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LC determination of impurities in methoxsalen drug substance: isolation and identification of isopimpinellin as a major impurity by atmospheric pressure chemical ionization LC/MS and NMR

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Abstract

A gradient elution LC method was developed to separate methoxsalen from three of its known impurities: isopimpinellin, bergapten, and ammidin. The method employs a methanol–6%THF (aq) mobile phase, phenyl column, and detection at 254 nm. The gradient LC procedure was applied to seven lots of methoxsalen from five different manufacturers. Six of the seven lots tested contained isopimpinellin as the major impurity at a concentration range of 0.2–2.5%. Identification of the impurity as isopimpinellin was accomplished by a combination of analytical and preparative LC, atmospheric pressure chemical ionization liquid chromatography/mass spectrometry, and NMR. Published by Elsevier B.V.

Keywords: Furanocoumarins; Methoxsalen; Isopimpinellin; Bergapten; Ammidin; Impurities; Liquid chromatography; Atmospheric ionization mass spectrometry; NMR spectroscopy

1. Introduction

In recent years there has been an increased awareness of the importance of setting minimum purity requirements for active pharmaceutical ingredients (APIs). The International Conference on Harmonization (ICH), a joint initiative by federal and private organizations in the United States, Japan and Europe to establish testing procedures for the quality, safety and efficacy of new drug entities, has issued a guideline entitled, 'Impurities in New Drug Substances' [1]. This document states that recurring impurities in new drug substances should be identified and their

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safety determined when they are present at a level of 0.1% (w/w) or greater. The Food and Drug Administration (FDA), a founding member of ICH, has published these guidelines in the Federal Register [2] and issued guidance documents to the pharmaceutical industry based on them [3–5].

The United States Pharmacopeia (USP), which sets quality standards for many drugs already approved and marketed, has introduced new, more stringent, purity requirements in the section entitled, *Other Impurities*, in the *General Notices and Requirements* section of the USP 24 [6]. In this section the USP states that the amount and identity of an impurity that is not detected by the monograph's chromatographic assay or purity test must be labeled in the certificate of analysis, and that the total amount of impurities (monograph detected impurities plus 'other impurities') may not exceed 2.0%. If an unlabeled impurity is present in a substance at greater than the 0.1% level, and it is not detected by the USP test, then that substance is at variance with the USP standard.

The FDA annually tests a wide range of APIs for compliance with USP specifications. The USP methods for many of these drugs consist of a titration procedure for potency and a thin-layer chromatographic (TLC) screening exam for purity or a chromatographic assay and no purity test at all. With the introduction of the 'Other Impurities' requirements in the USP, the FDA has undertaken studies to examine APIs for impurities that would not be detected using the USP tests.

The application of liquid chromatography/mass spectrometry (LC/MS) in conjunction with preparative LC and NMR has been used previously for the identification of impurities in APIs [7–13]. This laboratory has successfully employed these techniques for the isolation and identification of impurities in trimethoprim [14] and hydrochlorothazide [15] drug substances, and in the present report have employed them in the development of an impurity profile of the drug, methoxsalen.

Methoxsalen belongs to a class of compounds known as furanocoumarins, well known photoreactive complexes [16]. This drug is used in combination with ultraviolet light in a process known as PUVA to treat vitilgo, a disease in which skin color is lost [17]. It is also used for antineoplastic effects and for treating certain skin disorders, including alopecia, cutaneous T-cell lymphoma, excema, lichen planus, mycosis fungoides and psoriasis. In addition, a recent report has found that methoxsalen inhibits the enzyme, CYP2A6, which is responsible for the metabolism of nicotine [18]. The drug, when taken in combination with oral nicotine, can reduce the number of cigarettes smoked by about one quarter and decrease overall levels of tobacco smoke exposure by almost half in tobacco dependent individuals.

Numerous studies have indicated that furanocoumarins are carcinogenic [19], and their ability to intercalate into DNA in the presence of long wave UV light accounts for their mutagenicity [20]. Linear furanocoumarins have been shown to exhibit varying levels of phototoxicity, with isopimpinellin having the least photosensitizing activity [21].

The structure of methoxsalen and related furanocoumarins is presented in Fig. 1. Along with methoxsalen; psoralen, bergaptin, ammidin, pimpinellin and isopimpinellin are found in a variety of plant species and a number of methods have been described for analyzing these compounds [22–26]. While gas chromatography has proven to be most effective due to its speed and resolution [27], reverse phase LC methods have been less successful. In one case long analysis times precluded the analysis of large numbers of samples [25], while other LC methods do not achieve baseline resolution of all the compounds of interest, particularly bergapten and isopimpinellin [22–24,26]. Because these compounds are of low polarity, resolution is often difficult to achieve on reverse phase columns in short analysis times.

In this study, a gradient LC method has been developed to separate methoxsalen from its three major impurities. The method was used to examine seven lots of methoxsalen from five different manufacturers, resulting in the detection of a major impurity in six of the seven lots. This impurity was not detected by the USP test. Isolation and identification of this impurity was accomplished with a combination of analytical and preparative LC, atmosphere pressure chemical ionization (APCI) LC/MS and NMR.

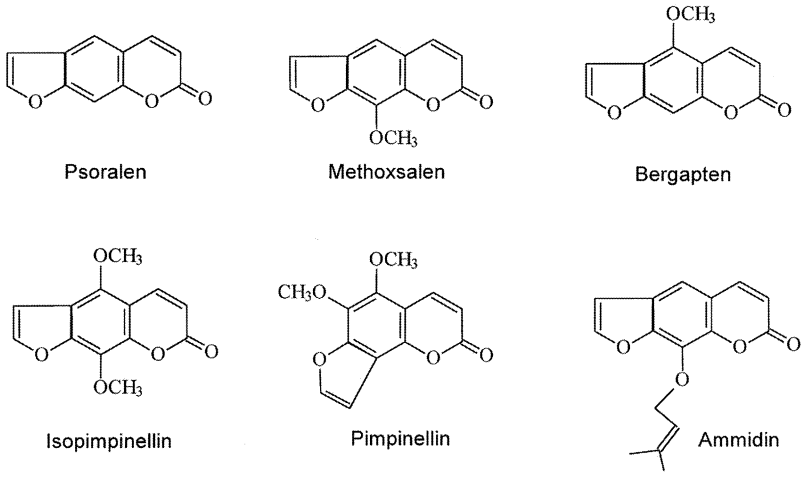

Fig. 1. Structures of related furanocoumarins.

2. Experimental

2.1. Apparatus

2.1.1. Liquid chromatographic systems

(1) *Analytical:* Two Shimadzu model LC-10AS pumps, a SPD-10AV UV–visible detector, a SCL-10A system controller, a SIL-10A auto-injector, a FRC-10A fraction collector, and a CLASS-VP data system (Shimadzu Scientific Instruments, Princeton, NJ) were used. Flow rate, 1.0 ml/min; detector wavelength, 254 nm; detector sensitivity, 4 AU/V; injection volume, 20 μl; column temperature, ambient.

(2) *Preparative:* Two Shimadzu model LC-8A pumps, a SPD-10AV UV–visible detector, a SCL-10A System Controller, a SIL-10A auto-injector, a FRC-10A fraction collector, and a CLASS-VP data system (Shimadzu Scientific Instruments) were used. Flow rate, 25.0 ml/min; detector wavelength, 247 nm; detector sensitivity, 1 AU/V; injection volume, 2000 μl; column temperature, ambient.

2.1.2. Chromatographic columns

(1) *Analytical:* Zorbax-SB-Phenyl (4.6 × 150 mm) 5 μm particle size (Phenomenex UK Ltd, Chesire, UK).

(2) *Preparative:* Beckman Ultrasphere ODS (22.5 × 250 mm), 5 μm particle size (Beckman Instruments, Fullerton, CA).

(3) *LC/MS:* Beckman Ultrasphere ODS (4.6 × 150 mm), 5 μm particle size (Beckman Instruments).

2.1.3. LC/MS system

(1) *HPLC:* Two Shimadzu model LC-10AD pumps, an SPD-10AV UV–visible detector, an SCL-10A system controller, and a SIL-10A auto-injector were used. Operating parameters: flow rate, 1.0 ml/min; detector wavelength, 247 nm; detector sensitivity, 2 AU/V; injection volume, 20 μl; column temperature, ambient.

(2) *Mass spectrometer:* Finnigan LCQ equipped with an Atmospheric Pressure Chemical Ionization (APCI) probe operated in positive ion mode. The operating parameters for on-line LC/MS analyses (1 ml/min) were: capillary temperature 250 °C; vaporizer temperature, 450 °C; capillary voltage, 0 V; tube lens offset, −15 V; discharge current, 5 μA; sheath gas flow rate, 80; aux gas flow rate, 10. Each 25-min analysis was broken up into two segments, with two scan events per segment. Segment 1 (first 15 min): scan event 1, full scan positive ion, 100–500 m/z; scan event 2, MS/MS on parent ion at m/z 217.1 $[M+H]$, isolation width = 2.0 m/z, activation amplitude = 35%, activation $Q = 0.250$, activation time = 30 ms, range = 55–220 m/z, scan time = 3 μs, maximum injection time = 500 ms. Segment 2 (last 10 min): scan event 1, full scan positive ion, 100–500

m/z; scan event 2, MS/MS on parent ion at m/z 247.1 [$M+H$], same parameters as above except scan range = 65–250 m/z.

2.1.4. ^{1}H and ^{13}C NMR spectroscopy

NMR spectra were obtained with a Varian INOVA spectrometer operating at 399.96 MHz (^{1}H) and 100.58 MHz (^{13}C) at 25.0 °C. ^{1}H spectra employed 32 scans; ^{13}C used 35 000.

2.1.5. Rotary evaporator

A Buchler Digital Rotary Evaporator (Cole–Palmer Instrument Co., Vernon Hills, IL) was utilized to evaporate solvent. The condensing fluid, a mixture of ethylene glycol/water (50:50, v/v), was maintained at 0 °C with the aid of a Neslab Endocal circulating bath.

2.1.6. Filters

Nuclepore® Filinert™ (PTFE) membrane filters, 13 mm, 0.45 μm porosity. (Apple Scientific, Chesterland, OH.)

2.2. Reagents

2.2.1. Solvents

LC grade solvents were used for all mobile phases. Acetonitrile and methanol were purchased from Burdick & Jackson (Muskegon, MI), and tetrahydrofuran was purchased from Fisher Scientific (Fair Lawn, NJ).

2.2.2. Standards

Isopimpinellin and ammidin were purchased from Indofine Chemical Company (Belle Mead, NJ). Bergapten was purchased from ICN Biomedicals, Inc. (Aurora, OH).

2.2.3. Mobile phase

(1) *Analytical:* Solvent A, 6% THF (aq); solvent B, methanol. Gradient for analytical LC analysis: 0 min, 44% B; 21 min, 44% B; 40 min, 65% B; 45 min, 44% B; 50 min, 44% B.

(2) *Preparative:* Solvent A, H_2O; solvent B, CH_3CN. Gradient for preparative LC: 0 min, 25% B; 49 min, 25% B; 60 min, 50% B; 65 min, 25% B.

(3) *LC/MS:* Solvent A, H_2O; solvent B, CH_3CN. Gradient for LC/MS: 0 min, 15% B; 10 min, 15% B; 15 min, 30% B; 25 min, 30% B; 30 min, 40% B; 40 min, 40% B; 40.1 min, 15% B.

2.3. Samples

Samples from Manufacturers B and D were labeled 'Methoxsalen' and were obtained from pharmaceutical companies through the FDAs Drug Product Surveillance Program. Methoxsalen samples labeled as A, C, and E were purchased from Acros Organics (Fairlawn, NJ), Aldrich Chemical Co. (Milwaukee, WI), and Alfa Aesar (Ward Hill, MA), respectively, and were labeled as 99%. Samples were recently synthesized and were within their expiration dates at the time of testing. Samples were stored at ambient conditions and were not dried prior to testing.

2.3.1. Sample preparation: analytical LC, LC/MS, preparative LC, and NMR

(1) *Analytical LC and LC/MS:* 1 mg/ml. Approximately 10 mg of sample was accurately weighed and dissolved into 10 ml of acetonitrile–water (50:50, v/v), and 20 μl was analyzed using the LC method.

(2) *Preparative LC:* 25 mg/ml. Approximately 250 mg of sample from Manufacturer B was dissolved into 10 ml of acetonitrile–water (80:20, v/v), filtered through a 0.45 μm filter, and 2000 μl was analyzed using the LC method. The unknown impurity was isolated by fraction collection at approximately 50–53 min. The mobile phase was removed by rotary evaporation at 40 °C, in vacuum (<20 mmHg). Isolates were dried in vacuum (<20 mmHg) at 60 °C for 3 h.

(3) *NMR:* ca. 3 mg/ml. A total of 20 isolates were combined and dissolved in 0.75 ml of $CDCl_3$ (99.99 at.%, Aldrich Chemical Co.) and placed in a thin-walled 5 mm NMR tube (Wilmad Glass Company).

3. Results and discussion

The chromatogram of a standard mixture of methoxsalen, isopimpinellin, bergapten, and ammidin, at 10 μg/ml concentration, is presented in Fig. 2. An efficient separation of the four furano-

coumarins was achieved using a methanol–6%THF (aq) linear gradient (44%→65% MeOH) on a Zorbax-SB-Phenyl column (4.6 mm × 15 cm), with a flow rate of 1 ml/min. The main obstacle in chromatographing these compounds was achieving the difficult separation of isopimpinellin and bergapten while simultaneously ensuring that ammidin would elute within a reasonable analysis time. Higher percentages of methanol or the use of a step gradient allowed for the earlier elution of ammidin but had a detrimental effect on the separation of isopimpinellin and bergapten. The final gradient ensured that these two compounds were well separated, while allowing for the elution of ammidin within a 35-min analysis window. A phenyl column was chosen because it afforded improved peak shapes and resolution over other reverse phase packings that were tested.

The gradient system was then used to screen methoxsalen from different manufacturers for the presence of the above impurities. Typical LC profiles of these materials are presented in Fig. 3. Two lots of methoxsalen drug substance were collected from each of two pharmaceutical companies, Manufacturers B and D, and tested using the USP TLC method [28] for chromatographic purity. The TLC method did not detect an impurity in any of the four lots from the two manufacturers. When these samples were reexamined using the gradient LC method, all four lots were found to contain significant amounts of one impurity (Manufacturer B: 0.7 and 0.3%; Manufacturer D: 0.2 and 0.2%), but were still below the USP 1.0% limit. The retention time of the impurity (14.9 min) matched the retention time of isopimpinellin in the standard chromatogram (Fig. 2). Additional methoxsalen was purchased from Manufacturers A, C, and E in order to compare impurity profiles from different sources. All the manufacturers, with the exception of Manufac-

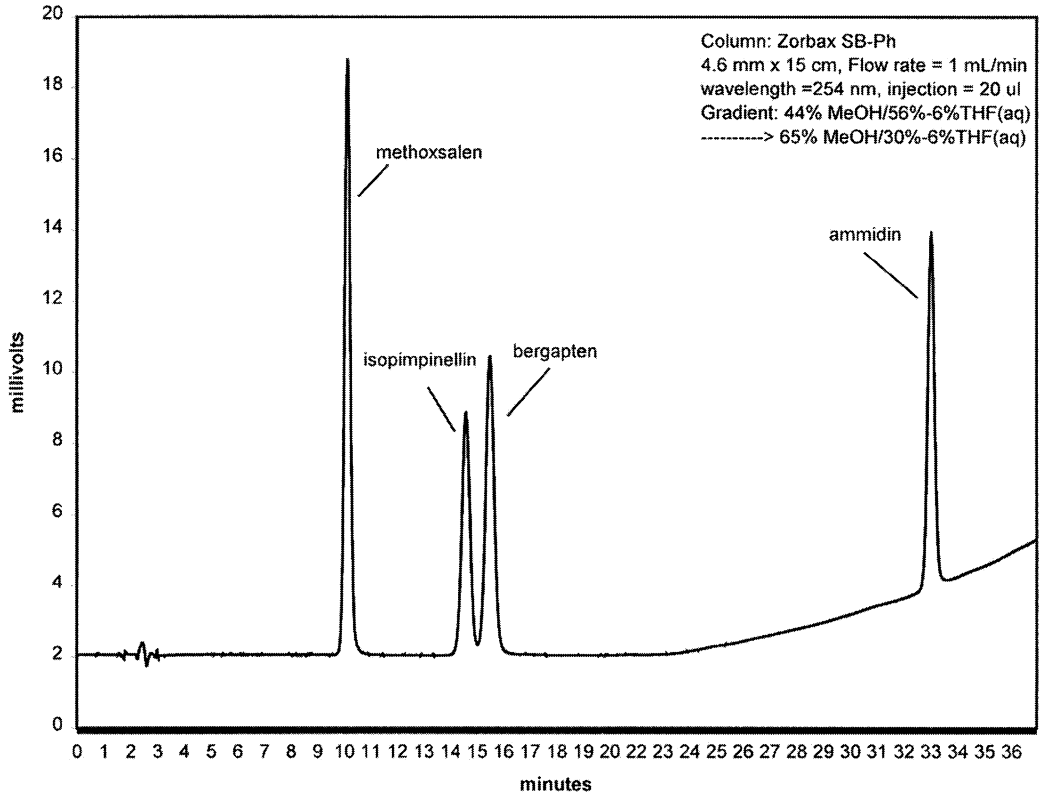

Fig. 2. LC chromatogram of a standard mixture of furanocoumarins at 10 μg/ml.

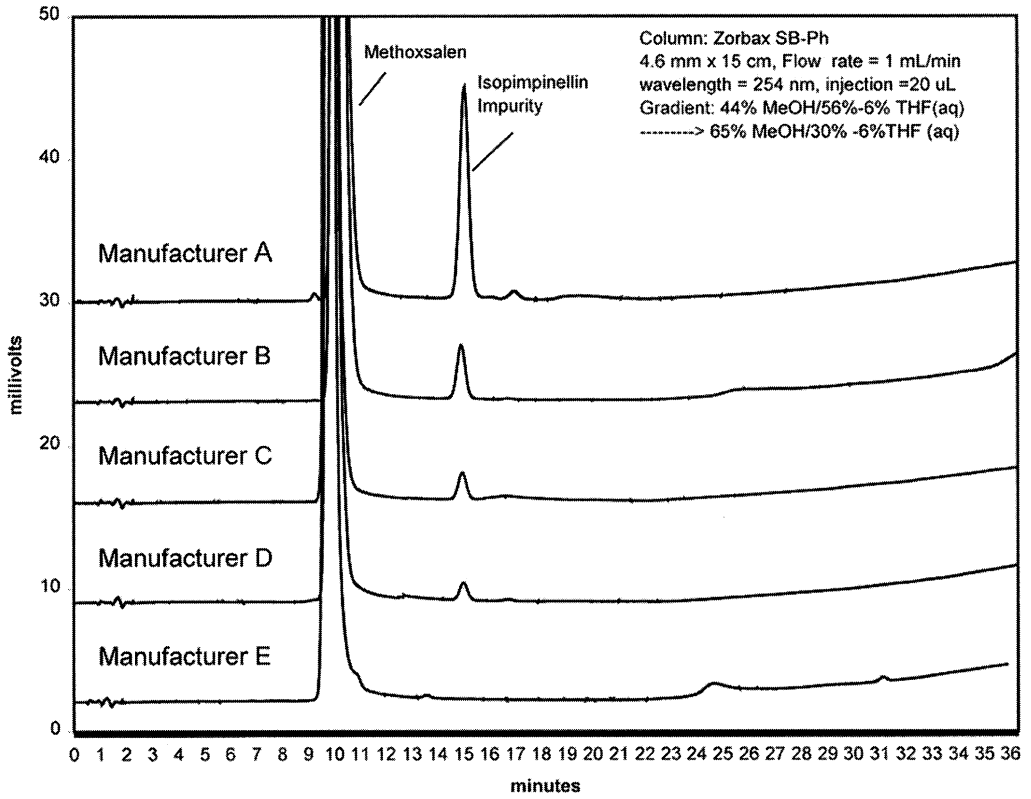

Fig. 3. LC profiles of methoxsalen (1 mg/ml) from five different manufacturers.

turer E, were found to contain the same impurity in significant concentration, with Manufacturer A containing 2.5%. A summary of the impurity level found in all the lots examined is presented in Table 1.

3.1. LC/MS and LC/MS/MS of methoxsalen, impurity, isopimpinellin

The on-line positive ion APCI LC/MS and LC/MS/MS data for methoxsalen, its impurity, and a reference standard of isopimpinellin are shown in Figs. 4–6.

Methoxsalen (MW = 216) eluted at 12.0 min and its mass spectrum (Fig. 4a) exhibited a protonated molecular ion $[M+H]^+$ at m/z 217 and a very weak fragment ion at m/z 202, $[(M+H)–CH_3]$. The LC/MS/MS spectra of methoxsalen (Fig. 4b) produced a major product ion at m/z 202, $[(M+H)–CH_3]$ plus additional ions at m/z 189, 185, 173, and 161.

Table 1
Area percent isopimpinellin impurity found in methoxsalen from different sources.

Manufacturer	% Isopimpinellin
A	2.5
B (Lot 1)	0.7
B (Lot 2)	0.3
C	0.3
D (Lot 1)	0.2
D (Lot 2)	0.2
E	0.0

The impurity eluted at 17.2 min and its mass spectrum (Fig. 5a) exhibited a protonated molecular ion $[M+H]$ at m/z 247 (MW = 246) and fragment ions at m/z 232 and m/z 217. The LC/

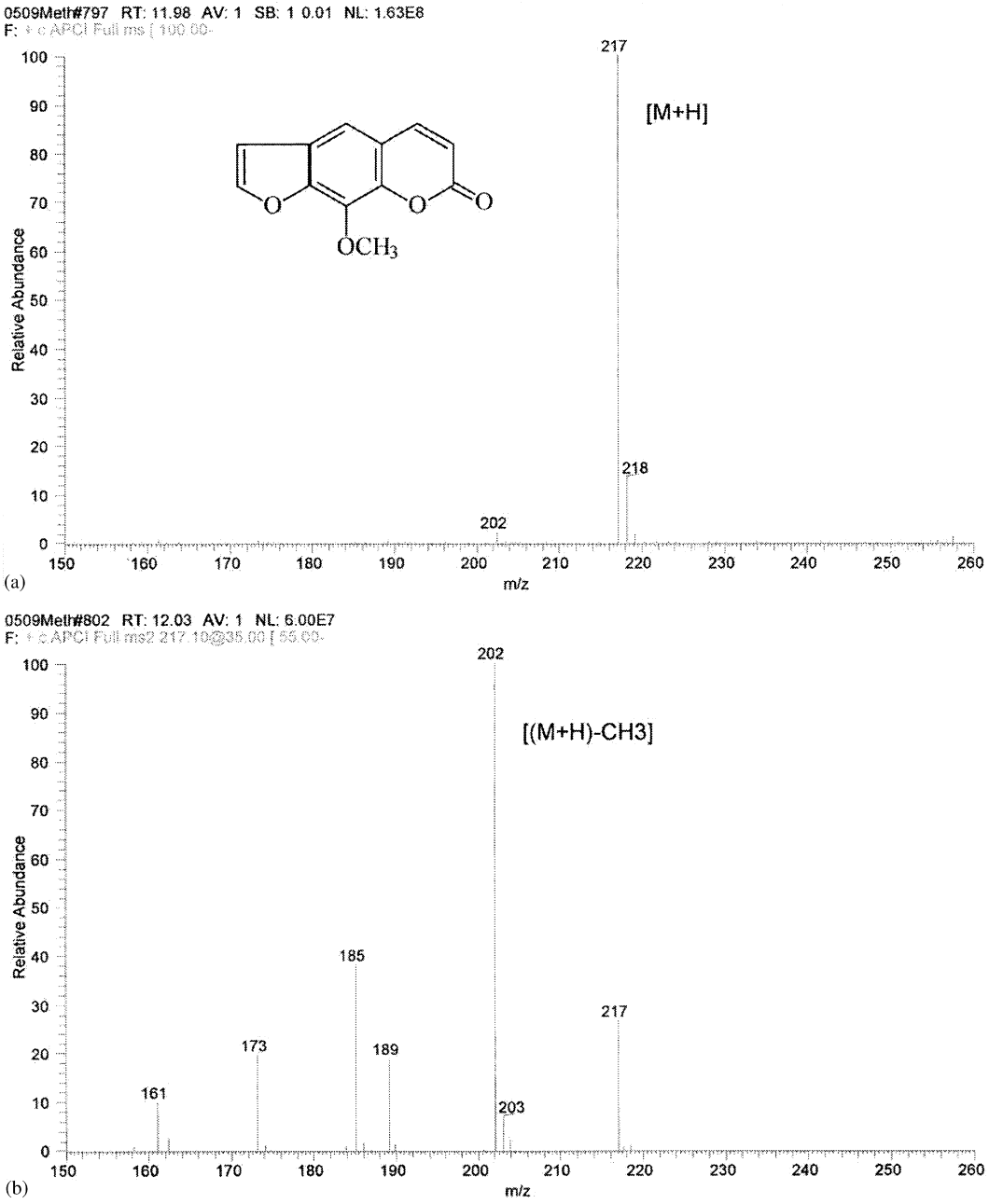

Fig. 4. APCI mass spectra of methoxsalen: (a) LC/MS and (b) LC/MS/MS.

MS/MS spectrum of the impurity (Fig. 5b) produced one major product ion at *m/z* 232, and a very minor product ion at *m/z* 217.

The retention time for isopimpinellin reference standard (MW = 246) was 17.2 min, its mass spectra (Fig. 6a) exhibited a protonated molecular ion $[M+H]$, at *m/z* 247 and fragment ions at *m/z* 232, $[(M+H)–CH_3]$ and 217, $[(M+H)–OCH_3+H]$. The LC/MS/MS spectrum of isopimpinellin reference standard (Fig. 6b) produced one major

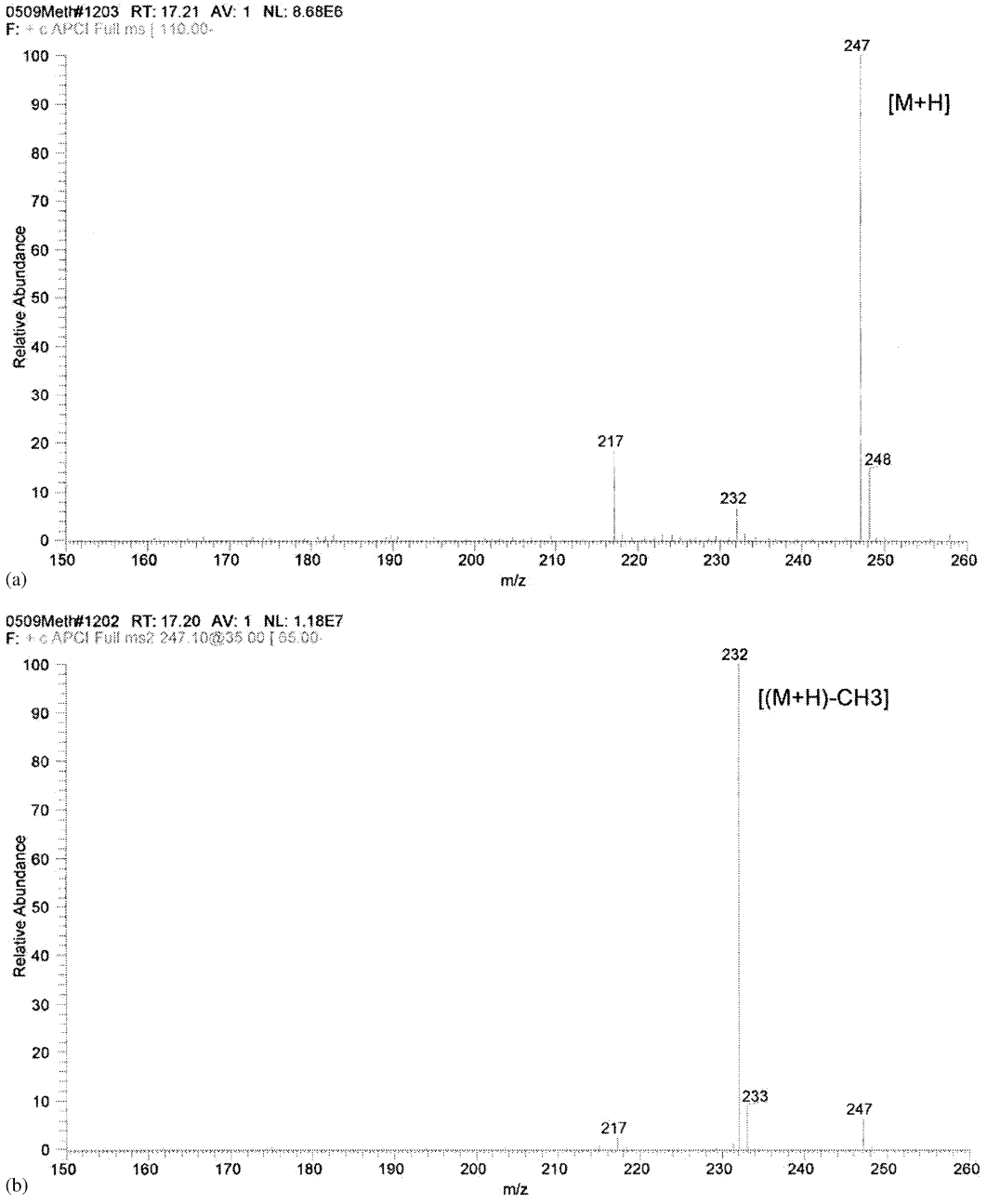

Fig. 5. APCI mass spectra of methoxsalen impurity: (a) LC/MS and (b) LC/MS/MS.

product ion at m/z 232, $[(M+H)-CH_3]$, and a weak product ion at m/z 217, $[(M+H)-OCH_3+H]$.

Both the retention time and the mass spectral data of the impurity agreed with that of the isopimpinellin reference standard.

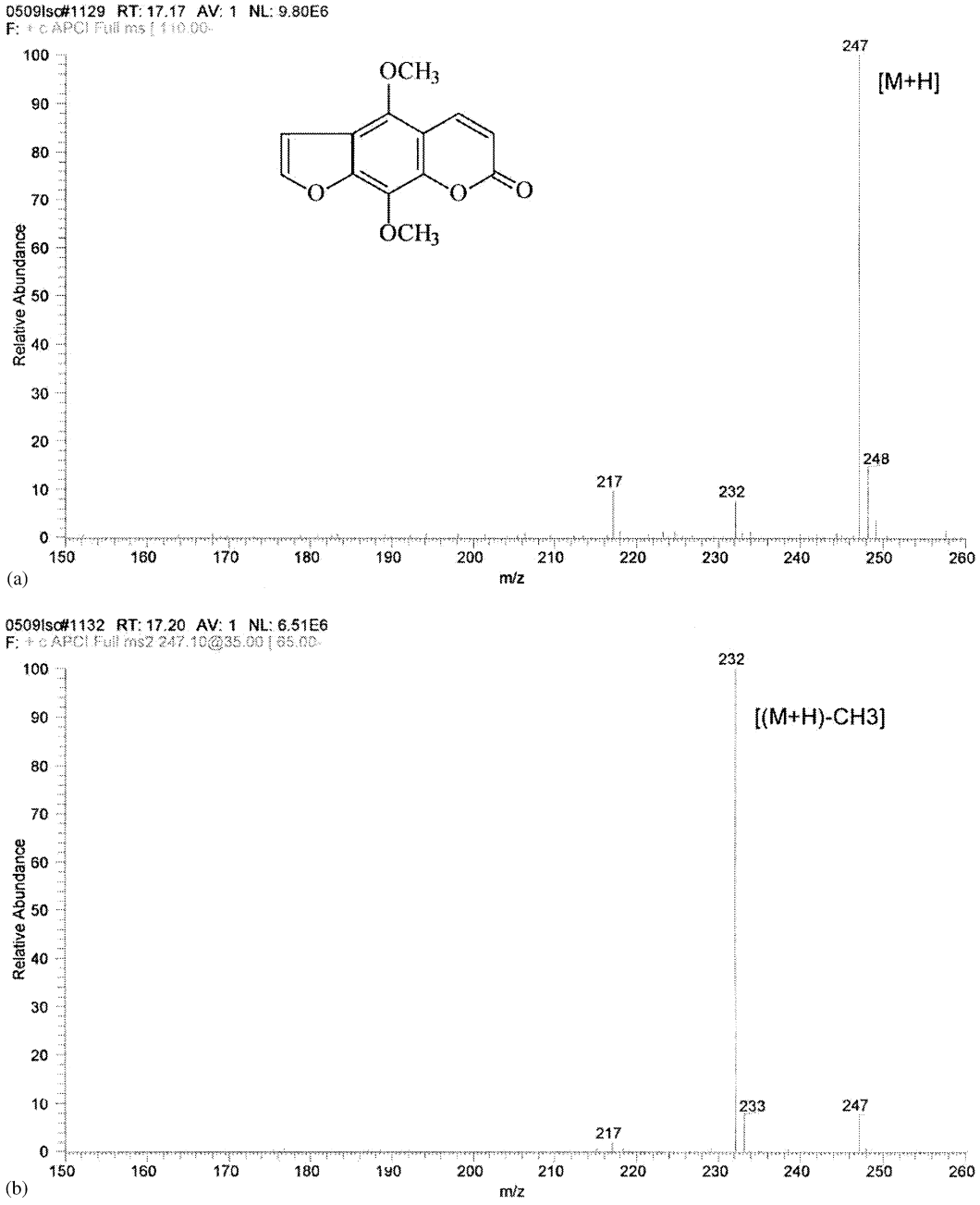

Fig. 6. APCI mass spectra of isopimpinellin: (a) LC/MS and (b) LC/MS/MS.

3.2. Isolation of the impurity and NMR analysis

The LC and mass spectral data suggests that isopimpinellin is the impurity present in six of the seven lots of methoxsalen that were tested. However, the lack of a pimpinellin standard precluded absolute assignment. Therefore, the impurity was isolated by preparative chromatography in order

to perform NMR spectroscopy for unequivocal identification.

Isolation of the impurity was accomplished by preparative LC on a Beckman Ultrasphere column (column dimensions: 250 × 22.5 mm, flow rate: 25 ml/min). The impurity fraction was collected and the solvents eliminated by rotary evaporation at 40 °C. A total of 20 isolates were combined and dried at 60 °C for 3 h.

The ^{1}H NMR spectra of the authentic isopimpinellin (referenced to TMS at 0.00 ppm): δ 8.124 (d, 1H), δ 7.630 (d, 1H), δ 7.003 (d, 1H), δ 6.289 (d, 1H), δ 4.174 (s, 3H), and δ 4.167 (s, 3H) and that of the isolated impurity (Fig. 7) were virtually identical with respect to chemical shifts, coupling constants, and integration. A notable feature of these spectra is the near coincidence (δ 4.174 and 4.167) of the methoxyl proton singlets (3H each). The remainder of the spectrum consisted of two pairs of coupled doublets (1H each): δ 8.124 and 6.289, $J = 10.0$ Hz (6 member ring protons), and δ 7.630 and 7.003, $J = 2.4$ Hz (five member ring protons). Similarly, the ^{13}C NMR chemical shifts of isopimpinellin (referenced to $CDCl_3$ at δ 77.297): δ 160.648 (s), δ 150.219 (s), δ 145.321 (s), δ 139.568 (s), δ 128.210 (s), δ 115.032 (s), δ 113.102 (s), δ 107.876 (s), δ 105.267 (s), δ 61.916 (s), and δ 61.047 (s) were identical to the isolated impurity. Both showed the anticipated 13 carbon signals between δ 61.0 and 160.6 with exactly the same chemical shifts. The complete coincidence of both the ^{1}H and ^{13}C spectra firmly establishes the impurity as isopimpinellin.

4. Conclusions

In this study a gradient LC method was developed to separate methoxsalen from three of its known impurities: isopimpinellin, bergapten, and ammidin. The method achieves baseline resolution of all the compounds of interest in less than 35 min, and affords better peak shapes and resolution over other methods described in the literature. The

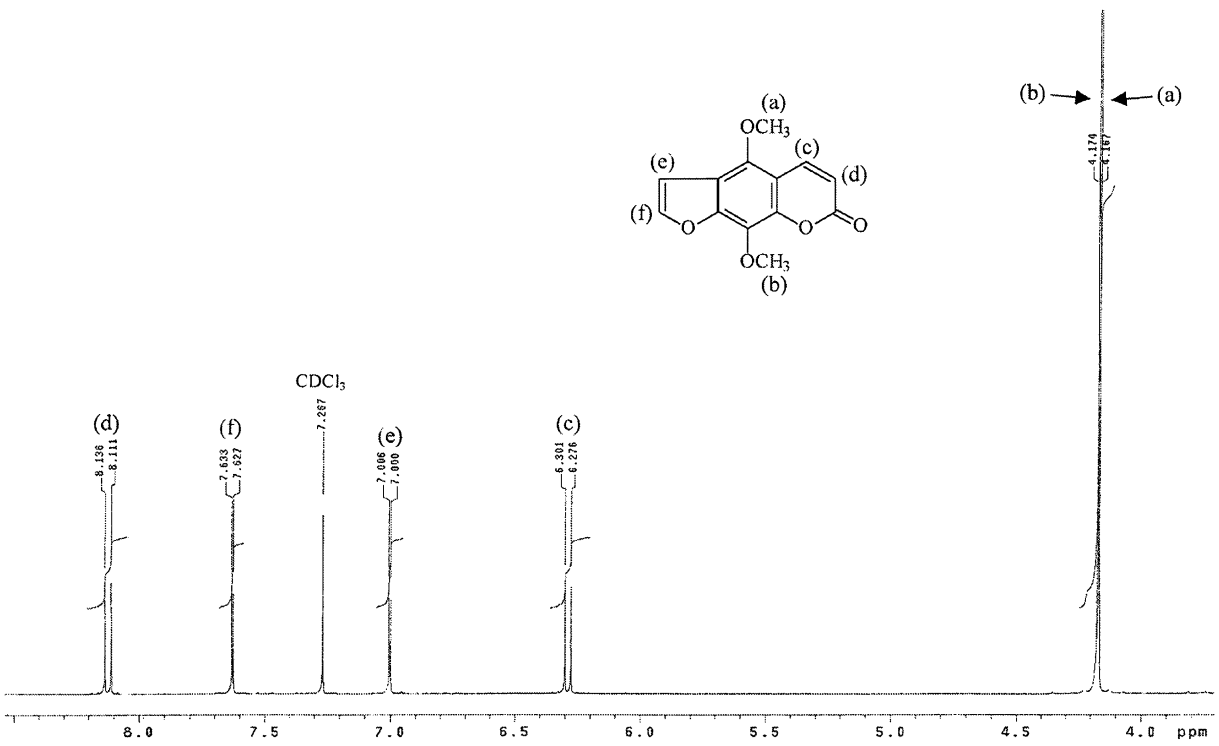

Fig. 7. ^{1}H NMR spectrum of methoxsalen impurity.

gradient HPLC procedure was applied to seven lots of the drug substance from five different manufacturers. Six of the seven lots tested contained isopimpinellin as the major impurity at a concentration range of 0.2–2.5%. Identification of the impurity as isopimpinellin was accomplished by a combination of analytical and preparative LC, APCI LC/MS, and NMR. This impurity was not detected by the USP chromatographic purity test in any of the lots tested due to co-elution with the parent compound.

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