Development and Validation of a HPLC Method for 4,7-Phenanthroline-5,6-Dione I and Identification of Its Major Impurity by HPLC–MS–APCI

Ghulam A. Shabir* and **Nigel J. Forrow**
Abbott Laboratories, R&D, Range Road, Witney, Oxon OX29 0YL, U.K.

Abstract

In this study, the development and validation of an analytical method for the assay of 4,7-phenanthroline-5,6-dione I (dione I) using high-performance liquid chromatography (HPLC) and the determination of its synthetic impurities by employing the method in HPLC–mass spectrometry with atmospheric-pressure chemical ionization and photodiode-array UV detection is reported. The results show that dione I is eluted as a spectrally pure peak resolved from its impurities. 5-Bromo,4-7-phenanthroline is identified as the main impurity. This is supported by elemental analysis of the dione I, which demonstrated the presence of bromine. Validation parameters such as specificity and selectivity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), ruggedness, stability, and system suitability, which are evaluated for this method. The LOD and LOQ are 2.0 µg/mL and 50 µg/mL with a 0.50% relative standard deviation (%RSD), respectively. The calibration curves showed good linearity over the concentration range of 0.05–1.50 mg/mL. The correlation coefficient is > 0.9991 in each case. The %RSD values for intra- and interday precision studies are < 0.40%.

Introduction

4,7-Phenanthroline-5,6-dione **I**, (dione **I**) or more commonly phanquinone (phanquone), is an important heterocyclic quinone, which is known historically to be active against protozoa, amoebae, and bacteria (1) (Figure 1). The compound has recently been suggested to be of use in the prevention and treatment of Alzheimer's disease (2,3), ocular macular (4), macular degenerative (5), and prion diseases (6) together with pathological conditions influenced by matrix metalloproteinases (7). Dione **I** is a starting material for phenanthroline ligands (8–10), which are useful for the preparation of complexes of transition metals, particularly ruthenium (11). The quinone cofactor methoxatin can be replaced by dione **I** in glucose dehydrogenase while maintaining enzyme activity (12). Our interest in dione **I** stems from its possible use in biosensor electrodes as a redox mediator for the cofactor NADH (13).

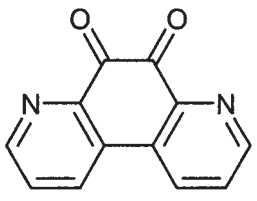

Figure 1. Chemical structure of dione **I**.

A simple method for the synthesis of dione **I** is sought, which is reportedly available via 5-methoxy-4,7-phenanthroline, which is derived from a double Skraup reaction of 2-methoxy-1,4-phenylenediamine hydrosulfate (10,14) or its diacetyl derivative (8). Avoiding the notorious Skraup synthesis, converting the commercially available 4,7-phenanthroline to dione **I** was successful, using oxidation conditions previously applied to the 1,10-isomer (15–17). However, it was suspected that the use of $KBr\text{-}HNO_3\text{-}H_2SO_4$ would result in the formation of brominated 4,7-phenanthrolines as byproducts (18). The goal of this study was to develop a high-performance liquid chromatography (HPLC) assay method for dione **I** to assess its purity, while also applying HPLC–mass spectrometry (MS) to the identification of any impurities. Dione **I** has been previously assayed in biological material by gas–liquid chromatography (19), and it has also been used as a label for amino acids to facilitate their analysis by HPLC (20). An isomer of dione **I** has been studied by HPLC and LC–MS (21). Herein, our efforts in developing and validating an HPLC method for this important compound and our further studies of its impurity profile by HPLC–MS–atmospheric-pressure chemical ionization (APCI) are described.

Experimental

Chemicals and reagents

All chemicals and reagents used in experimental work were of

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

HPLC-grade. Acetonitrile, orthophosphoric acid, triethylamine, and potassium dihydrogen orthophosphate were purchased from Merck (Darmstadt, Germany). Water was purified with a Millipore Milli-Q system (Watford, U.K.). Dione I was synthesized by two routes: direct oxidation of 4,7-phenanthroline (15) by $KBr–HNO_3–H_2SO_4$; or via the Skraup synthesis of 5-methoxy-4,7-phenanthroline followed by oxidation (10,14). 5-Bromo-4,7-phenanthroline was prepared according to the literature (18). Compounds were characterized by elemental analysis, proton NMR, and MS (electron impact, positive mode), as performed by Warwick Analytical Services (Coventry, U.K.).

HPLC instrumentation

A PerkinElmer HPLC system (Norwalk, CT) equipped with a model series 200 UV–vis detector, series 200 LC pump, series 200 autosampler, and series 200 peltier LC column oven was used to chromatograph the solutions. Separation was achieved using XTerra RP-18 column (150- × 4.6-mm i.d., 5-µm particle size) from Waters (Elstree, U.K.). The data were acquired via PerkinElmer TurboChrom Workstation data acquisition software, Version 6.1.0 using PE Nelson series 600 LINK interfaces (Wellesley, MA).

The mobile phase consisted of an aqueous solution of 10mM phosphate buffer (pH 3.05) and acetonitrile (85:15, v/v) containing 0.1% triethylamine. The pH was adjusted with orthophosphoric acid. HPLC settings were as follows: flow rate, 0.5 mL/min; injection volume, 2 µL; column temperature, 40°C; and detector wavelength, 254 nm.

HPLC–MS instrumentation

HPLC–MS analysis was performed using a Waters ZQ2000 single quadrupole MS, equipped with a Waters Alliance 2690 separations module to a 996 Waters photodiode-array (PDA) detector system. A XTerra MS C18 column (50- × 2.1-mm i.d., 3.5 µm particle size) was used. The mobile phase consisted of an aqueous solution of 10mM ammonium acetate and acetonitrile (90:10, v/v). A 2-µL aliquot of a 0.40 mg/mL solution of dione **I** was introduced by the HPLC into the MS via flow injection in a mobile phase. The sample was ionized using APCI in the positive ionization mode under the following source conditions: source temperature, 120°C; capillary potential, 3.0 kV; sampling cone potential, 50 V; cone gas flow, 100 L/h; and APCI probe temperature, 500°C. Mass spectra were obtained over the scan ranges, 100–500 Da, at a rate of 0.5 scan/s. A wavelength range of 210–400 nm was used for the PDA detector.

Preparation of the standard and sample solutions

All sample and standard solutions at 0.40 mg/mL were prepared by dissolving approximately 40 mg of dione **I** in 100 mL of mobile phase.

Preparation of calibration solutions

Standard solutions of dione **I** were prepared at concentrations of 0.05, 0.25, 0.50, 0.75, 1.00, and 1.50 mg/mL in the mobile phase and injected in triplicate. Linear regression analysis was carried out on the standard curve generated by plotting the concentration of dione **I** versus UV–vis PDA peak area response.

Calculations

Identity

The chromatographic profile of the sample preparation must show the same general profile (peak presence and relative intensities) as that of the appropriate standard.

Related substances

The quantity of each impurity peak (the known impurity designated as peak B plus any other impurity peak) is calculated as an area percent versus the total area of all peaks in the chromatogram. Each impurity peak, plus the total area percent of all impurity peaks, must fall within the requirements in the specifications (Table I).

Assay

The area percent of the main dione **I** peak (designated as peak A) is determined regardless of the impurities. The area percent must fall within the specifications (Table I).

Purity

The purity of the dione **I** drug substance is calculated in relation to the reference standard using the area of the main peak A:

$$\text{Peak Purity} = \frac{\text{mg/100 of standard} \times \text{total area of peak A from sample}}{\text{area of peak A from standard} \times \text{mg/100 mL of sample}} \qquad \text{Eq. 1}$$

Results and Discussion

Separation of dione I and its impurity

Efficient chromatography and high sensitivity was achieved by using acetonitrile containing 10mM potassium dihydrogen phosphate containing 0.1% triethylamine as the mobile phase with varying detection wavelengths based on the response of the main component. The main peak tailed badly on some C18 columns with these mobile phases, but this was minimized by adding triethylamine. The amount of organic modifier was adjusted so that the assay run time could be reduced for faster analysis of the dione **I** samples. A chromatogram illustrating the separation of dione **I** and the one potential impurity is illustrated in Figure 2, confirming specificity with respect to the dione **I**. The chromatogram of the blank is displayed in Figure 3.

The remaining chromatographic conditions listed in the HPLC

Table I. Limits for Chromatographic Purity of Dione I and Its Impurity at 254 nm

	Assay	Related substances
Peak assignment	1(A)*	2(B)*
Retention time (min)	5.16	6.90
Limit as percent total area	c ≤ 96.0%	≤ 2.0% b + all other impurities. No other peak area can exceed 2.0%

* A, dione **I**; B, impurity 1.

experimental section were chosen because the lower flow rate of 0.5 mL/min did not have the potential problems associated with elevated back pressures. The UV–vis PDA detector was set at 254 nm, and the UV maximum was set for dione **I**.

The column temperature was held at 40°C, although separations at 30°C and 35°C indicated that slight variations in temperature did not have a significant effect on retention, resolution, or peak shape. The injection volume of 2 µL and sample concentration of 0.40 mg dione **I** per mL in the mobile phase were chosen to simplify sample preparation (further dilution is not needed). This concentration of dione **I** allows both assay (of the main component) and purity evaluation (of trace impurity). At this concentration, the dione **I** peak is well within the linear range for UV detection, and trace components are readily detectable.

System suitability testing was performed to determine the accuracy and precision of the system by making six injections of a solution containing 0.40 mg of dione **I**/mL. All peaks were well resolved, and the precision of injections for all peaks was acceptable. The percent relative standard deviation (%RSD) of the peak area responses was measured. The %RSD of peak areas averaged 0.30% (n = 6), the tailing factor (T) for each peak of the dione **I** was 1.12, and theoretical plate number (N) was 3,240.32. The resolution between each peak was > 2.40, and retention time (t_R) variation %RSD was 0.3% for six injections.

For assessment of method robustness within a laboratory, a number of chromatographic parameters were varied, 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.

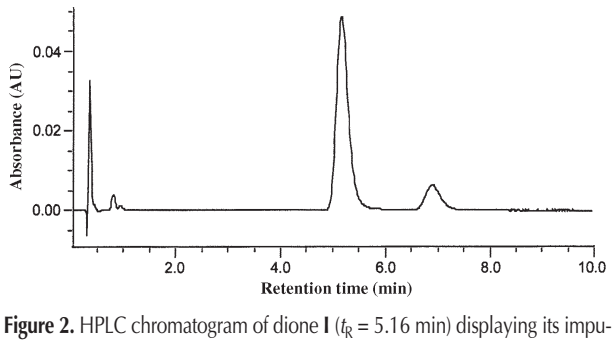

Figure 2. HPLC chromatogram of dione **I** (t_R = 5.16 min) displaying its impurity (t_R = 6.90 min).

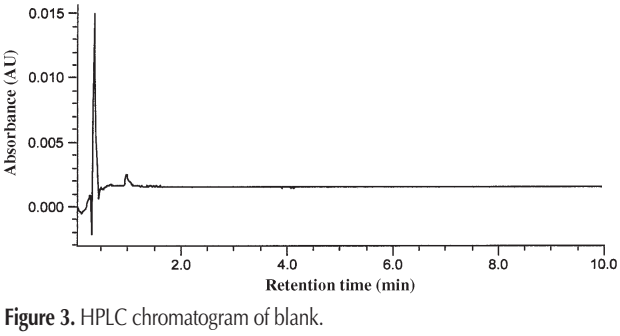

Figure 3. HPLC chromatogram of blank.

Table II. Chromatographic Results for Dione I and Its Impurity

Compound	t_R (min)	Peak area (V s)	Peak area (%)
Dione **I**	5.16	9.31	98.93
Impurity 1	6.90	7.64	0.81

Identification of impurities by HPLC–MS–APCI

Good separation of dione **I** was achieved by HPLC, and the resulting chromatogram is shown in Figure 2. The chromatographic run yielded two major peaks that are detailed in terms of area percent in Table II. The peak at 5.16 min is because of dione **I**. The second peak at (6.90 min) is thought to be a brominated impurity. Elemental analysis of some dione samples demonstrated the presence of approximately 0.3% Br. These samples had been produced via direct oxidation of 4,7-phenanthroline with

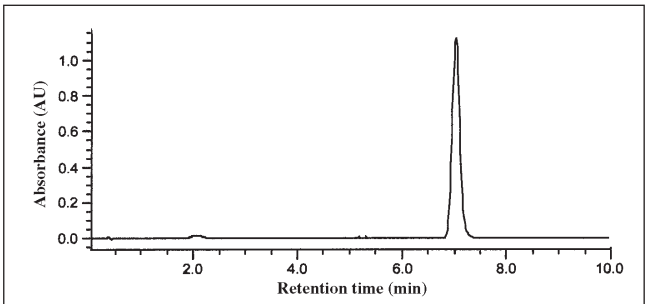

Figure 4. HPLC chromatogram of 5-bromo-4,7-phenanthroline (impurity).

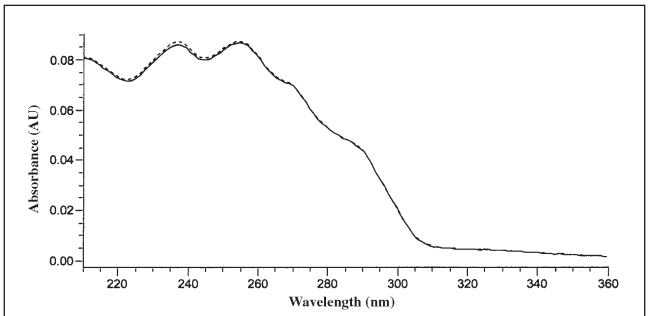

Figure 5. Photodiode-array UV match spectra of the middle of the peak corresponding to the retention times of main compound of dione **I** and impurity 1.

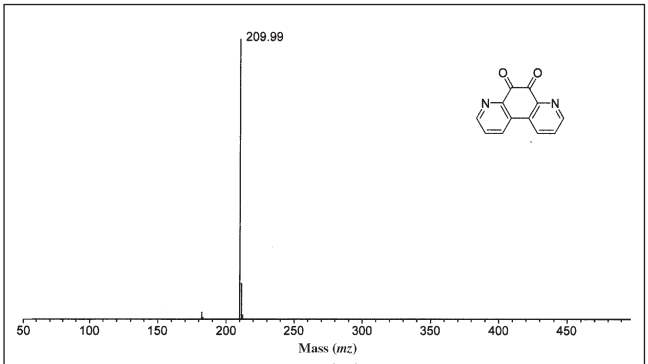

Figure 6. Mass spectrum of dione **I**.

$KBr–HNO_3–H_2SO_4$. In contrast, a sample of dione **I** prepared via 5-methoxy-4,7-phenanthroline was bromine-free. Consequently, a standard sample of 5-bromo-4,7-phenanthroline, the chromatogram displayed in Figure 4, was prepared by a literature procedure (18). The UV spectra of the potential impurities and the dione **I** are shown in Figure 5.

It was not possible to identify the chemical structure of this impurity by HPLC with UV detection. For this purpose, HPLC–MS was selected. The full scan HPLC–MS spectra of dione **I** and its impurity were measured in the mass range *m/z* 100–650. The dione **I** displayed a single peak at 5.16 min, which corresponds to the molecular mass at *m/z* 209.99 and an impurity peak (6.90 min) at *m/z* 258.88. The mass spectra of each peak ion chromatogram are shown in Figures 6 and 7, respectively. The impurity peak B at 6.90 min was identified as 5-bromo-4,7-phenanthroline by both HPLC (Figure 4) and MS data, with the $[M+H]^+$ ion pair at *m/z* 258.88 and 260.86 (Figure 7) being consistent with the presence of Br because of the known 1:1 isotope cluster for ^{79}Br and ^{81}Br. Interestingly, the dione **I** displayed an M^+ ion rather than an $[M + H]^+$ ion as previously found for its 1,10-isomer (21) and, indeed, the previous bromo compound. The reason for this difference is unknown, but the result is reproducible because external mass spectral analysis (electron impact, positive mode) also yielded an M^+ ion for the dione **I**.

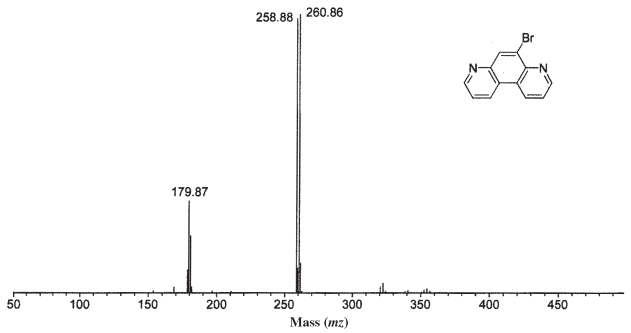

Figure 7. Mass spectrum of main impurity of 5-bromo-4,7-phenanthroline.

Table III. Validation of the HPLC Method for the Determination of Dione I

Validation criterion	Concentration range (mg/mL)	Results
Linearity	0.05 -1.50	$y = 221070x + 116721$
($n = 3$; $k = 6$)		$r^2 = 0.9991$
LOD		2.0 µg/mL
LOQ		50 µg/mL
Precision		
(A) Intra-day		
(%RSD; $n = 6$)	0.40	0.30
(B) Inter-day		
(%RSD; 2 days; $n = 6$)	0.05	0.12
	0.50	0.23
	1.50	0.17
Accuracy		
(Recovery ± %SD; $n = 6$)	0.05	98.65 ± 0.117
	0.50	99.21 ± 0.070
	1.50	98.73 ± 0.007
Stability	0.05	$t = 0.56$
	0.50	$t = 0.32$
	1.50	$t = 1.02$

Validation of the chromatographic method

Linearity

Linearity was studied using six solutions in the concentration range 0.05–1.50 mg/mL ($n = 3$). The regression equation was found by plotting the peak area (y) versus the dione **I** concentration (x) expressed in mg/mL. The correlation coefficient ($r^2 = 0.9991$) obtained for the regression line demonstrates that there is a strong linear relationship between peak area and concentration of dione **I** (Table III).

Limit of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) tests for the procedure were performed on samples containing very low concentrations of analyte. LOD is defined as the lowest concentration of analyte that can be detected above baseline noise. Typically, this is three times the noise level. LOQ is defined as the lowest concentration of analyte that can be reproducibly quantitated above the baseline noise with a signal to noise ratio of 10. In this study, the LOD was 2.0 µg/mL and the LOQ was 50 µg/mL and %RSD 0.50% ($n = 3$). A good match of the UV spectra of the 5-bromo,4-7-phenanthroline impurity was obtained at the LOD with those for other concentrations (Figure 5, Table IV).

Table IV. Chromatographic Parameters of Dione I and Its Impurity and Matching Spectral Results at LOD Concentration Levels

Compound	t_R (min)	Purity (%)	Points across peak	Purity match angle* (°)	Purity threshold angle† (°)	Lamada max
A	5.16	99.26	18	4.211	2.570	254.6
B	6.90	0.69	15	2.432	0.527	235.8

* Purity match angle = the spectral contrast between the two peaks.
† Purity threshold angle = the spectral difference attributed to noise and solvent effects.

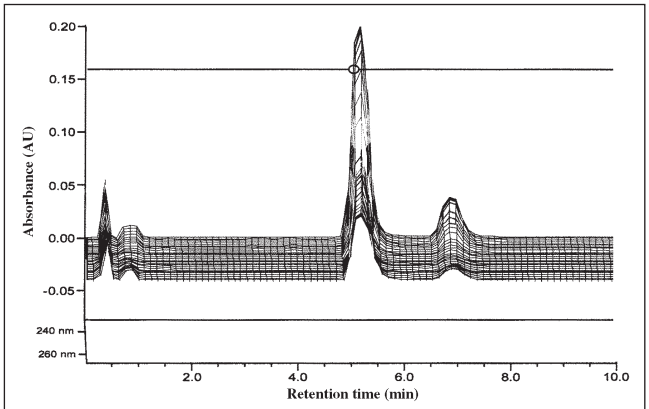

Figure 8. Three-dimensional chromatogram of the mixture of dione **I** and one of its impurity.

Precision

The precision of the chromatographic method, reported as %RSD, was estimated by measuring repeatability (intraday assay precision) on six replicate injections at 100% test concentration (0.40 mg/mL) and time-dependent (interday variation) on three replicate injections at three different concentrations (0.05, 0.50, and 1.50 mg/mL). The %RSD values presented in Table III were less than 0.40% in all cases and illustrated good precision for the analytical method.

Accuracy

The accuracy of an analytical method is determined by how close the test results obtained by that method come to the true value. It can be determined by application of the analytical procedure to an analyte of known purity (for the drug substance) or by recovery studies, where a known amount of standard is spiked in the placebo (for the drug product). In this study, a number of different solutions were prepared with a known added amount of dione **I** and injected in triplicate. Percent recoveries of response factor (area and concentration) were calculated as can be seen in Table III, and it is evident that the method is accurate within the desired range.

Stability of solutions

The stability of standard solutions was studied at concentrations of 0.05, 0.50, and 1.50 mg/mL. These solutions were stored for at least 24 h at 25°C, away from direct sunlight and reanalyzed by following the proposed method. A *t*-test ($P = 0.06$; $n = 6$) was performed to demonstrate that no significant degradation of the substance occurred (see Table III).

Specificity and selectivity

The three-dimensional PDA chromatogram (Figure 8) demonstrates a good separation of the dione **I** peak A ($t_R = 5.16$ min) from the impurity ($t_R = 6.90$ min). A wavelength of 254 nm was found to be the most effective compromise to accomplish the detection and quantitation of all impurities and the main component of dione **I** in a single run. The impurity and dione **I** peak area were adequately resolved from each other; typical resolution values for the dione **I** peak are > 2.39. This method demonstrates acceptable specificity.

Forced degradation studies were performed to evaluate the specificity of dione **I** and its impurity under four stress conditions (heat, UV light, acid, and base). Solutions of dione **I** were exposed to 50°C for 1 h, UV light using a Mineralight UVGL-58 light for 24 h, acid (1M HCl) for 24 h, and base (1M NaOH) for 4 h. A summary data of the stress results is shown in Table V. It is evident from Figure 2 that the method has been able to separate the peaks of the degraded products from that of the dione **I**. This was further confirmed by peak purity analysis on a PDA UV detector.

Table V. Assay (%) of Dione I Under Stress Conditions

Stress conditions	Sample treatment	t_R (min)	Assay (%)	Peak area (V s)
Reference	Fresh solution	5.16	99.19	9.27
Acid	1M HCl for 24 h	5.15	99.24	9.27
Base	1M NaOH for 4 h	5.16	99.26	9.30
Heat	50°C for 1 h	5.16	99.15	9.24
Light	UV light for 24 h	5.15	99.12	9.28

Conclusion

An HPLC method for the identity, assay, and purity evaluation of dione **I** and its synthetic impurities has been successfully developed. Studies have demonstrated that the method is rugged. It has also been extensively validated. 5-Bromo-4,7-phenanthroline was identified as main impurity by applying the method in HPLC–MS–APCI mode. This impurity is only present in dione **I** prepared via direct oxidation of 4,7-phenanthroline with $KBr–HNO_3–H_2SO_4$. Dione **I** obtained via oxidation of 5-MeO–4,7-phenanthroline is bromine-free.

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