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Validation of a reversed-phase HPLC method for 1,10-phenanthroline-5,6-dione and analysis of its impurities by HPLC–MS

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Abstract

A reversed-phase HPLC analytical method for the assay of 1,10-phenanthroline-5,6-dione (I) has been developed and validated. A C18 column (150 × 4.6 mm; 5 μm) was employed together with a mobile phase of methanol–water (50:50, v/v) containing 0.1% triethylamine. UV detection was performed at 254 nm. Dione (I) eluted as a spectrally pure peak resolved from its impurities allowing the method to be applied to the purity evaluation of samples obtained via two synthetic routes. In addition, 4,5-diazafluoren-9-one (V) was identified as the main impurity by employing the method in HPLC–MS mode with photodiode array UV detection.

Keywords: 1,10-Phenanthroline-5,6-dione; HPLC; HPLC–MS; Method development; Method validation; Synthetic impurities

1. Introduction

The phenanthroline-5,6-diones (I)–(III) are a useful class of heterocyclic *o*-quinone compounds (Fig. 1). Historically, in the 1950s, they were first found to be of use because of their activity against protozoa, amoebae and bacteria [1] The 4,7-isomer, also known as phanquone, proved to be the most active and was marketed as a treatment for amoebic dysentery under the tradename Entobex (Ciba Pharmaceuticals, now Novartis).

Further interest [2–4] in compounds (I)–(III) was stimulated by the similarity of their structures and reactivity to that of the pyrroloquinoline quinone derivative methoxatin or PQQ (IV), which has been identified as a cofactor in various bacterial dehydrogenase enzymes. In particular, PQQ (IV) and the analogues (I)–(III) [4] together with transition metal complexes of (I) [5] have been suggested as catalysts for the regeneration of the cofactor NAD in the presence of oxygen. Latterly, electrochemical oxidation was substituted for aerial oxidation in these catalytic systems [6]. The quinones (I)–(III) are useful as NADH mediators in biosensors containing NAD-dependent enzymes for analytes such glucose or D-3-hydroxybutyrate [7,8]. Compound (I) has found

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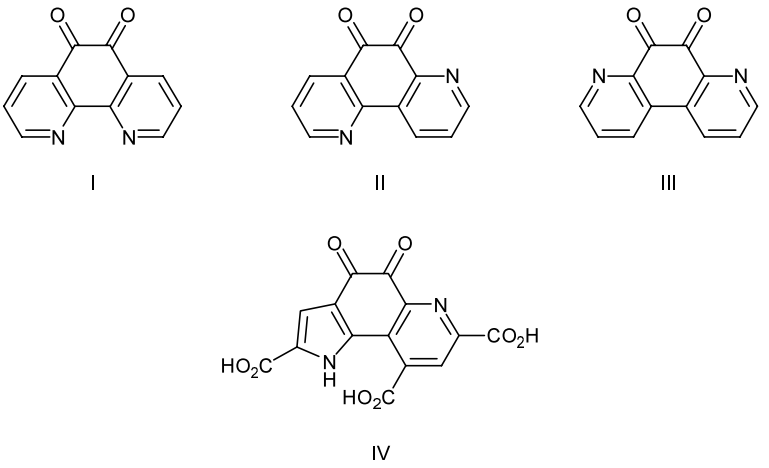


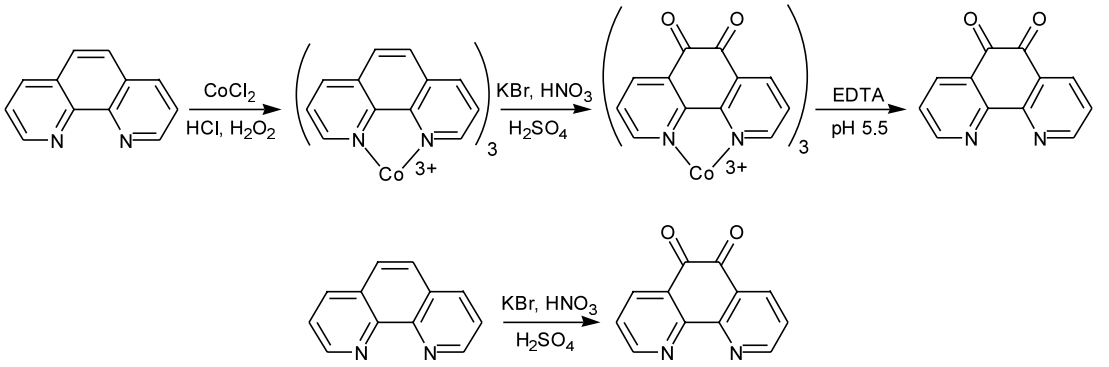
Fig. 1. Heterocyclic *o*-quinone compounds.

widespread use as a starting material for the preparation of phenanthroline ligands for transition metals, particularly ruthenium.

The 1,10-isomer (I) was first reported by Smith and Cagle [9] in 1947 as a minor by-product (2% yield) of the nitration of 1,10-phenanthroline. Early attempts to prepare (I)–(III) via the direct oxidation of the parent phenanthroline using reagents such as chromic acid, selenium dioxide, vanadium pentoxide, iodic acid and periodic acid were unsuccessful [10]. However, Druey and Schmidt [11] did achieve the first syntheses of (I)–(III) in good yield by oxidising the appropriate 5-methoxy phenanthroline derivative with a concentrated nitric/sulphuric acid mixture at 120 °C. This route is also described in a patent [12] assigned to Ciba Ltd. A detailed method has been reported [13] for the case of the 4,7-isomer (III). The 1,10-isomer (I) can also be obtained by nitric acid oxidation of 5-amino-1,10-phenanthroline derived from the 5-nitro derivative [14,15]. Direct oxidation of 1,10-phenanthroline, as a cobalt complex, was first achieved in 1970 by adding potassium bromide to the nitric/sulphuric acid oxidising medium [13]. Latterly, the use of the $KBr/HNO_3/H_2SO_4$ mixture was applied successfully in the preparation of (I) from 1,10-phenanthroline in one step by several groups [16–18]. Compounds (II) and (III) can be similarly prepared using more vigorous reaction conditions. The one-step oxidation and three-step cobalt complexation routes to (I) are illustrated in Scheme 1.

We were interested in assessing the purity of the quinone (I) prepared by the two main synthetic routes [13,16] and identifying any impurities, where possible, to evaluate its suitability for use as a mediator to NADH in biosensors for diabetics. A number of potential impurities were expected from an examination of the literature concerning the reactivity of the phenanthroline-5,6-diones (Fig. 2). Thus, compound (I) is known [11,19] to undergo alkaline decarboxylation to afford 4,5-diazafluoren-9-one (V). Here, (V) may be obtained during the neutralisation of highly acidic reaction mixtures in the preparation of (I). The use of $KBr/HNO_3/H_2SO_4$ reaction medium may result in the formation of 5-bromo-1,10-phenanthroline (VI) [16,20] and 5-nitro-1,10-phenanthroline (VII) [9,21] as by-products. Under certain oxidation conditions, the 1,10-isomer (I) can be converted to 2,2′-bipyridine-3,3′-dicarboxylic acid (VIII) or 1*H*-cyclopenta[2,1-*b*:3,4-*b*′]dipyridine-2-5-dione (IX) as reported by Baxter et al. [22].

Impurity profiling is an important issue in pharmaceutical analysis, particularly during product development and quality control. The standard requirements of such an impurity method [23] are that all likely synthetic and degradative



Scheme 1. Synthesis of 1,10-phenanthroline-5,6-dione (I).

impurities are resolved from each other and the main drug, and [24] that the impurities can be monitored at the 0.1% (w/w) level or below. Guidance for controlling impurity levels in drug substances has been developed by the International conference on harmonisation (ICH) [25]. Normally, synthetic impurities are discovered during routine HPLC analysis of the drug substance [26]. An impurity profile of a synthetic drug may require the use of complementary chromatographic methods such as HPLC/diode array UV and HPLC–MS to permit the observation of non-UV absorbing synthetic impurities.

The combination of MS and photodiode array detection (PDA) provides a powerful tool for controlling 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.

GLC [27] has been used previously for the determination of phanquone (III) in biological material. Here, in this article, we describe a

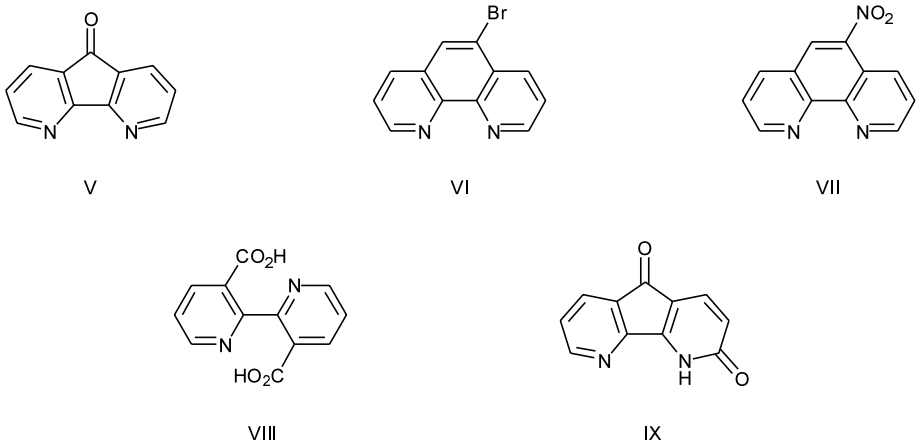


Fig. 2. Potential impurities in 1,10-phenanthroline-5,6-dione (I).

validated HPLC method for the assay of the quinone (I) and the application of HPLC–MS for identification of impurities in this compound.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were of the highest purity. HPLC-grade methanol and triethylamine were obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, Watford, UK). 1,10-Phenanthroline-5,6-dione (I) was prepared by the oxidation of 1,10-phenanthroline itself [16] and its cobalt complex [13]. Anhydrous 1,10-phenanthroline (I) was supplied by Lancaster Synthesis (Lancaster, UK). 4,5-Diazafluoren-9-one (V) was synthesised according to a literature method [11]. 5-Nitro-1,10-phenanthroline (VII) was obtained from Aldrich (Gillingham, UK).

2.2. HPLC–MS instrumentation

HPLC–MS analysis was performed using a Waters ZQ2000 single quadrupole mass spectrometer, a Waters Alliance 2690 Separations Module to a 996 Waters PDA detector system (Waters, Elstree, UK). A stainless steel C18 column (250 × 2.0 mm i.d., 5 μm particle size) was used. A 2μl aliquot of a 0.6 mg/ml solution of 1,10-phenanthroline-5,6-dione (I) was introduced by the HPLC system into the mass spectrometer via flow injection in a mobile phase of methanol:water (50:50, v/v) at 0.8 ml/min. The sample was ionised by positive-ion electrospray ionisation (ESI) probe in the positive ion mode using atmospheric pressure chemical ionisation (APCI) under the following source conditions: source temperature, 100 °C; capillary potential, 3.0 kV; sampling cone potential, 30 V. Mass spectra were obtained over the scan range 100–650 Da at a rate of 1 scan per s with 15.0 resolution and wavelength range 210–400 nm.

2.3. HPLC instrumentation

The HPLC system consisted of a Waters Alliance 2690 Separations Module to a 996 Waters PDA Detector. Chromatographic separation was achieved isocratically in reversed-phase columns of the following type: Luna column C18 (150 × 4.60 mm i.d.) from Phenomenex UK (Macclesfield, UK), packed with silica, lowest silanol activity (100 Å, 5 μm particle size, surface area 400 m^2/g, 17.5% carbon loading) was used. The mobile phase comprised methanol:water (50:50, v/v) containing 0.1% triethylamine and was filtered through a 0.45μm paper filter (Type HUHP, Millipore) before use. Degassing of the mobile phase was carried out continuously with an on-line degasser. Flow rate was 0.8 ml/min. The system was equilibrated for approximately 30 min. The PDA detector was set at 254 nm. The column temperature was held at 40 °C. The chromatographic run time was 10 min. The control of the HPLC system and data collection was by a Compaq computer equipped with WATERS MILLENIUM[32] software (version 3.20).

2.4. Preparation of the standard and sample solutions

All sample and standard solutions at 0.6 mg/ml were prepared by dissolving approximately 60 mg of 1,10-phenanthroline-5,6-dione (I) in 100 ml HPLC grade methanol.

2.5. Linearity assessment

Linearity experiments were performed by preparing dione (I) standard solutions in the range 0.04–1.60 mg/ml in methanol and injected in duplicate. Linear regression analysis was carried out on the standard curve generated by plotting the concentration of dione (I) versus peak area response.

2.6. Calculations and other HPLC determinations

2.6.1. 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.

2.6.2. Related substances

The quantity of each impurity peak (the known impurities designated as peaks (a) and (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 of the specifications (Table 1).

2.6.3. Assay

The area percent of the main dione (I) peak, designated as peak (c), is determined in a similar manner to the impurities. The area percent must fall within the specifications (Table 1).

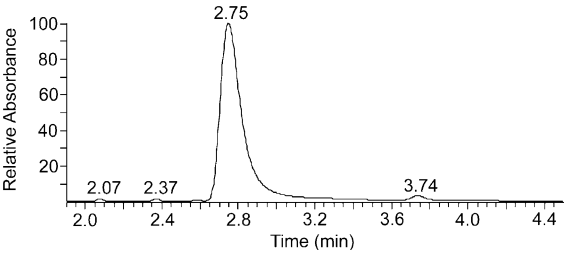


Fig. 3. HPLC chromatogram of crude 1,10-phenanthroline-5,6-dione (I) displaying its impurities. This material was produced via the one-step oxidation method and is not crystallised.

2.8. Precision (repeatability and reproducibility)

The precision of the method was investigated by performing ten determinations of the same batch of dione (I) at 100% of the test concentration by only one operator. The repeatability (within-run precision) was evaluated by only one analyst within 1 day, whereas reproducibility (between-run precision) was evaluated for 2 different days.

$$\text{Percent purity} = \frac{\text{mg/100 ml of reference standard} \times \text{total area of peak 3 from sample}}{\text{area of peak 3 from reference standard} \times \text{mg/100 ml of sample}}$$

2.6.4. Purity

The purity of the dione (I) drug substance is calculated in relation to the reference standard using the area of the main peak (3):

2.7. Forced degradation studies

Solutions of dione (I) were exposed to 50 °C for 1 h, UV light using a Mineralight UVGL-58 light for 24 h, acid (1 M HCl) for 24 h and base (1 M NaOH) for 4 h.

2.9. Stability of analytical solutions

Test solutions of three batches of dione (I) were prepared using the conditions cited in Section 2.4. They were chromatographed at the beginning and after 24 h. The stability of dione (I) and the mobile

Table 1
Limits for the chromatographic purity of 1,10-phenanthroline-5,6-dione (I) and its impurities at 254 nm

	Related substances		Assay
Peak assignment	1(a)[a]	2(b)[a]	3(c)[a]
RT (ca. min)	1.4	2.1	2.5
Limit as percent total area	≤2.0%	≤2.0%	
	a+b+all other impurities. No other peak area can exceed 2.0%	≤4.0%	c≥96.0%

[a] a, impurity 1; b, impurity 2; c, 1,10-phenanthroline-5,6-dione (I).

Table 2
Chromatographic results for 1,10-phenanthroline-5,6-dione (I) and its impurities

Compounds	RTs (min)	Peak area (μV s)	Peak area (%)
Impurity 1	2.07	25 404	0.64
Impurity 2	2.37	23 658	0.59
1,10-Phenanthroline-5,6-dione (I)	2.75	3 725 059	93.21
Impurity 3	3.74	107 954	2.70

phase were calculated by comparing area response and area percent of two standards at 0.6 mg/ml over time.

3. Results and discussion

3.1. Identification of impurities by HPLC–MS

Good separation of 1,10-phenanthroline-5,6-dione (I) from its synthetic impurities was achieved by HPLC as demonstrated by the chromatogram displayed in Fig. 3. The chromatographic run yielded four major peaks that are detailed by area percent in Table 2. Here, the data is from the analysis of a sample of crude (I) derived from the one-step oxidation of 1,10-phenanthroline.

The first two peaks at (2.07 and 2.37 min) are minor impurities while the third (3.74 min) is the major impurity. The peak at 2.75 min is identified as that due to 1,10-phenanthroline-5,6-dione (I) since its UV spectrum matches that of a known sample of (I) as shown in Fig. 4.

It was not possible to identify the chemical structure of the impurities by HPLC alone. For this purpose, HPLC–MS was selected to obtain structural information. The full scan HPLC–MS spectra of dione (I) and its impurities were measured in the mass range m/z 100–650. Dione

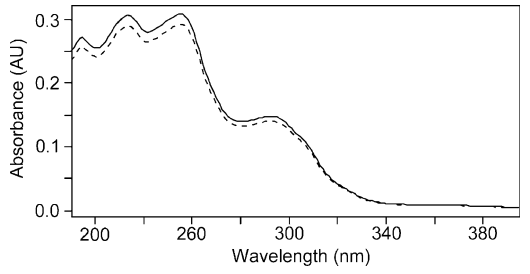


Fig. 4. PDA UV match spectra of the middle of the peak corresponding to the RT of the main component of 1,10-phenanthroline-5,6-dione (I) and a reference sample. See Section 2.3 for HPLC conditions.

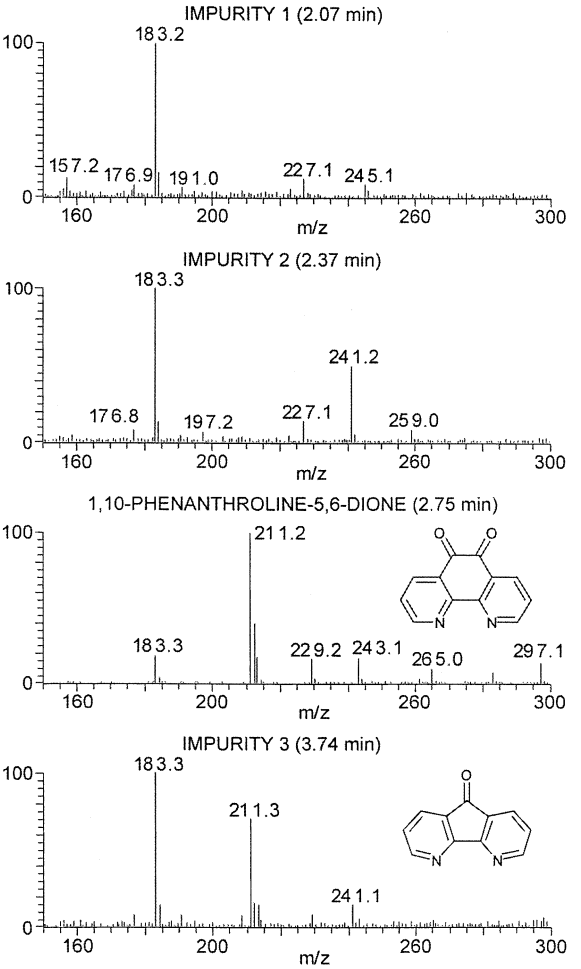


Fig. 5. Mass spectra (x-axis: relative abundance) of 1,10-phenanthroline-5,6-dione (I) and its impurities 1–3.

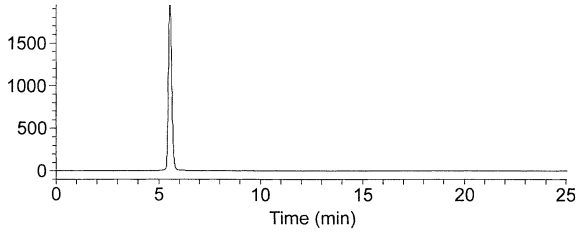


Fig. 6. HPLC chromatogram of 4,5-diazafluoren-9-one (V), (impurity 3).

(I) displayed a single peak at 2.75 min with a corresponding protonated molecular ion of mass *m*/*z* 211.2. Impurity peak 3 at 3.74 min was assigned to 4,5-diazafluoren-9-one (V) on the basis of both MS (Fig. 5) and HPLC (Fig. 6) data. Again, a protonated molecular ion of mass *m*/*z* 183.3 was observed in the mass spectrum.

The chemical structure of impurities 1 and 2 could not be identified from the HPLC and HPLC–MS data. However, the ions at *m*/*z* 183.3 and 241.2 for the two impurities (2.07 and 2.37 min, Fig. 5) do not correspond with the other suspected impurities such as 1,10-phenanthroline, 5-bromo-1,0-phenanthroline (VI), 5-nitro-1,10-phenanthroline (VII), 2,2′-bipyridine-3,3′-dicarboxylic acid (VIII) or 1*H*-cyclopenta[2,1-*b*:3,4-*b*′]dipyridine-2-5-dione (IX).

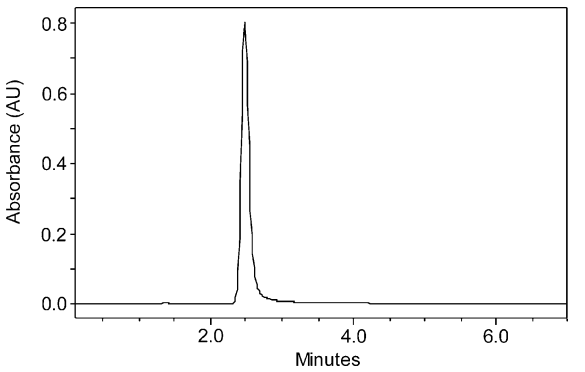


Fig. 7. HPLC chromatogram of 1,10-phenanthroline-5,6-dione (I) and its potential impurities. This material was produced via the three-step route and is crystallised.

3.2. Syntheses

1,10-Phenanthroline-5,6-dione (I) was prepared according to the two methods in Scheme 1. Direct oxidation has the advantage in being a rapid one-step route from the readily available starting material 1,10-phenanthroline. As such, this method has proved very popular in the literature. In contrast, the cobalt complexation route is longer with two additional steps involving initial formation of the cobalt-phenanthroline compound and then final decomplexation of the product (I) using EDTA. Overall yields are moderate at 35–40% based on 1,10-phenanthroline. However, the direct oxidation step proceeds more cleanly and the cobalt-phenanthroline dione complex is isolated directly by precipitation with perchlorate ion. There is no requirement to neutralise the strongly acidic reaction mixture with the consequent risk of forming 4,5-diazafluoren-9-one (V). As such, the three-step route to (I) affords a purer product where (V) is completely absent (Fig. 7). The crude dione (I) obtained from the one-step method can be purified by crystallisation from hot methanol solution which is filtered prior to cooling. The resulting crystallised material contains a reduced level of (V) as detected by HPLC. The unidentified impurities 1 and 2 appeared in samples of dione (I) produced by both synthetic methods.

3.3. HPLC method development and optimisation

Efficient chromatography and high sensitivity was achieved by using methanol–water as the mobile phase with varying detection wavelengths, based on the response of the active. However, the main peak tailed badly on some C18 columns with these mobile phases. Addition of triethylamine minimised the tailing. The amount of organic modifier was adjusted so that the assay run time could be reduced for faster analysis of samples. A chromatogram illustrating the separation of 1,10-phenanthroline-5,6-dione (I) and the two potential impurities is illustrated in Fig. 7 confirming specificity with respect to dione (I).

The remaining chromatographic conditions listed in HPLC experimental section were chosen for the following reasons: the lower flow rate of 0.8

ml/min was chosen because of the potential problems associated with elevated back pressures. The PDA UV detector was set at 254 nm, λ_{max} for dione (I).

Column temperature was held at 40 °C although separations at 30 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 0.6 mg 1,10-phenanthroline-5,6-dione/ml in methanol were chosen to simplify sample preparation (further dilution is not needed). This concentration allows both assay (of the main component) and purity evaluation (of trace impurities). The peak for dione (I) is well within the linear range for UV detection, and trace components are readily detectable.

A system suitability test was developed for the routine application of the assay method. Prior to each analysis, the chromatographic system must satisfy suitability test requirements (resolution and repeatability). Peak-to-peak resolution, between each peak measured on a reference solution, must be above 1.0. The percent relative standard deviation (%R.S.D.) of the response factor (area:-mass ratio) for dione (I) sample peaks was determined from six replicate injections of the reference solutions and is required to be less than 2.0%.

System suitability testing was performed to determine the accuracy and precision of the system from six replicate injections of a solution containing 0.6 mg 1,10-phenanthroline-5,6-dione/ml. All peaks were well resolved and the precision of injections for all peaks was acceptable. The %R.S.D. of the peak area responses was measured, giving an average of 0.62 ($N = 6$). The tailing factor (T) for each dione (I) peak was 1.61 and the theoretical plate number (N) was 3035.45. The resolutions between each peak were > 1.2 and the %R.S.D. of retention time (RT) was $\leq$ 2% for six injections.

Selectivity was also studied over extended time using several columns and many different batches of mobile phase. Relative RT ranges (RT of peak of interest/RT of 1,10-phenanthroline-5,6-dione) were as follows: a = 0.45; b = 1.20; c = 1.60. These data indicate that the RT windows for each impurity are unique and do not overlap. For rugged separations such as this, impurity identification could be based on relative RT alone. It would not be necessary to inject an authentic standard of each impurity to confirm identification. Overall selectivity was established through determination of purity for each impurity peak using the PDA UV detector.

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 column from different lots. In all cases, good separations were always achieved, indicating that the method remained selective for all components under the tested conditions.

Table 3
Linearity assessment of the HPLC method for the assay of 1,10-phenanthroline-5,6-dione (I) employing the analytical working standard dissolved in methanol

Concentration of dione (I) (mg/ml)	Concentration as percent of 0.6 mg/ml of dione (I)	Dione (I) peak area as mean of three injections (μV s) (day 1)	Dione (I) peak area as mean of two injections (μV s) (day 2)
0.0400	4.0	225 761	241 740
0.1000	10	577 405	616 281
0.2002	20	1 126 188	1 401 765
0.4004	40	2 574 365	3 063 222
1.0009	100	6 561 498	8 541 076
1.6016	160	10 458 276	13 686 533
Correlation coefficient	0.9998		0.9996
Intercept (%)	−90		−26
Equation for regression line	$y = 7E+06x-90\,335$		$y = 9E+06x-263\,239$

3.4. Validation of the method

3.4.1. Linearity

The linearity of the method should be tested in order to demonstrate a proportional relationship of response versus analyte concentration over the working range. It is usual practice to perform linearity experiments over a wide range of the nominal concentration of analyte [28]. This gives confidence that the response and concentration are proportional and consequently ensures that calculations can be performed using a single reference standard, rather than the equation of a calibration line.

Good linearity of this method was seen in the concentration range 0.04–1.60 mg/ml. Table 3 displays an assessment of the linearity results.

Table 4
Recovery studies

Analyte level (percent of target)	Recovery (%) ($n = 3$)
10	100.00
20	100.00
40	100.00
100	99.79
160	99.76

3.4.2. Accuracy/recovery studies

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 a known added amount of dione (I) and injected in triplicate. Percent recoveries of response factor (area/concentration) were calculated as shown in Table 4.

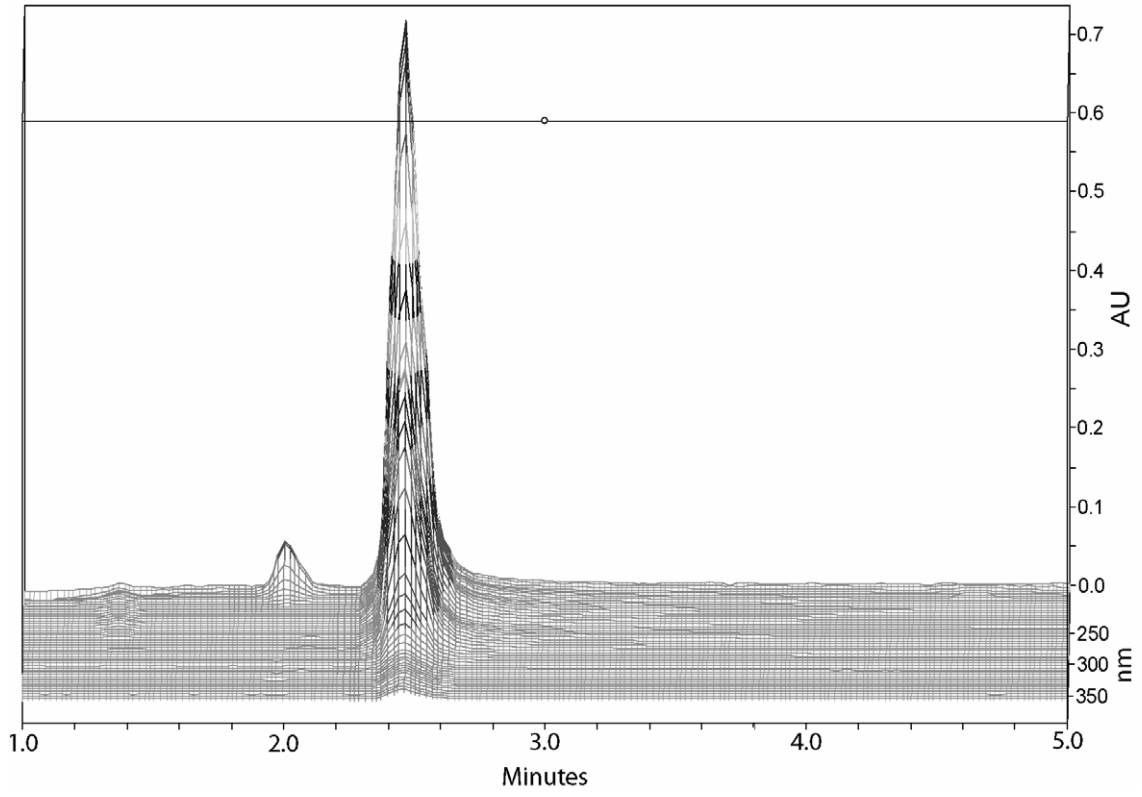


Fig. 8. Three-dimensional chromatogram of the mixture of 1,10-phenanthroline-5,6-dione (I) and two of its impurities.

Table 5
Assay (%) of 1,10-phenanthroline-5,6-dione (I) under stress conditions

Stress conditions	Sample treatment	RT (min)	Assay (%)	Peak area (μV s)
Reference	Fresh solution	2.35	99.91	8 582 691
Acid	1 N HCl for 24 h	2.30	99.94	8 753 142
Base	1 N NaOH for 4 h	2.35	99.50	8 697 192
Heat	50 °C for 1 h	2.35	99.80	8 630 907
Light	UV Light for 24 h	2.30	99.25	7 189 035

3.4.3. Specificity/selectivity

The PDA three-dimensional chromatogram (Fig. 8) demonstrates a good separation of the dione (I) peak (c) (RT = 2.5 min) and from the impurities (RT = 1.4 and 2.0 min) and of the impurities from each other. A wavelength of 254 nm was found to be the most effective compromise to accomplish the detection and quantification of the two impurities and the main dione (I) component in a single run.

The impurities and the dione (I) peaks are adequately resolved from each other; typical resolution values for the dione (I) peak are $\geq$ 1.5. This method demonstrates acceptable specificity.

Forced degradation studies were performed to evaluate the specificity of dione (I) and its impurities under four stress conditions (heat, UV light, acid, base). A summary of the stress results is shown in Table 5.

It is evident from Fig. 7 that the method has been able to separate the peaks due to the degraded products from that of the dione (I). This was further confirmed by peak purity analysis on a PDA UV detector.

3.4.4. Precision (repeatability and reproducibility)

Percent recoveries were obtained from within- and between-run precision studies (Table 6). The %R.S.D. values for the within run and between run precision studies are $<1\%$, thereby confirming that the method has acceptable precision.

3.4.5. Limit of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) tests for the procedure were performed on samples containing very low concentrations of analyte. LOD is defined as the lowest amount of analyte that can be detected above baseline noise. Typically, this is three times the noise level. LOQ is defined as the lowest amount of analyte that can be quantified reproducibly above the baseline noise with a signal to noise ratio of 10. In this study, the LOD was 2.0 and the LOQ was 200 μg/ml and R.S.D. 0.45% ($n = 3$). An excellent match of the UV spectra of the impurities obtained at the LOD with those obtained at other concentrations (Fig. 4, Table 7) was observed.

3.4.6. Stability of analytical solutions

The stability of 1,10-phenanthroline-5,6-dione (I) solutions was investigated. The solutions were stable during the investigated 24 h and the %R.S.D. was in between 0.09 and 0.19% for RTs. Standard solutions stored in a capped volu-

Table 6
Comparison of within and between-run precision studies for 1,10-phenanthroline-5,6-dione (I)

Injection #	Recovery day 1 (%)	Recovery day 2 (%)
1	99.93	100.00
2	99.88	98.62
3	99.87	99.95
4	99.86	99.84
5	99.84	99.94
6	99.83	99.83
7	99.83	99.83
8	99.81	99.95
9	99.80	99.95
10	99.79	99.95
Average	99.84	99.79
%R.S.D.	0.04	0.44
Total average	99.82	
Total %R.S.D.	0.035	

Table 7
Chromatographic parameters of 1,10-phenanthroline-5,6-dione (I) and its impurities and matching spectral results at LOD concentration levels

Compound	RT (min)	Purity (%)	Points across peak	Purity match angle[a] (°)	Purity threshold angle[b] (°)	λ_{max}
a	1.367	0.07	19	5.166	3.049	217.0
b	2.095	0.12	16	3.512	0.643	214.7
c	2.454	99.81	136	0.412	0.222	254.7

[a] Purity match angle, the spectral contrast between the two peaks.
[b] Purity threshold angle, the spectral difference attributed to noise and solvent effects.

Table 8
Stability of 1,10-phenanthroline-5,6-dione (I) in solution ($n = 4$)

Time (h)	Area (%R.S.D.)	Height (%R.S.D.)	Recovery (%)	Percent of initial
0	0.40	0.003	99.88	
24	0.37	0.008	99.78	99.35

metric flask on a laboratory bench under normal lighting conditions for 24 h, were shown to be stable with no significant change in dione (I) concentration over this period (Table 8). This is indicated (0.6% changes in area between $T = 0$ and $T = 24$ h). Based on these data that show quantitative recovery through 24 h, solutions of dione (I) can be assayed within 24 h of preparation.

4. Conclusions

A reversed-phase HPLC method for the assay and purity evaluation of 1,10-phenanthroline-5,6-dione (I) obtained via two synthetic routes is described. The method has been demonstrated to be rugged and has been extensively validated. Application of the method in HPLC–MS mode resulted in the identification of 4,5-diazafluoren-9-one (V) as the major impurity in dione (I) prepared via the one-step oxidation of 1,10-phenanthroline. In contrast, the impurity (V) was absent from samples of dione (I) produced using a three-step cobalt complexation route from 1,10-phenanthroline. As such, this latter method is preferred for the synthesis of high purity samples of dione (I). Two minor impurities apparent in dione (I) synthesised via both routes remained unidentified.

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