Development and Validation of a Liquid Chromatography–Mass Spectrometry Method for the Determination of 4,5-Diazafluoren-9-one

Ghulam A. Shabir*

Abbott Laboratories, Range Road, Witney, Oxon OX29 0YL, UK

Abstract

A new liquid chromatography–mass spectrometry (LC–MS) method is developed and validated for the identification and determination of novel 4,5-diazafluoren-9-one compound. The method employs a Waters XTerra RP-18 column (150 mm x 4.6 mm, i.d. 5 µm) with a mobile phase comprised of a (50:50, v/v) mixture of deionized water containing 0.2% acetic acid (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min, at 35°C. The detection is performed with photodiode-array (PDA) set at 210–400 nm and single quadropole mass spectrometer with electrospray ionization (ESI) positive ion mode. The chromatographic separation is achieved in less than 3 min. The linearity is established over the concentration range of 0.1–0.5 mg/mL (r^2 = 1.000). The mean RSD values for intra- and inter-day precision studies are < 2%. The recovery of 4,5-diazafluoren-9-one ranged between 99.84 and 99.97%. The limits of detection and quantitation are determined to be 0.58 and 0.1 mg/mL, respectively.

Introduction

4,5-Diazafluoren-9-one (Figure 1B) is a useful co-product of the 1,10-phenanthroline (Figure 1A). It can be synthesized by oxidation of 1,10-phenanthroline with alkaline potassium permanganate (KM_nO_4) in a potassium hydroxide (KOH) solution (1-3). 4,5-Diazafluoren-9-one ($C_{11}H_6N_2O$) has hitherto been a difficulty accessible compound, its only synthesis being from the action of aqueous alkali on 1,10-phenanthroline,5-6-quinone by way of a benzilic acid type rearrangement analogous to the formation of fluorenone from phenanthrenequinone (4).

Various analytical procedures for characterization of 4,5-diazafluoren-9-one, including elemental analysis, UV–vis, IR (5), NMR spectroscopy (6,7), X-ray crystallography, and FTIR spectrophotometry (8) have been reported in the literature. However, liquid chromatography (LC) or LC–mass spectrometry (MS) method has not been reported in the literature.

LC is a universal separation technique that is capable of separating both volatile and non volatiles without the need for derivatization. Combined chromatographic and spectrometric techniques and in particular LC–MS have been contributing to the progress in life sciences in general (9). In recent years, LC–MS has been extensively adopted in the field of biology, biochemistry, structural biology, and biomedicine (10-18). It is, therefore, promising and useful to develop an LC–MS method for the assay of 4,5-diazafluoren-9-one compound.

In this work for the first time, a simple, rapid, specific, and sensitive LC–MS (with ESI^+ ion mode) novel assay method for the separation, identification, and determination of 4,5-diazafluoren-9-one compound is reported. There are various types of ionization sources that can be used as the interface between the LC eluent and the mass spectrometer. The two most common sources are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Both of these source types are now standard equipment on mass spectrometers that are used for LC–MS apparatus. For both ESI and APCI, the ionization occurs at atmospheric pressure, so these sources are often referred to as atmospheric pressure ionization (API) sources. For both ESI and APCI, some combination of high voltage and heat is used to provide the ionization that is needed to produce the ions that are assayed by the MS system. In ESI, the high voltage field (3–5 kv) produces nebulization of the column effluent resulting in charged droplets that are focused toward the mass analyzer. These droplets get smaller as they approach the entrance to the mass analyzer: as the droplets get smaller, individual ions emerge in a process referred to as "ion evaporation"—these ions are then separated by the MS system (19). In APCI, heat is used to vaporize the column eluent and then a corona discharge is used to ionize solvent molecules, which then produce the analyte ion via chemical ionization mechanism (19).

Finally, the developed analytical method was validated to assess the validity of research data means determining whether the method used during the study can be trusted to provide a genuine, account of the intervention being evaluated. As a best

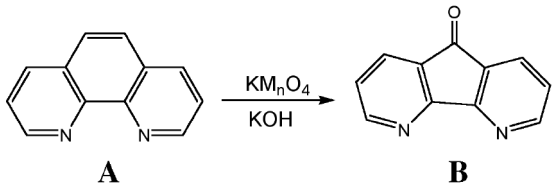

Figure 1. Synthesis of 4,5-diazafluoren-9-one.

* Author to whom correspondence should be addressed: email ghulam.shabir@abbott.com.

practice (20–22), in the subsequent investigation, the new LC–MS method was validated (23) using pre-approved protocol and validated LC–MS system.

Experimental

Chemicals and reagents

Methanol (HPLC-grade) and acetic acid were obtained from Merck (Darmstadt, Germany). 4,5-Diazafluoren-9-one reference standard and sample (98% pure) were purchased from Aldrich (St. Louis, MO). Distilled water was de-ionized by using a Milli-Q system (Millipore, Bedford, MA).

LC conditions

A Waters (Elstree, UK) Alliance 2690 Separations Module LC system, consisting of a vacuum degasser, a quaternary solvent pump, an autosampler with a column oven and a photo diode-array (PDA) detector, all controlled by a Empower software, was used. A reversed-phase XTerra RP-18 (Waters) column (150 × 4.6 mm, particle size 5 µm) was used for separation. The mobile phase comprized of a (50:50, v/v) mixture of deionized water containing 0.2% acetic acid (solvent A) and methanol (solvent B). The flow rate was 1 mL/min, the injection volume was 4 µL, and the temperature was set at 35°C. Chromatograms were recorded at 243 nm using UV detector. The second LC system used was PerkinElmer (Norwalk, CT) equipped with a module series 200 UV detector, series 200 LC pump, series 200 autosampler, and series 200 peltier LC column oven. The data were acquired via PE TotalChrom Workstation data acquisition software (version 6.3.1) using PE Nelson series 600 LINK interfaces.

Table I. Results Obtained from the FIA Tests of the MS Parameters

MS parameter	Studied range	Optimal value
Capillary voltage	2–4 kv	3.5 kv
Cone voltage	50–70 v	60 v
Extractor voltage	2–10 v	5 v
Source temperature	50–120°C	80°C
Cone temperature	10–30°C	20°C
Desolvation temperature	150–350°C	200°C
Desolvation gas flow	100–400 L/h)	260 L/h
Cone gas flow	30–65 L/h	60 L/h

Table II. Experimental Domain of the Factors During Robustness Testing

Factor	Experimental domain	Optimal value
Sample solvent	Mobile phase: methanol–water	Methanol
Analytical columns (Different lots)	C_{18}–C_{18}	C18
Percent organic solvent	45–55	50
Flow rate (mL/min)	0.8–1.2	1.0
Injection volume (µL)	2–6	4
Column T (°C)	30–40	35
Wavelength (nm)	239–247	243

MS conditions

A Waters Micromass ZQ2000 single quadropole mass spectrometer (MS) with an ESI and APCI interfaces, all controlled by Empower software (version 1.0), was used. Operating conditions of the ESI interface in positive mode, which were obtained after making flow injection analysis (FIA) tests of the MS parameters, are reported in Table I. Full-scan LC–MS spectra were obtained by scanning from *m/z* 100 to 500 da.

Preparation of the standard and sample solutions

For each sample, approximately 30 mg of 4,5-diazafluoren-9-one were weighed into separate 100-mL volumetric flasks followed by the addition of 70 mL of HPLC-grade methanol. The resulting mixture was sonicated for about 5 min to aid dissolution of 4,5-diazafluoren-9-one. Volume was made up to 100 mL with HPLC-grade methanol and was mixed well manually.

Linearity assessment

Linearity experiments were performed by preparing 4,5-diazafluoren-9-one standard solutions in the range 0.1–0.5 mg/mL in methanol and injected in triplicate. Linear regression analysis was carried out on the standard curve generated by plotting the concentration of 4,5-diazafluoren-9-one versus peak area response.

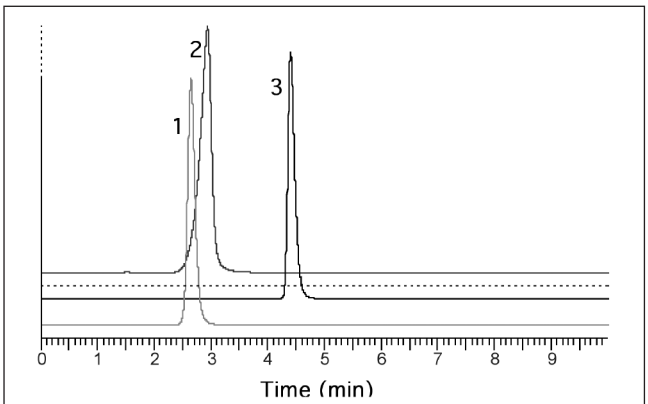

Figure 2. LC chromatograms obtained from different C_{18} columns: 1, Xterra; 2, Luna; 3, HyperClone.

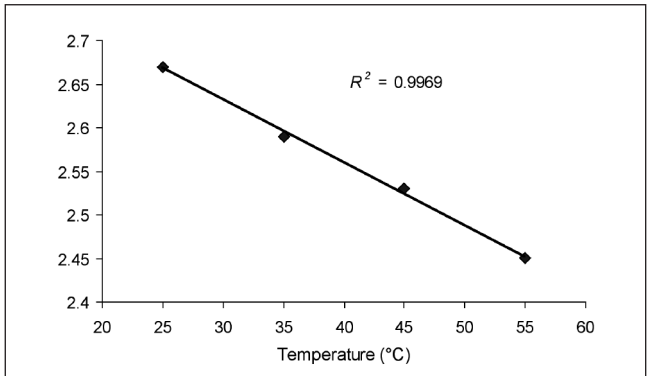

Figure 3. Effect of LC column temperature on the retention times.

Results and Discussion

Liquid chromatography analysis

Prior to the coupling with the mass spectrometer, an optimization of the LC separation was carried out using photodiode array UV detector.

Acetic acid in water, ammonium formate, and ammonium acetate buffers were studied, selecting the first one because less analysis time and better separation were obtained with the addition of methanol.

Initially, three analytical columns were tried in order to reach acceptable specificity and selectivity. I first exploited Luna C_{18} (150 × 4.6 mm, 5-µm) and HyperClone (ODS) C_{18} (250 × 4.6 mm, 5-µm) phase columns from Phenomenex (Macclesfield, UK). The Luna column gave poor separation with long tailing at fronting of analyte peak with retention time 2.96 min. The HyperClone column gave good peak shape but retention time was 4.46 (min) for the 4,5-diazafluoren-9-one peak. Shift to XTerra RP-18 (150 × 4.6 mm, 5-µm) column (Waters) produces peak with superior band shape and column efficiency with shorter retention time (2.65 min) under the same conditions (Figure 2).

Some tests were made varying the temperature between 25°C and 55°C at 10°C steps, to study the influence of this parameter. The results showed that the variation of the temperature at 25-45°C did not significantly affect any of the chromatographic parameters and only increased the retention time of the analyte (Figure 3). A higher temperature (55°C) worsened the peak shape and selectivity (Figure 4), so 35°C was chosen as work temperature.

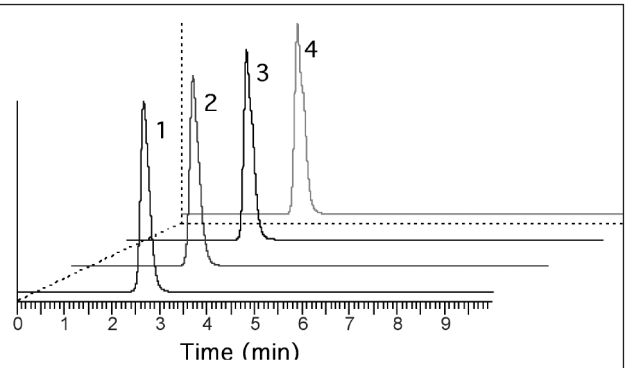

Figure 4. LC chromatograms of 4,5-diazafluoren-9-one obtained at different temperatures: (1) 25°C, RT, 2.67, (2) 35°C, RT, 2.57 (3) 45°C, RT, 2.53 and (4) 55°C, RT, 2.45 (min).

Table III. Linearity Assessment Data for the Assay of 4,5-Diazafluoren-9-one*

Concentration (mg/mL)	Concentration as percent of analyte target	Mean area (µVs, *n* = 3)	± SD	RSD (%, *n* = 3)
0.1	10	3374350	15926.80	0.47
0.2	25	6642282	16319.09	0.25
0.3	75	9921267	10500.98	0.11
0.4	100	13237998	16625.44	0.13
0.5	150	16217461	12561.90	0.08

* Correlation coefficient: r^2 = 1.000; Equation for regression line: y = 32281938.00x + 194090.20.

The choice of wavelength is essential to accomplish a sensitive and a selective chromatographic assay. The optimal wavelength for 4,5-diazafluoren-9-one detection was established using the scan range of 190 to 400 nm. It was shown that 243 nm were the optimal wavelength to maximize the signal (Figure 5).

To evaluate the quantitative nature of the analytical method, a series of samples with different amounts of 4,5-diazafluoren-9-one were run to investigate the best assay concentration. Using an XTerra RP-18 column, best concentration was assessed by injecting six-reference standard of 4,5-diazafluoren-9-one in the range of 0.01 to 1.0 mg/mL. The integrated peak areas were plotted versus amount injected. The calibration curve was found to be linear from concentration range 0.1 to 0.5 mg/mL with a correlation coefficient of 0.9999. On the bases of these data, the middle concentration of the linearity was chosen as a best concentration (0.3 mg/mL) for the assay.

The system suitability test was established from six replicate injections of a solution containing 0.3 mg 4,5-diazafluoren-9-one /mL. The percent relative standard deviation (RSD) of the retention time (min) and peak area were found to be less than 0.50%. The USP tailing factor, T_f, was 0.76, and column efficiency, N, was 6012 for 4,5-diazafluoren-9-one.

Robustness testing was performed during method development phase to optimize final LC conditions. An LC method must prove to be able to remain unaffected by small, but deliberate variations in method parameters, thus showing its own reliability during normal usage. It is advisable to simultaneously study the possible variations of method parameters in an interval chosen symmetrically around the optimized conditions. This

Table IV. Intermediate Precision Study Data for 4,5-Diazafluoren-9-one

	LC 1, Day 1		LC 2, Day 2	
Concentration (mg/mL)	Mean area (µVs, *n* = 3)	RSD (%, *n* = 3)	Mean area (µVs, *n* = 3)	RSD (%, n = 3)
0.2	5320180	0.27	6745738	1.08
0.3	7736782	0.35	10060231	0.24
0.4	10208667	1.01	13351243	0.24

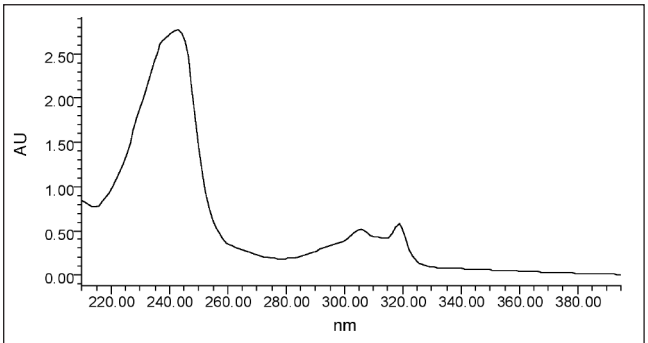

Figure 5. Photodiode-array UV spectra of the middle of the peak corresponding to the retention times of main compound of 4,5-diazafluoren-9-one.

interval represents the variations expected during method transfer and routine use in quality control testing. In this case, the seven selected parameters were the same considered in the optimization step. Their experimental domain is reported in Table II. This showed that the method for determination of 4,5-diazafluoren-9-one was reproducible and robust.

MS analysis

The first MS experiments to select the optimum MS parameters and the appropriate ions were carried out by FIA of 4,5-diazafluoren-9-one sample solutions, monitoring the MS intensity. Different ionization interfaces APCI and ESI modes (negative and positive ionization) were investigated. The chromatogram is generated from the ion abundances and mass spectra recorded in the positive-ion, ESI mode gave almost no loss of sensitivity compared to the APCI. The studied range for each ESI-MS parameter is shown in Table I. Best sensitivity was obtained for 4,5-diazafluoren-9-one compound using the conditions in Table II. The full scan MS spectrum of 4,5-diazafluoren-9-one was measured in the mass range *m/z* 100–500 da. The 4,5-diazafluoren-9-one displayed a single peak at 2.735 min, which corresponds to the molecular mass $[M+H]^+$ at *m/z* 183.04 (see Figure 6). In addition, Figure 7 shows a three-dimensional display of the photodiode array absorbance data obtained by LC–PDA-MS for 4,5-diazafluoren-9-one. Peak at *m/z* 183.04 is for 4,5-diazafluoren-9-one. The first dimension is LC retention time, second is *m/z,* and third is intensity.

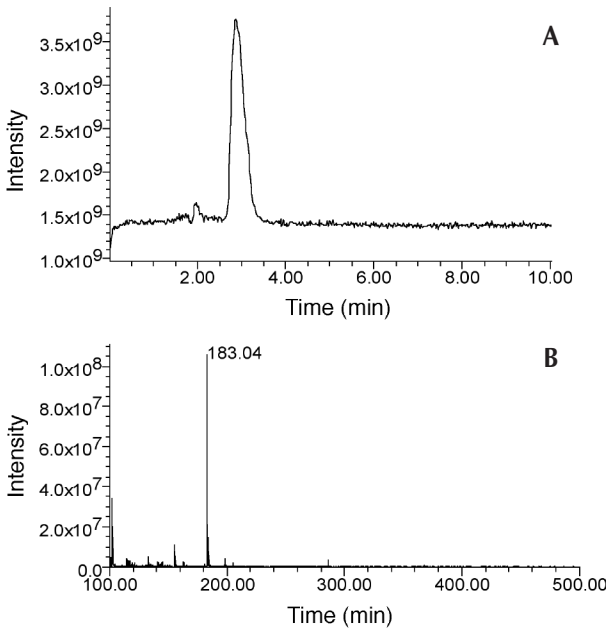

Figure 6. LC–MS chromatogram (A), ESI (–) MS spectrum of 4,5-diazafluoren-9-one, (B). The LC–MS retention time and the mass spectrum readily identify the peak eluting at 2.735 min as 4,5-diazafluoren-9-one.

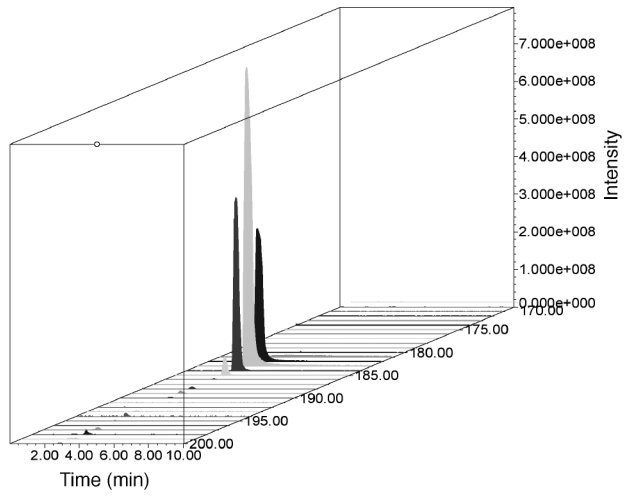

Figure 7. Three-dimensional display of the photodiode array absorbance data obtained by LC–PDA-MS for 4,5-diazafluoren-9-one. The first dimension is LC retention time, second is *m/z* and third is intensity.

Method validation

Great care was taken in the method development phase, which resulted in the subsequent validation being straightforward. The validation parameters performed were linearity, range, precision (repeatability and intermediate precision), accuracy, specificity, and limit of detection and quantitation.

Linearity and range

Linearity was studied in the concentration range 0.1 to 0.5 mg/mL ($n = 3$; $K = 5$) and the following regression equation was found by plotting the peak area (y) versus the 4,5-diazafluoren-9-one concentration (x) expressed in mg/mL:

$$y = 32281938.00x + 194090.20 \ (r^2 = 1.000) \qquad \text{Eq. 1}$$

The determination coefficient (r^2) obtained (Table III) for the regression line demonstrates the excellent relationship between peak area and the concentration of 4,5-diazafluoren-9-one.

Table V. Recovery Studies Data for 4,5-Diazafluoren-9-one from Samples with Known Concentrations

Sample	Concentration as % of analyte target	Mean recovery (%, $n = 3$)	RSD (%, $n = 3$)
1	10	99.84	0.03
2	25	99.96	0.04
3	75	99.95	0.01
4	100	99.97	0.02
5	150	99.96	0.05

Table VI. Force Degradation Studies Data for 4,5-Diazafluoren-9-one

Stress conditions	Sample treatment	RT (min)	Area (µVs)	Assay (%)
Reference	Fresh solution	2.69	6927585	99.86
Acid	1M HCl for 24 h	2.75	6862371	98.94
Base	1M NaOH for 4 h	2.64	6752936	98.51
Heat	50°C for 1 h	2.66	6692625	99.92
Light	UV Light for 24 h	2.66	6957040	99.92

Precision

The precision (repeatability and intermediate precision) of the chromatographic method, reported as % RSD, was estimated by measuring repeatability (intra-day assay precision) on 10 replicate injections at 100% test concentration (0.3 mg/mL). The RSD values for retention time (min) 0.04%, peak area 0.49%, and peak height were 0.48%.

The intermediate precision (inter-day variation) was studied using two LC systems over two consecutive days at three different concentration levels (0.2, 0.3 and 0.4) that cover the assay range (80–120%). Three replicate injections were injected for each solution. The chromatograms obtained using PerkinElmer (LC 1) and Waters (LC 2) are given in Figure 8 and 9, respectively. The RSD values for both instruments were ≤ 1.08% (Table IV) and illustrated the good precision of this analytical method.

Accuracy/recovery study

Recovery studies may be performed in a variety of ways depending on the composition and properties of the sample matrix. In the present study, a number of different solutions were prepared with known added amounts of 4,5-diazafluoren-9-one and injected in triplicate. Percent recoveries of response factor (area/concentration) were calculated as can be seen in Table V.

Table VII. Stability of 4,5-Diazafluoren-9-one in Solution (*n* = 3)

Time (h)	Area (%RSD)	Height (%RSD)	Recovery (%)	% of initial
0	0.62	0.09	99.95	
48	0.48	0.12	99.88	99.93

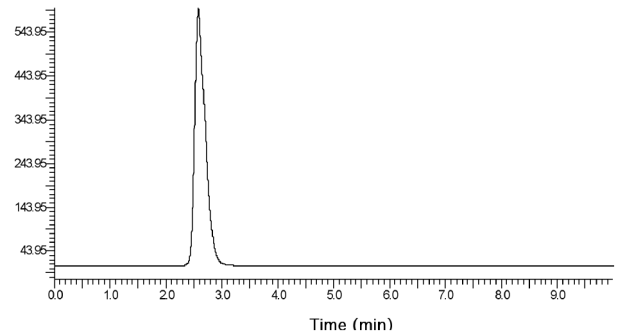

Figure 8. LC chromatogram of 4,5-diazafluoren-9-one eluted at 2.693 min obtained using PerkinElmer LC system during inter-day variation study.

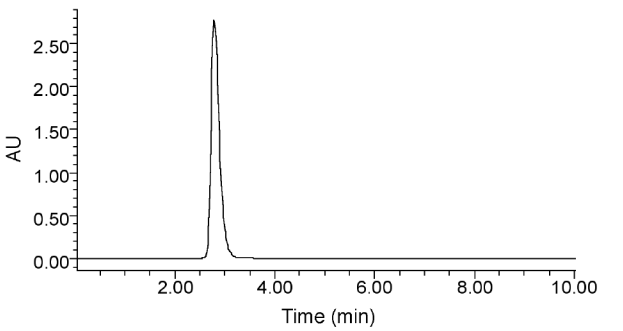

Figure 9. LC chromatogram of 4,5-diazafluoren-9-one eluted at 2.735 min obtained using Waters LC system during inter-day variation study.

Specificity/forced degradation studies

The LC–PDA three-dimensional chromatogram (Figure 10) demonstrates a good separation of the 4,5-diazafluoren-9-one. The three-dimensional data consist of UV absorption spectra from 210 to 400 nm for each point along the chromatogram. This method demonstrates acceptable specificity.

The forced degradation studies were performed to evaluate the specificity of 4,5-diazafluoren-9-one under four stress conditions (heat, UV light, acid, base). Solutions of 4,5-diazafluoren-9-one were exposed to 50°C for 1 h, UV light using a Mineralight UVGL-58 light for 24 h, acid (1M hydrochloric acid) for 24 h and base (1M sodium hydroxide) for 4 h. A summary of the stress results is shown in Table VI. It is evident from Figure 11 that the method has been able to separate the peak due to the degraded products from that of the 4,5-diazafluoren-9-one.

Limits of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) of 4,5-diazafluoren-9-one were estimated from the intercept ($\bar{a}$) of the regression line and the corresponding residual standard

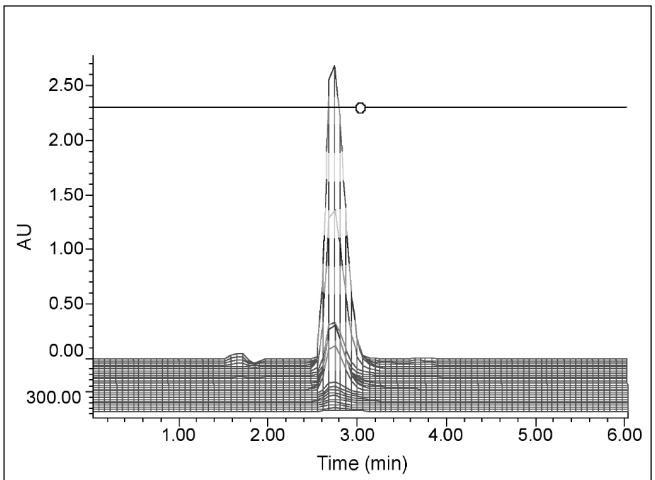

Figure 10. Three-dimensional display of the photodiode array absorbance data obtained by LC–PDA for a 4,5-diazafluoren-9-one.

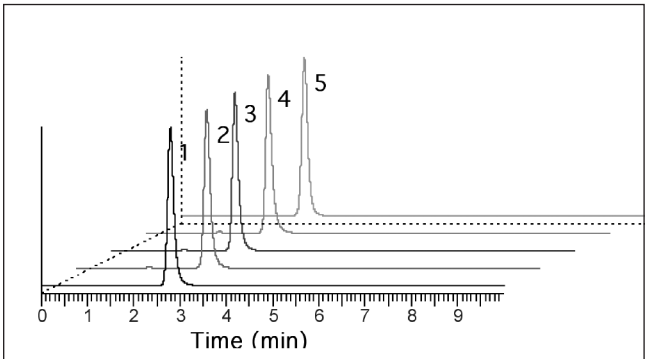

Figure 11. LC chromatograms obtained from forced degradation study: 1, fresh solution; 2, acid; 3, base; 4, heat at 50°C; 5, UV light.

deviation ($S_{y/x}$) (18). The responses at the LOD and LOQ were estimated by the following expressions respectively.

$$f(\text{LOD}) = \bar{a} + 3S_{y/x} \quad \text{Eq. 2}$$

$$f(\text{LOQ}) = \bar{a} + 10S_{y/x} \quad \text{Eq. 3}$$

Applying this method, LOD and LOQ for 4,5-diazafluoren-9-one were found to be 0.58 and 0.1 mg/mL, respectively.

Stability of analytical solutions

The stability of 4,5-diazafluoren-9-one solutions was investigated. The solutions were stable during the investigated 48 h and the RSD was in between 0.04 and 0.07% for retention times. Standard solutions stored in a capped volumetric flask on a laboratory bench under normal lighting conditions for 48 h, were shown to be stable with no significant change in 4,5-diazafluoren-9-one concentration over this period (Table VII). This is indicated (0.5% changes in area between $T = 0$ h and $T = 48$ h). Based on these data that show quantitative recovery through 48 h, solutions of 4,5-diazafluoren-9-one can be assayed within 48 h of preparation.

Conclusion

A new, simple, sensitive, and specific LC–MS method for the assay of 4,5-diazafluoren-9-one was developed. With the use of the Waters XTerra RP-18 column, 4,5-diazafluoren-9-one was well retained, with a good peak shape. A simple binary isocratic was used without adding buffer. The proposed method for its validity was successfully validated using validated (qualified) LC–MS system and results showed a good linearity, precision, and accuracy. The quantitation linear range was from 0.1 to 0.5 mg/mL with correlation coefficient (r^2) of 1.000. The mean RSD values for intra- and inter-day precision studies were < 2% in each case. The developed method could be satisfactorily applied as a routine procedure to identify and quantify 4,5-diazafluoren-9-one.

References

1. L.J. Henderson, F.R. Fronczek, and W.R. Cherry. Selective perturbation of ligand field excited states in polypyridine ruthenium(II) complexes. *J. Am. Chem. Soc.* **106:** 5876–79 (1984).
2. T. Zihou et al. Molecular assemblies of diazafluorenone Schiff-base amphiphiles. II. The vesicle and its molecular aggregation behavior. *Molecular Engineering.* **3:** 293–99 (1994).
3. O. Katsuhiko et al. Synthesis and properties of 9,9′-diaryl-4,5-diazafluorenes, A new type of electron-transporting and hole-blocking material in EL device. *Chem. Lett.* **33:** 276–77 (2004).
4. G. M. Badger and J.W. Cook. Chemistry of Carbon Compounds Vol. IIIB. E.H. Rodd, Ed. Elsevier, Amsterdam 1956, p. 1446.
5. M.S. Deshpande and A.S. Kumbhar. Mixed-ligand complexes of ruthenium (II) incorporating a diazo ligand: synthesis, characterization and DNA binding. *J. Chem. Sci.* **117:** 153–59 (2005).
6. G. Zhou and I.I. Harruna. Synthesis of ligand monomers derived from 4,5-diazafluoren-9-one. *Tetrahedron Lett.* **44:** 4617–19 (2003).
7. U. Siemeling and I. Scheppelmann. Cyclopentadienone-like behaviour of fluorenone and 4,5-diazafluoren-9-one. *Organometallics* **23:** 626–28 (2004).
8. S. Menon and M.V. Rajasekharan. A channel-forming polyiodide network in [Cu(dafone)3]I12. A tris chelate of defone and a new planar structure for the I122- ion (defone = 2,5-diazafluoren-9-one). *Inorg. Chem.* **36:** 4983–87 (1997).
9. E. Gelpi. Contributions of liquid chromatography–mass spectrometry to "highlights" of biomedical research. *J. Chromatogr. A* **1000:** 567–81 (2003).
10. A. Vera Francesca, L. Giovanna, P. Carlo, et al. Determination of protein phosphorylation sites by mass spectrometry: a novel electrospray-based method. *Rapid Commun. Mass Spectrom.* **19:** 3343–48 (2005).
11. H.Y. Ji, H.W. Lee, H. Kim, H.K. Kim, et al. Liquid chromatography-mass spectrometric analysis of compound K, a ginseng saponin metabolite, in rat plasma. *Anal. Lett.* **37:** 1307–18 (2004).
12. R. Buchalla and T.H. Begley. Characterization of gamma-irradiated polyethylene terephthalate by liquid-chromatography–mass-spectrometry (LC–MS) with atmospheric-pressure chemical ionization (APCI). *Radiat. Phys. Chem.* **75:** 129–37 (2006).
13. S.S. Singh and K. Sharma. Validation of LC-MS electrospray ionisation method for quantitation of haloperidol in human plasma and its application to bioequivalence study. *Anal. Chim. Acta.* **551:** 159–67 (2005).
14. K.P. Deventer, F. P. Van, W. Mikulcikova, T. Van, and F.T. Delbeke. Quantitative analysis of androst-4-ene-3,6,17-trione and metabolites in human urine after the administration of a food supplement by liquid chromatography/ion trap-mass spectrometry. *J. Chromatogr. B* **828:** 21–26 (2005).
15. M. Xu, G. Wang, H. Xie, R. Wang, et al. Determination of schizandrin in rat plasma by high-performance liquid chromatography–mass spectrometry and its application in rat pharmacokinetic studies. *J. Chromatogr. B* **828:** 55–61 (2005).
16. C.N. McEwen, R.G. Mckay, and B.S. Larsen. Analysis of solids, liquids, and biological tissues using solids probe introduction at atmospheric pressure on commercial LC–MS instruments. *Anal. Chem.* **77:** 7826–31 (2005).
17. T. Qian, Z. Cai, R.N.S. Wong, and Z-H. Jiang. Liquid chromatography/mass spectrometric analysis of rat samples for in vivo metabolism and pharmacokinetic studies of ginsenoside Rh2. *Rapid Commun. Mass Spectrom.* **19:** 3549–54 (2005).
18. M. Liu, Y. Hashi, Y.Y. Song, and J.M. Lin. Simultaneous determination of carbamate and organophosphorus pesticides in fruits and vegetables by liquid chromatography–mass spectrometry. *J. Chromatogr. A* **1097:** 183–87 (2005).
19. W.M. Niessen. Progress in liquid chromatograpy-mass spectrometry instrumentation and its impact on high-throughput screening. *J. Chromatogr. A* **1000:** 413–36 (2003).
20. G.A. Shabir, W.J. Lough, A. A. Shafique, and T.K. Bradshaw. Evaluation and application of best practice in analytical method validation. *J. Liq. Chromatogr. Rel. Technol.* **30:** 311–33 (2007).
21. G.A. Shabir. Validation of HPLC methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the U.S. Food and Drug Administration, the U.S. Pharmacopoeia and the International Conference on Harmonization. *J. Chromatogr. A* **987:** 57–66 (2003).
22. G.A. Shabir. Step-by-step analytical methods and protocol in the quality system compliance industry. *J. Validation Technol.* **10:** 314–24 (2004).
23. International Conference on Harmonization (ICH), Q2(R1): Validation of analytical procedures: Text and Methodology, May 1997.

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