m/z* 92 and 77; the main product ion of 94 was *m/z* 77. On the other hand, *m/z* 119 and 94 were not products of *m/z* 195 precursor ion; *m/z* 94 was not a product ion of *m/z* 119 precursor ion. These relationships between the MS^2 and MS^3 ions clearly indicate the presence of three independent fragmentation pathways that are summarized in Fig. 5. Based on the analyses, the unknown molecule would likely have an amino group due to the mass loss of 17 (NH_3) from *m/z* 212 to form *m/z* 195. The smallest fragment ion observed is *m/z* 77, which is most likely a

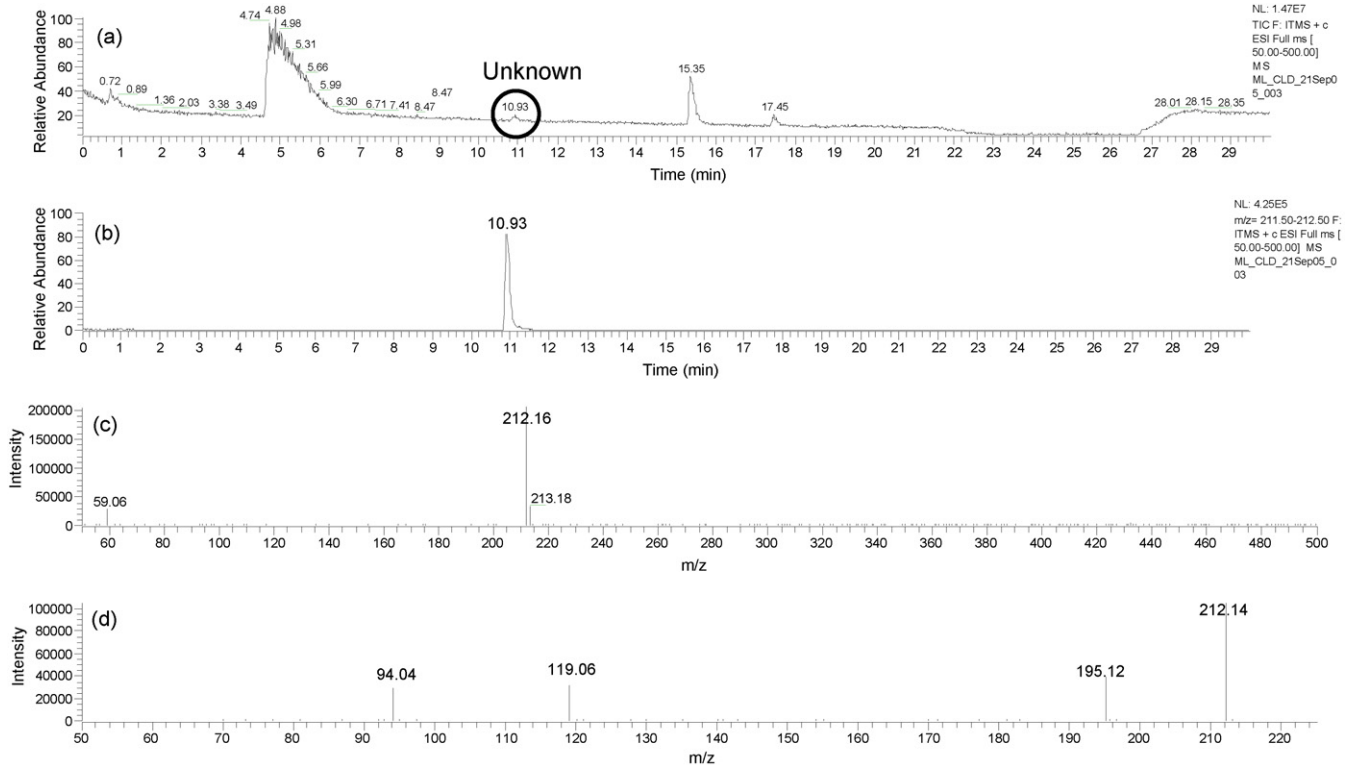


Fig. 3. LC–MS/MS analysis of the unknown species. (a) Total ion chromatogram; based on their UV spectra, the RT 4.9, 10.9 and 15.4 min peaks are assigned as active ingredient 1, the unknown species, and active ingredient 2. (b) Extracted ion chromatogram of *m/z* 212 ion. (c) The average of MS full scans from RT 9.8–10.8 min. (d) MS/MS on *m/z* 212 ion at a normalized collision energy of 30.

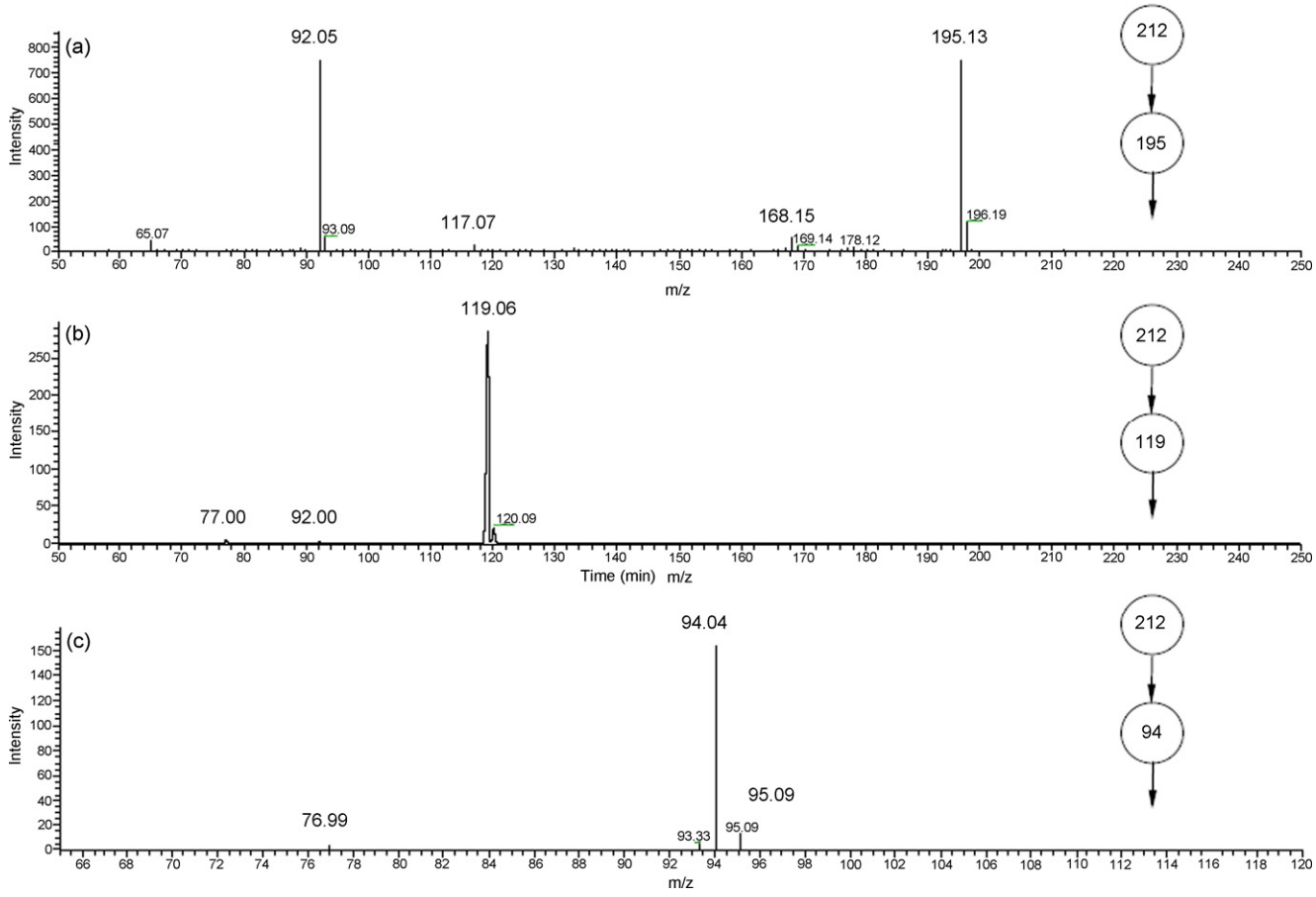


Fig. 4. LC–MS^3 analysis of the unknown species. The precursor ions are shown in the circled numbers on each panel.

benzene cation ($C_6H_5^+$), a common fragment usually produced from molecules containing monosubstituted phenyl group(s). It was noted that the summation of the *m/z* values from the two MS^2 fragments 119 and 94 is 213, which is the molecular weight of the unknown species plus two protons. Since the above analysis result showed that the *m/z* 77 ion could be produced from the two fragmentation pathways independently (Fig. 5), the unknown molecule must contain two separate phenyl groups. The difference between *m/z* 94 and *m/z* 77 is 17, suggesting that *m/z* 94 might be a protonated aniline $(C_6H_5NH_2 + H)^+$.

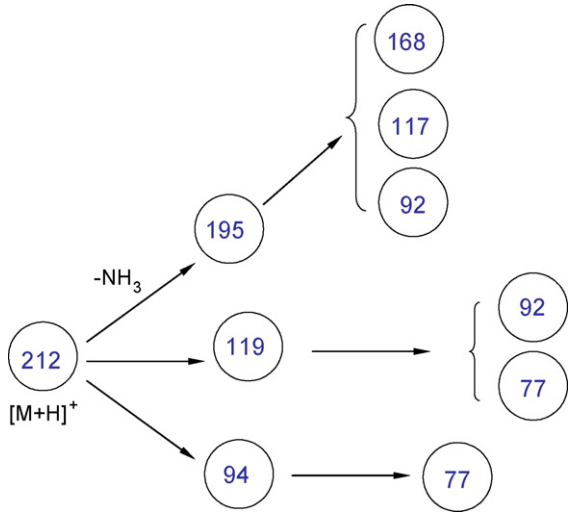
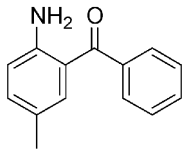
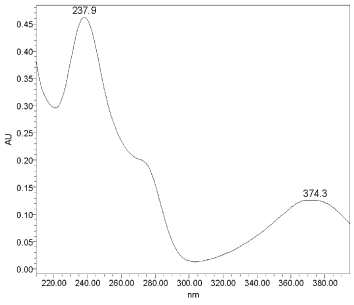
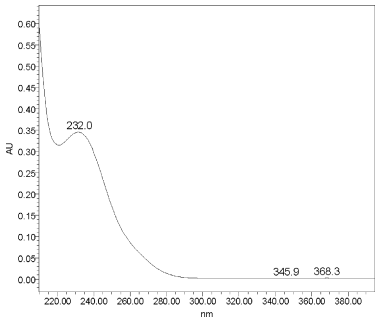
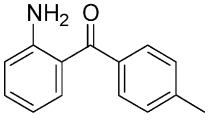
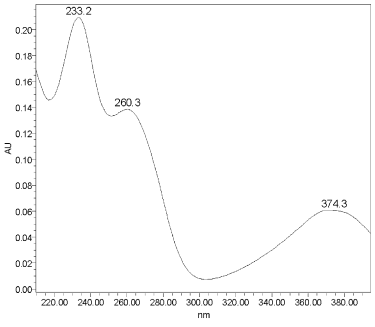
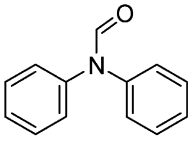
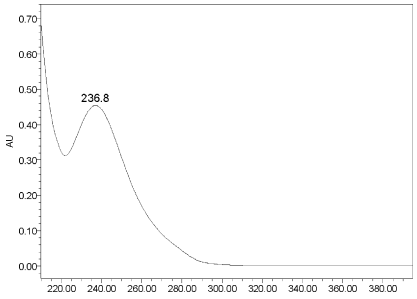
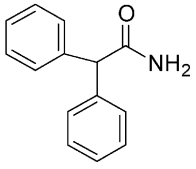
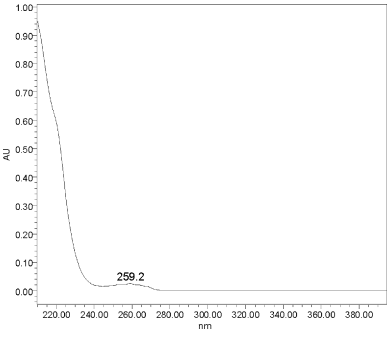
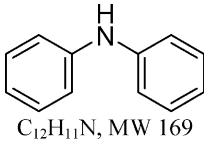
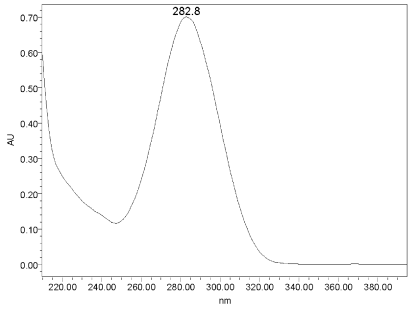
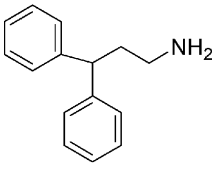
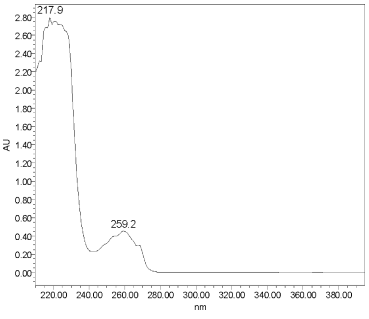
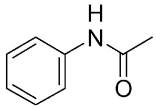
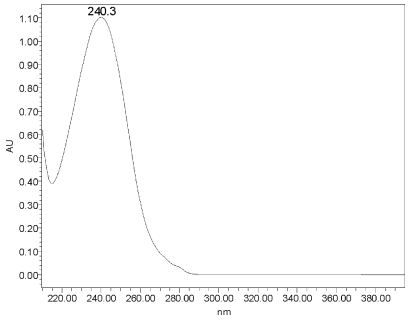


Fig. 5. Summary of the fragmentation pathways of the protonated unknown molecule.

3.3. LC–PDA/UV analysis of structurally related model compounds

The above LC–MS and MS^n analyses revealed a number of structural features, e.g., the unknown species should contain two phenyl groups, one amino group, and an odd number of nitrogen atom(s). The accompanying LC–PDA/UV analysis showed that the unknown species displayed a UV profile with a single maximum absorption at 236 nm which is consistent with the UV profiles of certain monosubstituted phenyl groups. Based on these analyses, a number of compounds that contain some or all of the structural features were examined by LC–PDA/UV as model compounds in order to determine the functional group connectivity in the unknown molecule. The model compounds examined and their UV spectra are summarized in Table 1. The model compounds, 2-amino-4-methylbenzophenone (**1**) and its isomer 2-amino-4′-methylbenzophenone (**2**), not only have the same molecule weight as the unknown species but also contain required functional groups. Nevertheless, they displayed completely different UV spectra as compared to the unknown species: an absorbance peak at ∼230–238 nm, a broad

Table 1
UV spectra of model compounds (**1–8**) that contain structural features related to the unknown species

Compounds	UV spectra	Compounds	UV spectra
2-Amino-4-methyl-benzophenone (1)  $C_{14}H_{13}NO$, MW 211		**N,N-Diphenyl-acetamide (5)**  $C_{14}H_{13}NO$, MW 211	
2-Amino-4'-methyl-benzophenone (2)  $C_{14}H_{13}NO$, MW 211		**N,N-Diphenyl-formamide (6)**  $C_{13}H_{11}NO$, MW 197	
2,2-Diphenyl-acetamide (3)  $C_{14}H_{13}NO$, MW 211		**Diphenylamine (7)**  $C_{12}H_{11}N$, MW 169	
3,3-Diphenyl-propylamine (4)  $C_{15}H_{17}N$, MW 211		**Acetanilide (8)**  C_8H_9NO, MW 135	

absorbance peak at ∼374 nm, and a shoulder peak at ∼260 nm. Another model compound, 2,2-diphenylacetamide (**3**), showed several weak absorbance bands at ∼240–270 nm which is typical for the alkyl-substituted phenyl group. The absence of the broad band at ∼370 nm in the UV of 2,2-diphenylacetamide (**3**) indicates that this band is most likely due to the direct attachment of the free amino group to an otherwise unsubstituted or alkyl-only substituted phenyl group as in the cases of the two benzophenone derivatives. Likewise, 3,3-diphenylpropylamine (**4**) also showed the characteristic weak absorbance band at ∼240–270 nm due to its alkyl-only substituted phenyl groups. On the other hand, *N*,*N*-diphenylacetamide (**5**), another model compound that meets the requirements for molecular weight and functional groups, was found to have a UV spectrum almost identical to that of the unknown species. Nevertheless, its retention time (17.5 min) was significantly longer than that of the unknown peak (∼11 min) under the reverse-phase elution condition as described in Section 2.2, suggesting the unknown species

should be much less hydrophobic than the model compound. Several other model compounds that are structure variations to *N*,*N*-diphenylacetamide (**5**) were subsequently investigated. *N*,*N*–Diphenylformamide (**6**), which has a less hydrophobic formic group, maintained the same UV absorbance feature of *N*,*N*-diphenylacetamide (**5**) but eluted earlier than **5** as expected. Removal of the formyl group from *N*,*N*-diphenylformamide (**6**) led to another model compound, diphenylamine (**7**), which had its UV maximum absorption shifting ∼30 nm toward the longer wavelength region. The results from these model compounds suggested that an electron-withdrawing group, such as carbonyl group, is necessary to keep the absorbance peak at the low wavelength region. Eliminating one phenyl group from *N*,*N*-diphenylacetamide (**5**) resulted in the model compound, acetanilide (**8**), which showed an absorbance peak only slightly shifting toward longer wavelength as compared to **5**, suggesting it might not be necessary for the unknown species to have its two phenyl groups connected to the same hetero atom (N in this case). Thus, the UV absorbance profile analyses of the aforementioned model compounds (**1**–**8**) provided a clear picture of how the functional groups would connect to each other in the unknown molecule: both of the two phenyl groups would need to connect to a hetero atom (not necessarily to the same atom), which should immediately bond to an electron withdrawing group.

3.4. High-resolution MS measurement

The accurate mass of the unknown species was determined to be 212.1196 from high-resolution MS analysis using a reference compound having an accurate mass of 240.1600. The elemental composition used during the chemical formula search was limited to C, H, N and O based on all structural information obtained. In addition, two criteria were applied in searching the matching chemical formulas: (1) the formula should have an odd number of nitrogen atom(s), and (2) the formula should have at least eight double bond equivalences (DBEs), since the unknown species should contain two phenyl groups with each one accounting for four DBEs. Even with a mass error tolerance of 200 ppm and with one nitrogen selected, only two formulas were found: $C_{15}H_{17}N$ (theoretical mass 212.1439, 115 ppm) and $C_{14}H_{13}NO$ (theoretical mass 212.1075, 57 ppm). The possible structure matches with these two formulas had been examined (Table 1) and no match was found. When the number of nitrogen was increased to 3 and 5 in the search while other criteria remained unchanged, the formula $C_{13}H_{14}N_3$ (theoretical mass 212.1188, 3.8 ppm) turned out to be the only hit. Considering the structure requirement that the two phenyl groups must connect to a hetero atom which itself is adjacent to an electron withdrawing group, 1,3-diphenylguanidine (**9**) was proposed to be the most likely candidate for the unknown species.

3.5. Confirmation of 1,3-diphenylguanidine as the unknown species

An authentic 1,3-diphenylguanidine (**9**) sample obtained from a commercial source was analyzed by LC–PDA/UV–MS and the results showed an exact match with the unknown species. The authentic **9** eluted at 11.2 min under the ammonium acetate method, and the UV spectrum displayed only one maximum absorbance peak at 236 nm. The MS^2 and MS^3 fragmentation patterns observed were also identical to those seen from the unknown species. The final confirmation was conducted by spiking authentic **9** to the working solution containing the unknown peak and the spiked solution was then examined under the original content uniformity test method of the drug product. As shown in Fig. 6, the spiking experiment confirmed that the unknown species is indeed 1,3-diphenylguanidine (**9**).

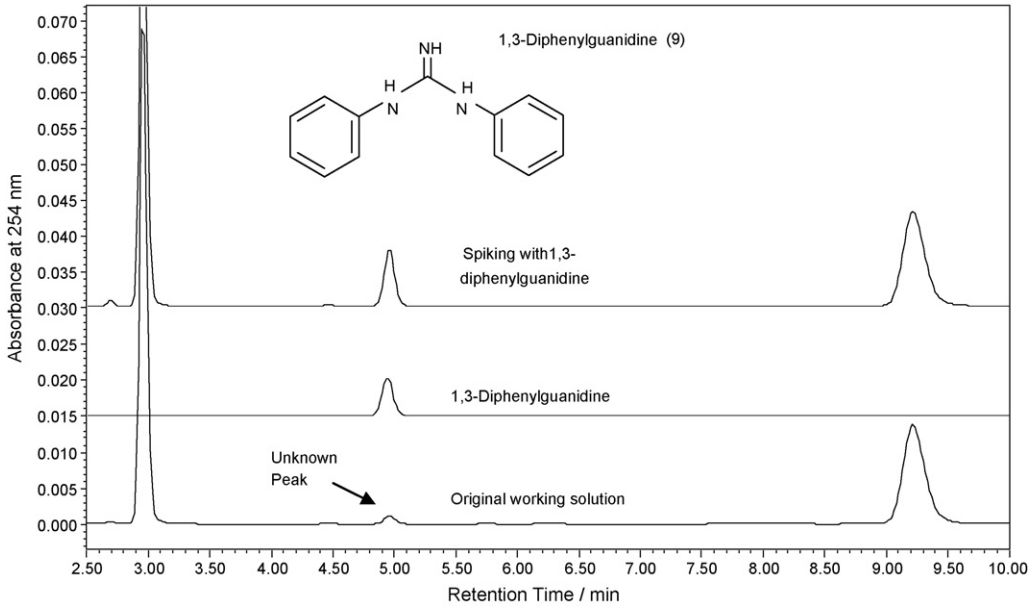
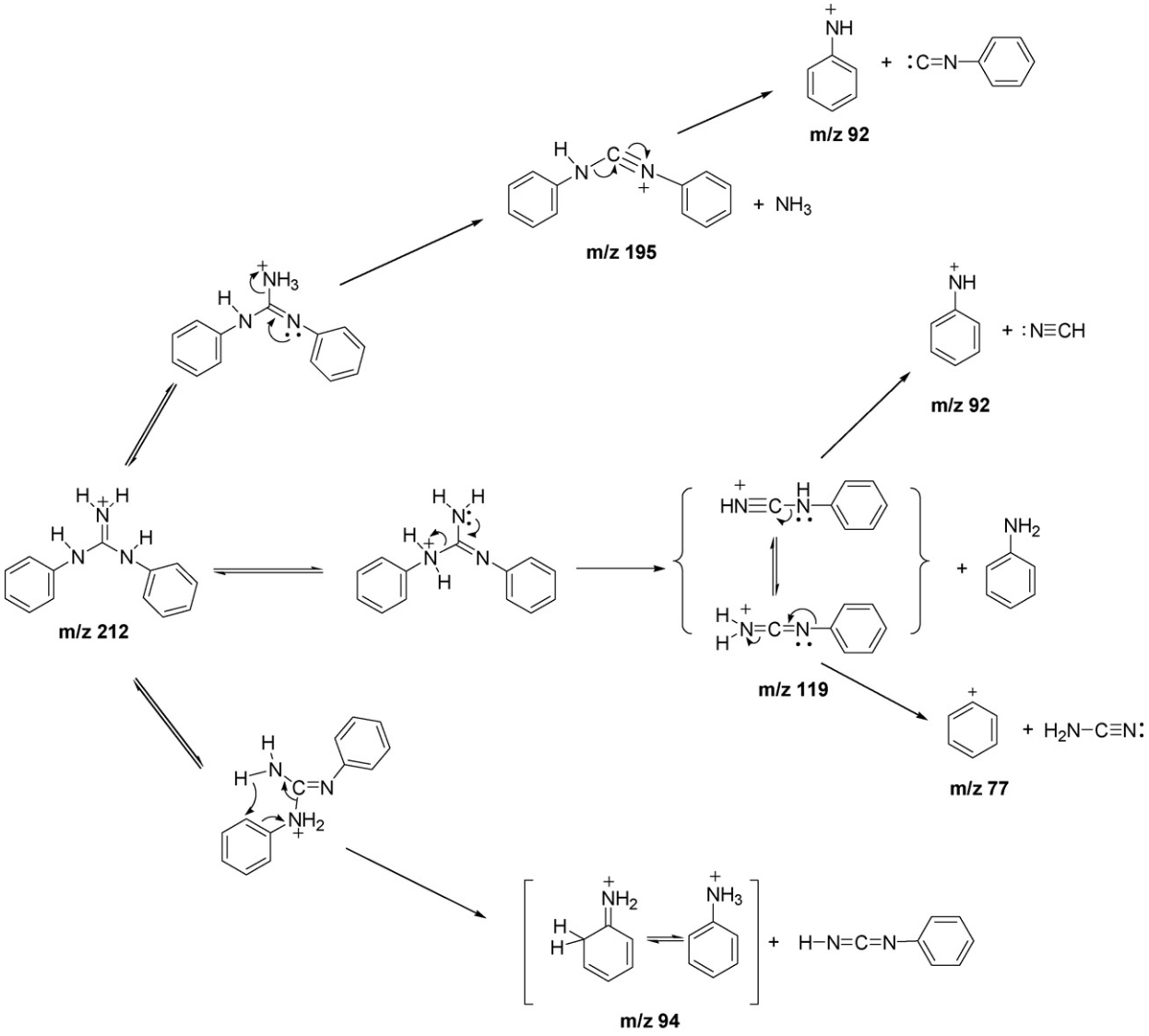


Fig. 6. HPLC Chromatograms of the spiking experiments using the original drug content uniformity test method. Bottom trace—the working solution contained the unknown peak at RRT 0.52. Middle trace—1,3-diphenylguanidine standard solution. Top trace—the working solution spiked with 1,3-diphenylguanidine standard.



Scheme 1. Proposed fragmentation pathways of the protonated 1,3-diphenylguanidine (**9**).

3.6. Proposed fragmentation pathway

The gas phase fragmentation pathway of the protonated 1,3-diphenylguanidine ion is proposed in Scheme 1. Due to the large proton affinity of the guanidine moiety (235.7 kcal/mol [6]), 1,3-diphenylguanidine possesses a good ionization efficiency during the electrospray ionization process, thus showing a strong and clean protonated molecular ion in its LC–MS spectrum. The protonation of the unsubstituted 2-guanidinyl nitrogen can lead to an NH_3 neutral loss, producing *m/z* 195 ion. The *m/z* 195 ion can fragment further at higher collision energies, producing *m/z* 92 ion and a neutral carbene species as MS^3 products. Protonation of either of the substituted 1- and 3-guanidinyl nitrogens followed by the C–N bond cleavage should result in *m/z* 119 ion and a neutral aniline; likewise, the rearrangement of a hydrogen from the unsubstituted 2-guanidinyl nitrogen can lead to a protonated aniline (*m/z* 94).

3.7. Source of the contamination

1,3-Diphenylguanidine (**9**) can be used as a base standard for acid titration and can also be used as a rubber-curing reagent [7]. After ruling out the possibility of using **9** as a titration standard in the laboratory where the original content uniformity was performed, the most likely origin of the contaminant was identified as derived from the safety filler of the pipette bulb used to transfer liquid during sample preparation. The vendor of the pipette bulb confirmed the presence of 1,3-diphenylguanidine (**9**) as a sealant component in the product.

4. Conclusion

This study prescribes a systematic approach which has been demonstrated effective in the successful structure elucidation of a pharmaceutical impurity resulting from an external contam-

ination in laboratory sample preparation using a combination of LC–PDA/UV, LC–MS^n, high-resolution MS measurement, and use of UV profile analysis on a number of structurally related model compounds. In particular, the UV profile analysis has been proven quite informative and complementary to the powerful LC–MS^n technique during the structure elucidation. The overall strategy presented in this paper can be used as a general strategy for identification of any unknown impurities in pharmaceutical analysis, in particular those resulting from sources that are not related to the active ingredients and excipients, e.g., external contaminants and extractable/leachable species.

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