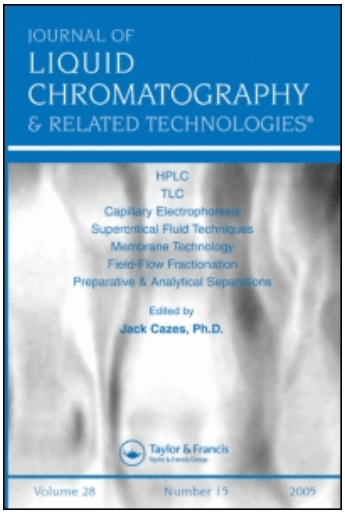


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Development and Validation of an LC Assay for Benzo[*f*]quinoline-5,6-dione and Identification of its Impurities by LC-MS

Ghulam A. Shabir[a]
[a] Abbott Diabetes Care, Abbott Laboratories, Witney, Oxfordshire, U.K.

To cite this Article Shabir, Ghulam A.(2008) 'Development and Validation of an LC Assay for Benzo[*f*]quinoline-5,6-dione and Identification of its Impurities by LC-MS', Journal of Liquid Chromatography & Related Technologies, 31: 3, 428 — 442
To link to this Article: DOI: 10.1080/10826070701780839
URL: http://dx.doi.org/10.1080/10826070701780839

Journal of Liquid Chromatography & Related Technologies®, 31: 428–442, 2008

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701780839

Development and Validation of an LC Assay for Benzo[*f*]quinoline-5,6-dione and Identification of its Impurities by LC-MS

Ghulam A. Shabir

Abbott Diabetes Care, Abbott Laboratories, Witney, Oxfordshire, U.K.

Abstract: A new analytical method based on reversed-phase high performanc liquid chromatography (HPLC) was developed and validated for the assay of benzo[*f*]quinoline-5,6-dione and the determination of its synthetic impurities by employing the method in liquid chromatography-mass spectrometry (LC-MS) with electrospray ionization (ESI) and photodiode array (PDA) UV detection. Separation was performed on a Waters Xterra C_{18} (150 mm × 4.6 mm, 5 μm) column. UV detection was performed at 262 nm. The results showed that benzo[*f*]quinoline-5,6-dione is eluted as a spectrally pure peak resolved from its impurities. 1*H*-indeno[2,1-*b*]pyridine-2,9-dion and benzo[*f*]quinoline are identified as the main impurities. The proposed method was extensively validated and satisfactory results were obtained in terms of linearity ($r^2 = 0.9997$) and precision (RSD < 0.61%) in all cases. The limits of detection and quantification were 2 μg/mL and 20 μg/mL, with a 0.88% RSD, respectively.

Keywords: Benzo[*f*]quinoline-5,6-dione, HPLC, LC-MS, Method development, Method validation, Synthetic impurities

INTRODUCTION

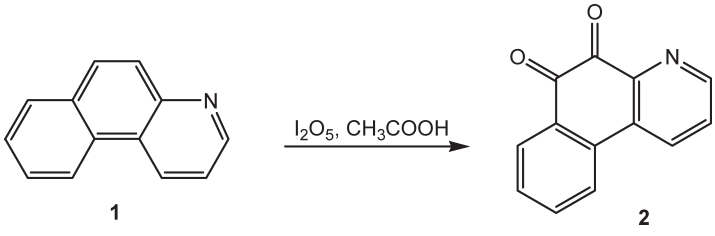
Benzo[*f*]quinoline-5,6-dione **2** (4-azaphenanthrene-5,6-dione), is an important heterocyclic o-quinone, which is known historically to be active against protozoa, amoebae, and bacteria.[1] Benzo[*f*]quinoline **1** and benzo[*f*]quinoline-5,6-dione **2** are starting materials for quinoline legends, which are useful for the preparation of complexes of transition metals.

Correspondence: Ghulam A. Shabir, Abbott Diabetes Care, Abbott Laboratories, Witney, Oxfordshire OX29 0YL, U.K.. E-mail: ghulam.shabir@abbott.com

Benzo[*f*]quinoline-5,6-dione was synthesized from benzo[*f*]quinoline by oxidation with iodine pentoxide (I_2O_5) in acetic acid (Scheme 1). A number of potential impurities were expected from an examination of the literature[2] concerning the reactivity of the benzo[*f*]quinoline-5,6-dione (Figure 1).

The purpose of this study was to develop a reversed-phase high performance liquid chromatography (HPLC) assay method for benzo[*f*]quinoline-5,6-dione to asses its purity, while also applying HPLC-electrospray ionization (ESI)-mass spectrometry (MS) to the identification of any impurities. Impurity profiling is a major issue in pharmaceutical and biomedical analysis, particularly during product development phase and quality control.

The standard requirements of such an impurity method[3] are that all likely synthetic and degradative impurities are resolved from each other and the main drug, and[4] that the impurities can be monitored at the 0.1% (w/w) level or below. A guideline for controlling impurity levels in drug substances has been developed.[5] Normally, synthetic impurities are discovered during routine HPLC analysis of the drug substances.[6] An impurity profile of a synthetic drug may require the use of complementary chromatographic methods such as HPLC/diode array UV and LC-MS to permit the observation of non-UV absorbing synthetic impurities. The combination of MS and PDA detection provides a powerful tool for controlling the quality in drug synthesis and for the identification of impurities. The PDA detector has the capability to acquire and store a great amount of spectral data from the UV absorbing compounds in chromatograms, thereby, making possible both spectral identification and individual analysis of the peak homogeneity/purity of each chromatographic peak. ESI, an atmospheric ionization technique, was used to generate gas phase ions by spraying analyte solution at high voltage. ESI has become one of the most important and powerful ionization techniques for MS, because of its effectiveness in detecting large biomolecules[7] and ease of use for interfacing liquid based separation techniques, such as LC[8,9] and capillary electrophoresis.[10–12] According to the literature search, this is the first report on HPLC based separations and identifications of impurities of this benzo[*f*]quinoline-5,6-dione compound. Herein, my effort in developing and validating an HPLC method for this important novel compound and my further studies of its impurity profile by LC-MS are described. The aim of the



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Scheme 1. Synthesis of benzo[*f*]quinoline-5,6-dione.

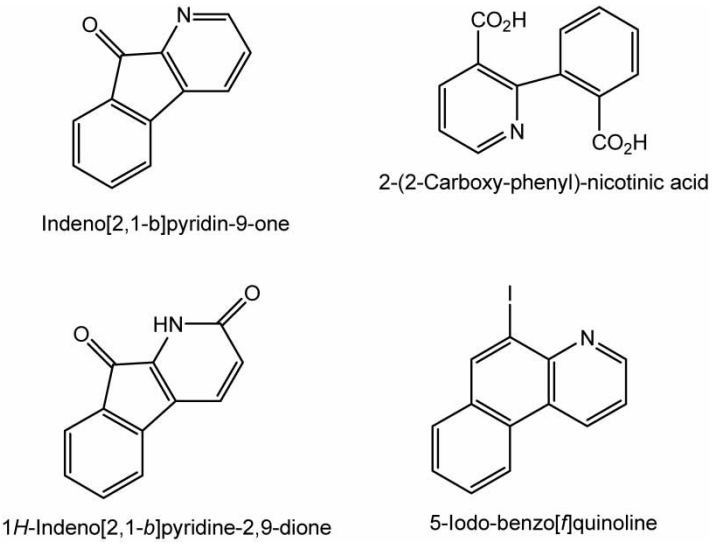

Figure 1. Structures of potential impurities in benzo[*f*]quinoline-5,6-dione.

validation work is to prove that the research data/results obtained in the analysis of benzo[*f*]quinoline-5,6-dione and its synthetic impurities are indubitable. This involves demonstration of aspects such as linearity, range, accuracy, precision, specificity, sensitivity, limit of detection, and limit of quantitation. The results obtained in these evaluations show that the method could be used reliably in routine analysis of benzo[*f*]quinoline-5,6-dione.

EXPERIMENTAL

Chemicals and Reagents

Acetonitrile (HPLC-grade) was obtained from Merck (Darmstadt, Germany). Ammonium formate (formic acid ammonium salt, $HCOONH_4$) was purchased from Sigma chemicals (St. Louis, MO, USA). Benzo[*f*]quinoline-5,6-dione was prepared by Charnwood Molecular (Loughborough, UK). Benzo[*f*] quinoline-5,6-dione standard was characterised in house (>92%, pure). Deionised distilled water was used throughout the experiment.

HPLC Conditions

A Waters 2695 Alliance Separations Module equipped with a 996 photodiode array detector (PDA) (Waters, Milford, MA, USA) was used. Separation was

achieved on a 150 mm × 4.6 mm, 5 μm particle size Waters Xterra C_{18} column. The mobile phase consisted of 15 mM ammonium formate (pH 3) as solvent A and acetonitrile as solvent B, which were applied in the following gradient elution: initial 0–12 min, linear change from A-B (95:5, v/v) to A-B (65:35, v/v); 12–16 min, linear change to A-B (50:50, v/v); 16–25 min, linear change to A-B (40:60, v/v). The flow rate was 0.8 mL/min and sample injection volume was 10 μL. The temperature was held constant at 32°C. Ultraviolet (UV) spectra were recorded in the 210–400 nm range and the chromatograms were extracted at 262 nm. The data was collected and analyzed by Waters Empower software.

LC-MS Conditions

LC-MS analysis was performed using a Waters Micromass ZQ 2000 single quadrupole mass spectrometer, equipped with a Waters Alliance 2695 Separations Module and 996 PDA (Waters, Milford, MA, USA). The same LC conditions were used in LC-MS analysis as described above in the HPLC conditions section. The sample was ionized using ESI in the positive ionization mode under the following source conditions: source temperature, 150°C; capillary potential, 3.0 Kv; sampling cone potential, 30 v; cone gas flow, 60 L/h; extractor, 3.0 v; RF lens, 0.5 v; desolvation temperature, 350°C; low mass resolution 15; high mass resolution 15; ion energy, 0.3 v; and multiplier 430 v. Mass spectra were obtained over the scan ranges, 2–800 da, at a rate of 0.5 scan per second. A wavelength range 210 to 400 nm was used for the PDA detector. In ESI, a high electrical voltage charges the eluent as it emerges from a nebulizer, producing an aerosol of charged droplets. As the solvent evaporates, the droplets shrink, developing a charge dense enough to eject ions from their surfaces (ion evaporation). The mass analyzer then sorts the singly or multiply charged ions by mass to charge (m/z) ratio.

Sample and Standard Preparation

All sample and standard solutions at 0.20 mg/mL were prepared by dissolving approximately 20 mg of benzo[*f*]quinoline-5,6-dione in 100 mL of acetonitrile.

Calculations and other HPLC Determinations

Related Substances

The quantity of each known impurity peak, plus any other impurity peaks, was calculated as an area percent versus the total area of all peaks in the chromatogram.

Purity

The purity of the benzo[*f*]quinoline-5,6-dione drug substance is calculated in relation to the reference standard using the area of the main benzo[*f*]quinoline-5,6-dione peak at 9.187 min Eq. (1).

$$\text{Peak purity} = \frac{\begin{array}{c}\text{mg/100 mL of reference standard} \\ \times \text{ total area of main analyte from sample}\end{array}}{\begin{array}{c}\text{Area of main analyte from reference standard} \\ \times \text{ mg/100 mL of sample}\end{array}} \quad (1)$$

RESULTS AND DISCUSSION

HPLC Analysis

Prior to the coupling with the mass spectrometer, an optimization of the liquid chromatographic separation was carried out using a photodiode array UV detector. Some important considerations have to be taken into account when a HPLC method is developed before it's coupling with mass spectrometry detection. For example, a volatile buffer of low conductivity (i.e., eclectic current below 50 μA) is required to avoid plugging of the dielectric capillary between the spray chamber and mass spectrometer, as well as to obtain a stable electrospray. Ammonium formate and ammonium acetate buffers were studied, selecting the first one because better peak shapes were obtained with the addition of acetonitrile. With this buffer, a systematic study of the pH effect was carried out between pH 2 and 4; finally, pH 3 was chosen because at this value the best resolution between the main component and its impurities occurred. The optimized buffer concentration was 15 mM, as it was found to provide a good compromise among peak shape, and analysis time.

Two analytical columns were tried in order to reach acceptable specificity and selectivity. I first exploited RP-8 columns, but the analyte was retained on these columns. The shift to RP-18 columns, among which Xterra column proved to be superior to others, exhibited better separation with shorter retention time (Figure 2), probably due to its polymeric octadecylsilane of less silanol sites with a wide pH range. The temperature of the analytical column was set to 32°C, because lower as well as higher temperatures worsened the resolution among main component and its impurities. The choice of wavelength is essential to accomplish a sensitive and a selective chromatographic assay. The optimal wavelength for benzo[*f*]quinoline-5,6-dione detection was established using two UV absorbance scans over the range of 190 to 400 nm, one scan of the mobile phase, and the second of the analyte in the mobile phase. It was shown that 262 nm were the optimal wavelength to maximize the signal.

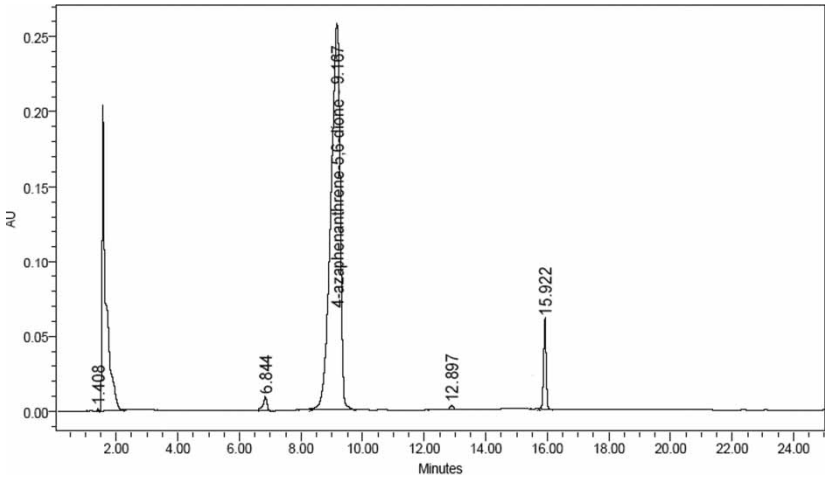

Figure 2. HPLC chromatogram obtained for benzo[*f*]quinoline-5,6-dione.

To evaluate the quantitative nature of the analytical method, a series of samples with different amounts of benzo[*f*]quinoline-5,6-dione were run to investigate the best assay concentration. Using a C_{18} column, the best concentration was assessed by injecting six reference standard of benzo[*f*]quinoline-5,6-dione in the range of 0.002 to 1.60 mg/mL. The integrated peak areas were plotted versus amount injected. The calibration curve was found to be linear from concentration range 0.02 to 1.40 mg/mL with a correlation coefficient of 0.9997. On the bases of these data, the best concentration (0.20 mg/mL) was chosen as a working concentration for the assay.

System suitability testing was performed to determine the accuracy and precision of the system from six replicate injections of a solution containing 0.20 mg benzo[*f*]quinoline-5,6-dione/mL. The relative standard deviation (RSD) of the retention time (min) and peak area were found to be less than 0.25%. The retention factor (also called capacity factor, k) was calculated using the equation $k = (t_r/t_0) - 1$, where t_r is the retention time of the analyte and t_0 is the retention time of an unretained compound; in this study, t_0 was calculated from the first disturbance of the baseline after injection and capacity factor value obtained was 9.03 for the benzo[*f*]quinoline-5,6-dione peak. The separation factor (α) was calculated using the equation, $\alpha = k_2/k_1$ where k_1 and k_2 are the retention factors for the first and last eluted peaks, respectively. The separation factor for the benzo[*f*]quinoline-5,6-dione peak obtained was 2.14. The plate number (also known as column efficiency, N) was calculated as $N = 5.54\ (t_r/w_{0.5})^2$ where $w_{0.5}$ is the peak width at half peak height. In this study, the theoretical plate number was 2826. Resolution is calculated from the equation $R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$. Where t_1 and t_2 are retention times of the first and second eluted

peaks, respectively, and t_{w1} and t_{w2} are the peak widths. The resolution for the benzo[*f*]quinoline-5,6-dione peak was >2.0.

For the determination of method robustness within a laboratory, a number of chromatographic parameters were determined which included flow rate, temperature, mobile phase composition, and columns from different lots. In all cases, good separations were always achieved, indicating that the method remained selective for all components under the tested conditions.

LC-MS Analysis

LC-MS experiments were performed to identify the chemical structure of impurities using the conditions described in the LC-MS conditions section. In my experience, the HPLC flow rate had to be decreased to 0.8 mL/min. However, with this low flow rate, while keeping same solvent system and gradient as those for HPLC-PDA method, the retention time will be slightly longer than HPLC-PDA. The full scan ESI-MS spectra of benzo[*f*]quinoline-5,6-dione and its impurities were measured in the mass range m/z 2-800 da. The benzo[*f*]quinoline-5,6-dione displayed a single peak at 9.187 min, which corresponds to the molecular mass at m/z 210.05 (Figure 3) and an impurity peak (12.897 min) at m/z 198.02 (Figure 4) and impurity peak (15.922) at m/z 180.08 (Figure 5). Both these impurity peaks at 12.897 and 15.922 min were identified by both LC and MS data with $[M + H]^+$ at m/z 197.0, as 1*H*-indeno[2,1-*b*]pyridine-2,9-dione and $[M + H]^+$ at m/z 179.0, as benzo[*f*]quinoline (Table 1). Figure 6 shows MS and PDA spectra for the impurity peak at 6.844 min; it remained unidentified.

Validation of the HPLC Method

The new developed analytical method was critically validated to assess the validity of the research as a means of determining whether the method used during the study could be trusted to provide a genuine account of the intervention being evaluated. As a best practice,[13–15] the method was validated in terms of linearity, precision (repeatability and intermediate precision), specificity, accuracy (recovery), limit of detection, and limit of quantification.

Linear Concentration Range

The linearity range of peak area response versus concentration for benzo[*f*]-quinoline-5,6-dione was studied from approximately 0.02 to 1.40 mg/mL. Six solutions were prepared corresponding to 20, 40, 60, 80, 100, and 140% of the nominal analytical concentration (0.20 mg/mL) and each one was injected in triplicate. The correlation coefficient, r^2, was 0.9997, slope 146444 and intercept was 167799 (Table 2).

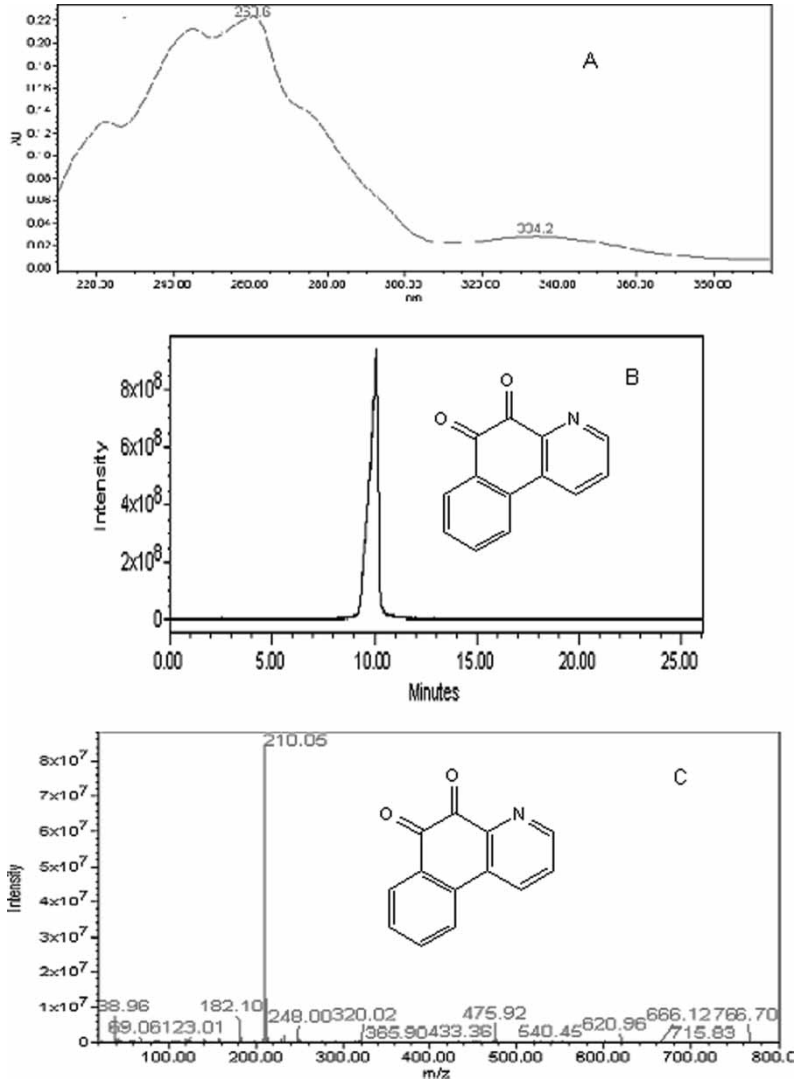

Figure 3. (A) PDA UV match spectra of the middle of the peak corresponding to the RT (9.187 min) of the main component of benzo[*f*]quinoline-5,6-dione and a reference sample; (B) HPLC-ESI-MS chromatogram; (C) mass spectrum (x-axis: relative abundance) obtained for benzo[*f*]quinoline-5,6-dione.

Precision (Repeatability and Intermediate Precision)

The precision of the analytical method was evaluated in terms of repeatability (intra-day precision) and intermediate (inter-day) precision. Repeatability was assessed injecting six replicate injections at 100% test concentration

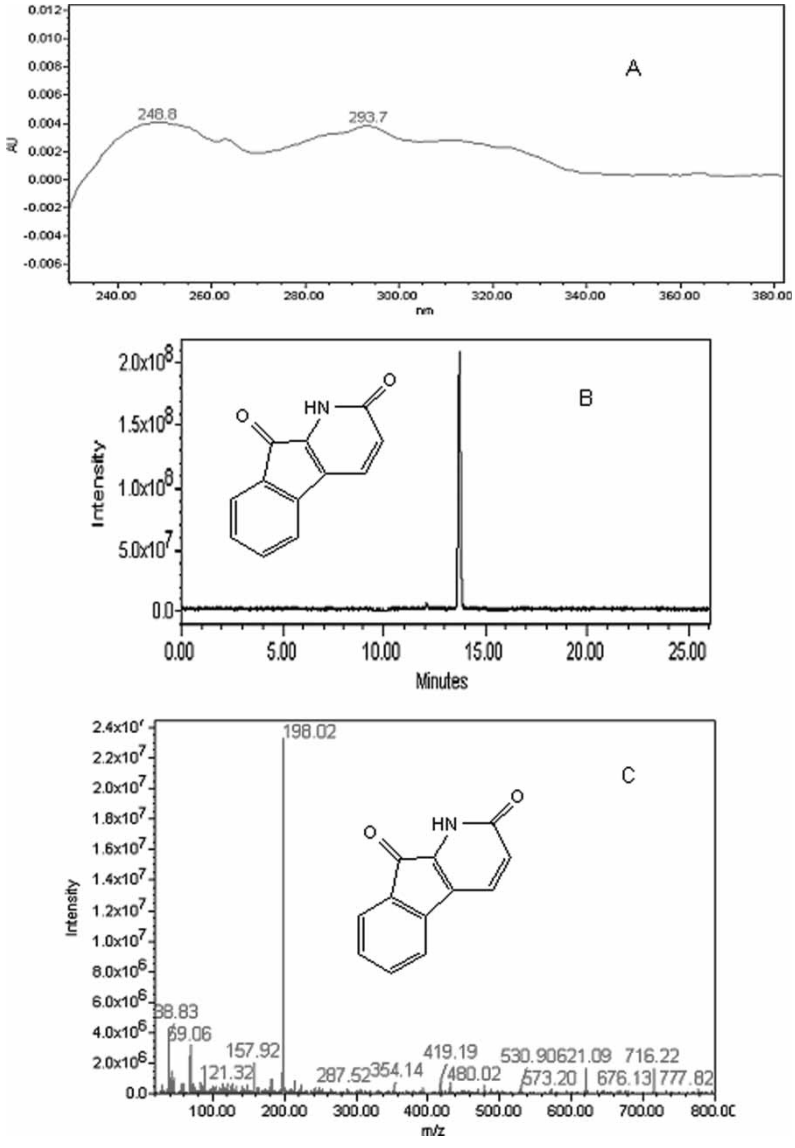

Figure 4. (A) PDA UV spectra; (B) HPLC-ESI-MS chromatogram; (C) mass spectrum (x-axis: relative abundance) of the impurity peak corresponding to the RT (12.897 min) obtained for impurity.

(0.20 mg/mL). The %RSD values for peak area and retention time presented in Table 3 was less than 0.57% in each case and illustrated good repeatability precision for the analytical method.

Intermediate precision (interday precision) was demonstrated by two analysts using two HPLC systems on different days and evaluating the peak

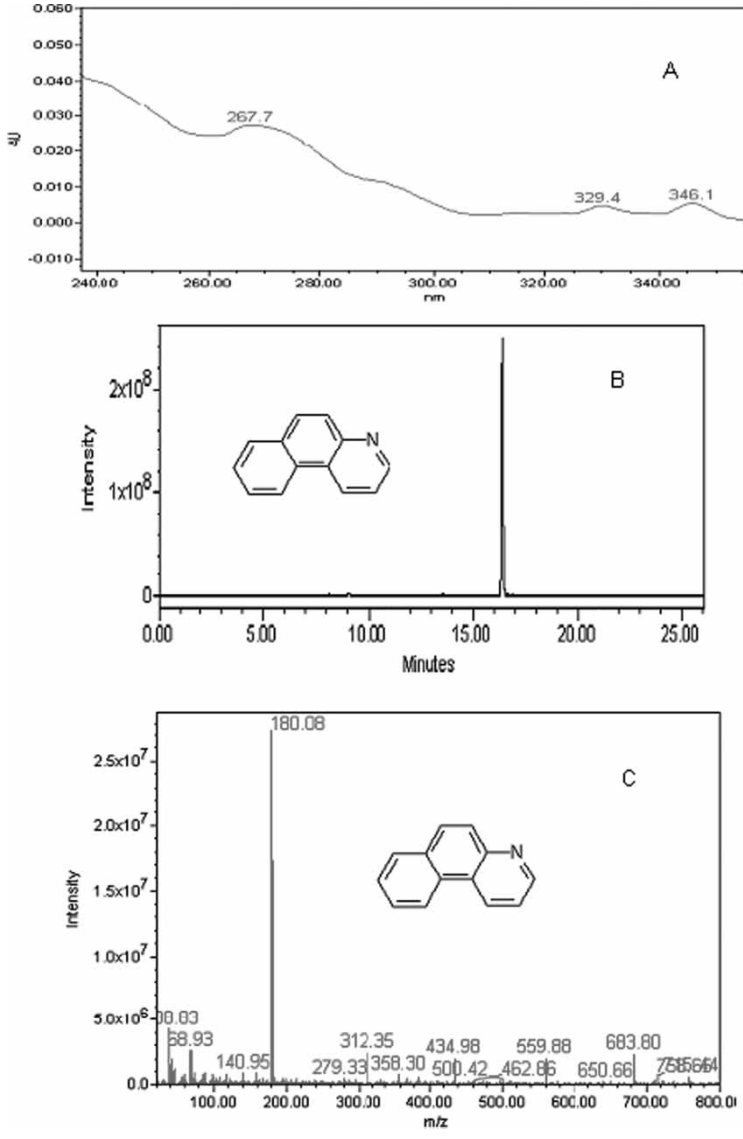

Figure 5. (A) PDA UV spectra of the impurity peak corresponding to the RT (15.922 min); (B) HPLC-ESI-MS chromatogram; (C) mass spectrum (x-axis: relative abundance) obtained for impurity.

area data across the HPLC systems at three concentration levels (0.04, 0.60, and 1.40 mg/mL) that cover the assay method range 0.02 to 1.40 mg/mL for benzo[*f*]quinoline-5,6-dione. The mean and %RSD across the HPLC systems and analysts were calculated from the individual peak area mean values at the 0.04,

Table 1. Chromatographic results for benzo[*f*]quinoline-5,6-dione and its impurities

Compound	Chemical name	Retention time (min)	Purity (%)	Mass (m/z)
Impurity 1	Unknown	6.844	1.16	Unknown
Benzo[*f*]quinoline-5,6-dione	—	9.187	89.21	210.05
Impurity 2	1*H*-indeno[2,1-*b*]pyridine-2,9-dione	12.897	0.34	198.02
Impurity 3	Benzo[*f*]quinoline	15.922	4.06	180.08

0.60, and 1.40 mg/mL of the test concentration. The %RSD values for both instruments and analysts were $<0.85\%$ (Table 4) for benzo[*f*]quinoline-5,6-dione and illustrated the good precision of the analytical method.

Specificity/Selectivity

Specificity can also be determined by measurement of peak homogeneity. Because the different techniques available in a PDA are not equally effective for the detection of possible impurities or interference in a chromatographic peak, the use of several techniques is recommended.

In this work the techniques used to validate the peak purity of the benzo[*f*]quinoline-5,6-dione were:

–Normalization and comparison of spectra from different peak sections:
–Absorbance of two wavelengths:

Both techniques showed that the peak corresponding the benzo[*f*]quinoline-5,6-dione studied presented a high level of purity. Also, a chromatogram of the blank was recorded under the same conditions and the signal obtained from this blank showed no interference from any other materials.

Forced degradation studies were also performed to evaluate the specificity of benzo[*f*]quinoline-5,6-dione under four stress conditions (heat, UV light, acid, base). Solutions of benzo[*f*]quinoline-5,6-dione were exposed to 50°C for 1 h, UV light using a Mineralight UVGL-58 light for 24 h, acid (1 M hydrochloric acid) for 24 h, and base (1 M sodium hydroxide) for 4 h. A summary of the stress results is shown in Table 5. It is evident from Figure 2 that the method has been able to separate the peaks of the degraded products from that of the benzo[*f*]quinoline-5,6-dione.

Accuracy/Recovery Studies

In order to test the efficiency of the analytical method, recovery studies at three known added concentration levels (0.04, 0.60, and 1.40 mg/mL) were carried

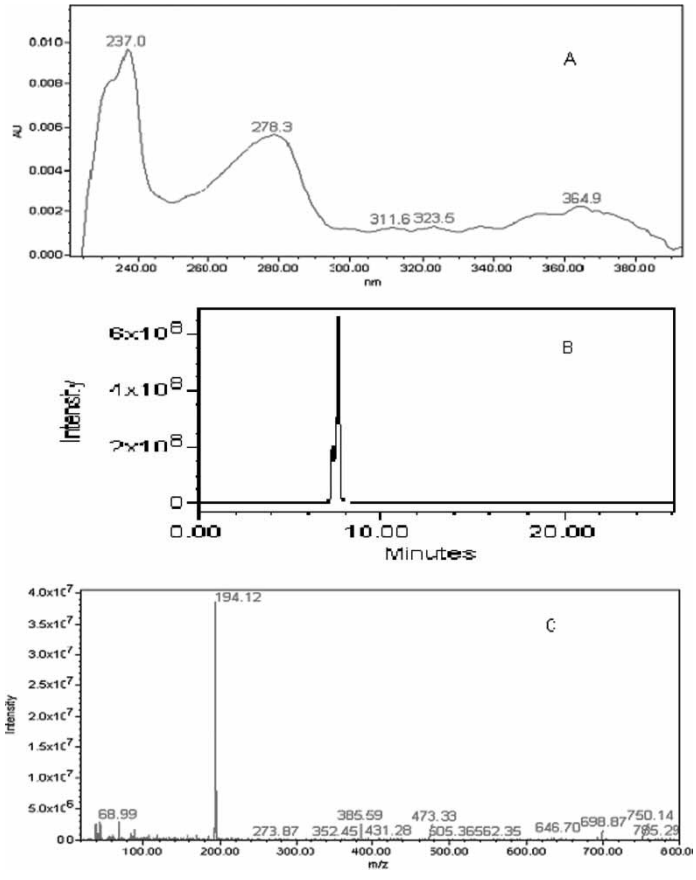

Figure 6. (A) PDA UV spectra of the impurity peak corresponding to the RT (6.844 min); (B) HPLC-ESI-MS chromatogram; (C) mass spectrum (x-axis: relative abundance) obtained for impurity.

out. Two replicates were prepared at each concentration level and each one was injected in triplicate. Mean recoveries higher than 88% were obtained in all cases with acceptable RSD. The results are shown in Table 2.

Limits of Detection and Quantification

The limit of detection (LOD) was considered as the minimum analyte concentration yielding a signal to noise ratio equal to three. The limit of quantification (LOQ) was adopted at the lowest analyte concentration yielding a signal 10 times greater than the noise. The LOD and LOQ values

Table 2. Validation results obtained for the HPLC assay of benzo[*f*]quinoline-5,6-dione

Validation criterion	Concentration range (mg/mL)	Results
Linearity ($n = 2$; $k = 6$)	0.02 to 1.40	$y = 146444x + 167799$ ($r^2 = 0.9997$)
Accuracy (Recovery ± %RSD; $n = 3$)	0.04 0.60 1.40	88.33 ± 0.65 90.33 ± 0.64 88.50 ± 0.65
LOD		(s/n = 3.3), 2.0 μg/mL
LOQ ($n = 6$)		(s/n = 10.3), 20.0 μg/mL

Table 3. Repeatability precision study data for benzo[*f*]quinoline-5,6-dione

Injection	Peak area (μV)	Retention time (min)
1	5308596	9.19
2	5312528	9.19
3	5311452	9.2
4	5302671	9.29
5	5327812	9.19
6	5382762	9.19
Mean	5324303	9.21
RSD (%)	0.56	0.44

Table 4. Intermediate precision studies data for benzo[*f*]quinoline-5,6-dione

	Analyst 1, day 1, HPLC 1			Analyst 2, day 2, HPLC 2		
Injection	0.04[a]	0.6	1.4	0.04	0.6	1.4
1	5352632[b]	5382372	5376291	5324562	5400236	5316282
2	5318324	5406276	5367286	5362861	5376235	5388117
3	5303271	5367428	5335172	5377242	5312872	5366245
Mean	5324742	5385359	5359583	5354888	5363114	5356881
RSD (%)	0.47	0.36	0.40	0.51	0.84	0.69

[a]Concentration (mg/mL).
[b]Peak area (μVs).

Table 5. Specificity results of benzo[*f*]quinoline-5,6-dione under stress conditions

Stress conditions	Sample treatment	Retention time (min)	Area (μVs)	Assay (%)
Reference	Fresh solution	9.19	5315628	89.76
Acid	1M HCl for 24 h	9.19	5329526	89.26
Base	1M NaOH for 4 h	9.19	5308364	89.12
Heat	50 °C for 1 h	9.20	5326538	89.01
Light	UV Light for 24 h	9.20	5301672	88.62

for benzo[*f*]quinoline-5,6-dione were found to be 2.0 μg/mL (s/n = 3.3) and 20.0 μg/mL (s/n = 10.3), with RSD 0.88% for six injection replicates, respectively.

CONCLUSIONS

A new, sensitive, specific, and robust reversed-phase HPLC method for the identity, assay, and purity evaluation of benzo[*f*]quinoline-5,6-dione and its synthetic impurities is described. The research methodology and data for its validity has been extensively validated and showed good linearity, precision, and accuracy. 1*H*-Indeno[2,1-*b*]pyridine-2,9-dione and benzo[*f*]-quinoline were identified as main impurities by applying the method in LC-MS mode. The developed method could be satisfactorily applied as a routine procedure to identify and quantify benzo[*f*]quinoline-5,6-dione and its synthetic impurities.

ACKNOWLEDGMENTS

I would like to thank Jon Salmon and Phil Durban at Abbott Diabetes Care UK, Abbott Laboratories as well as Susan Tall at Abbott Diabetes Care Inc., California for reviewing this research for publication.

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Received April 3, 2007
Accepted May 16, 2007
Manuscript 6114