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Development and validation of HPLC-DAD methods for the analysis of two novel iron chelators with potent anti-cancer activity

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Abstract

Di-2-pyridylketone isonicotinoyl hydrazone (PKIH) and di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT) novel iron chelators which possess marked anti-cancer activity *in vivo*. However, further progress in the development of these drug candidates requires precise and convenient methods for their qualitative and quantitative analysis. The aim of this study was to develop and validate HPLC methods suitable for the purity and stability evaluation of Dp44mT and PKIH and subsequently to employ these methods in stress tests addressing their chemical stability. The chromatographic analyses of both chelators were accomplished *via* HPLC using a Discovery HSF5 column (25 cm × 4 mm; 5 μm). For separation of Dp44mT and its synthetic precursors, the mobile phase was composed of a mixture of 2 mM EDTA and acetonitrile in a ratio 60:40 (v/v). A desirable separation of PKIH from its synthetic precursors was achieved with a mixture of 0.01 M phosphate buffer (pH 3.0), methanol and acetonitrile in a ratio of 65:21:14 (v/v/v) with the addition of EDTA (2 mM). In order to confirm the utility of these HPLC methods for measuring these drugs and their stability, Dp44mT and PKIH were subjected to chemical stress tests. These experiments showed that Dp44mT was relatively stable against hydrolytic degradation, but quite sensitive to oxidation. On the other hand, PKIH was slightly sensitive to acid-catalyzed hydrolysis, but it was relatively stable under other tested conditions. Furthermore, these studies confirmed the utility of these methods not only for appropriate evaluation of purity but also stability. The analytical methods developed and validated in this study, as well as the basic data on the chemical stability, should further support the development of both these novel anti-cancer chelators as promising drug candidates.

Keywords: Di-2-pyridylketone isonicotinoyl hydrazone; PKIH; Di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone; Dp44mT; Iron chelator; HPLC; Stability

1. Introduction

Despite enormous scientific effort over many decades, cancer remains an important clinical problem. New drugs with novel mechanisms of anti-tumor action could considerably boost the current therapeutic regimens and the prognosis of patients suffering from cancer. Due to the extensive demand for iron (Fe) during proliferation and metabolism, tumor cells have been shown to be markedly more sensitive to Fe-deprivation than normal cells [1–4]. Therefore, the principle of selective Fe-depletion is a unique strategy for novel anti-cancer drug development.

The anti-proliferative effects of iron chelators were first documented *in vitro* using the drug desferrioxamine (DFO), which is currently indicated for the treatment of Fe overload disease [2,4]. Many subsequent studies in a wide variety *in vitro* and in clinical trials have demonstrated the anti-tumor activity of DFO [5,6]. However, the limited permeability of this compound across membranes into intracellular compartments [7,8] together with extremely short half-life hampered its use as an effective anti-cancer drug [2].

Apart from DFO, intriguing anti-cancer activity was demonstrated in the thiosemicarbazone class of Fe chelators. To date, the most significant progress has been made with 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP or Triapine®) [9–14] which is currently in the Phase II of the clinical evaluation [2]. Other chelators tested for anti-tumor activity include tachpyridine [15,16], O-trensox [17], ICL-670A

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[18], diethylenetriaminepentaacetic acid [6] and aroylhydrazone chelators such as those of the pyridoxal isonicotinoyl hydrazone class [19,20]. Structure-activity relationship investigations focused on the development of novel cell-permeable iron chelators of the aroylhydrazone class were performed [19]. These studies identified key classes of chelators with high Fe chelation efficacy and anti-proliferative activity, with compounds belonging to the 2-hydroxy-1-naphthyladehyde benzoyl hydrazone series being particularly effective (e.g., chelator 311) [19]. Subsequent studies based upon these observations identified the di-2-pyridylketone isonicotinoyl hydrazone (PKIH) series of chelators which possessed more potent anti-proliferative activity along with the minimal anti-proliferative effects on normal cells [21,22]. Further systematic studies of the structure-activity relationships within this group revealed that the di-2-pyridyl moiety is important for pronounced anti-tumor activity both *in vitro* and *in vivo* [23,24]. Hence, this moiety was introduced into the basic chemical structure of thiosemicarbazones and thus a novel group of thiosemicarbazone analogs was developed, namely the di-2-pyridyl thiosemicarbazone (DpT) chelators [23,24]. Among the DpT series of ligands, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT), was shown to be the most effective in terms of anti-tumor activity. Interestingly, its anti-proliferative efficacy was comparable or many fold greater than doxorubicin, one of the most potent clinically used anti-neoplastic agents [23,24]. The mechanism of action of these DpT chelators relates to their ability to bind intracellular Fe and redox cycle to generate cytotoxic free radicals [21,23,25,26].

As described above, previous investigations recognized both Dp44mT and PKIH (Fig. 1) as potent anti-proliferative agents which deserved to be further intensively studied. Nevertheless, progress in the development of these drug candidates requires modern analytical methodologies appropriate for the qualitative and quantitative evaluation of these compounds. These methods are also fundamental to the pharmacological development of these agents as clinically useful anti-tumor drugs [27].

One of the important functions of the modern analytical methods is to reliably assess the stability of both drug substances and pharmaceutical formulations. Since inherent chemical stability of the drug molecule largely determines the stability of the final pharmaceutical product, regulatory authorities require novel chemical entities (NCE) to undergo extensive chemical stability evaluation (stress testing) following the internationally accepted guidelines of the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use [28]. The purpose of these chemical stress tests is to reveal the inherent stability of the novel molecule, to recognize potential degradation pathways as well as to identify likely degradation products. Moreover, the analyses of the samples subjected to chemical stress testing are useful for confirming the stability indicating power of the analytical methods [28]. To date, no information about the stability of either Dp44mT or PKIH are available in the literature.

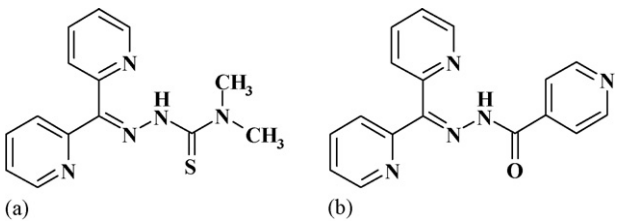

Fig. 1. The chemical structure of Dp44mT (a) and PKIH (b).

Despite the promising anti-proliferative efficacy of both Dp44mT and PKIH no modern analytical methods suitable for their analyses are available in the literature. However, RP-HPLC methods for the analyses of structurally related aroylhadrazone iron chelators with lower anticancer potential [19] but more pronounced iron mobilizing, antioxidative and cardioprotective effects [29–32] have been recently developed [33–35]. These isocratic methods employed C18 reverse phase HPLC columns and different mobile phases with addition of EDTA to prevent artificial formation of complexes with free or loosely bound metals within chromatographic systems [33–35]. With respect to Dp44mT, the only analytical methods for the analysis of structurally related compounds are those focusing on Triapine [36,37].

The aim of this study was to develop and validate HPLC methods suitable for the purity and stability evaluation of Dp44mT and PKIH. In order to evaluate the stability-indicating power of the HPLC methods and to obtain basic data on the stability of these new substances, both Dp44mT and PKIH were subjected to stress conditions recommended by the ICH.

2. Experimental

2.1. Chemicals

Dp44mT and PKIH were synthesized by Schiff condensation and characterized as described previously [22,23,26]. The precursors of these chelators, namely 2,2′-dipyridylketone, 4,4-dimethyl-3-thiosemicarbazide and isoniazid (PKIH) were obtained from Sigma-Aldrich (Munich, Germany). EDTA, $NaH_2PO_4{\cdot}2H_2O$, phosphoric acid, hydrochloric acid, sodium hydroxide and hydrogen peroxide were purchased from Sigma–Aldrich (Munich, Germany). Methanol and acetonitrile were obtained from Penta (Prague, Czech Republic).

2.2. Chromatographic systems and conditions

The chromatographic system LC 20A (Shimadzu, Duisburg, Germany) consisted of a DGU-20A3 degasser, LC-20 AD pumps, a SIL-20 AC autosampler, a CTO-20AC column oven, a SPD-M10AVP UV/Vis detector and a CBM-20AC communication module. Peak purity analysis was carried out using SPD-M20A Photodiode Array Detector. The chromatographic data were analysed using LC solution software, version 1.21 SP1 (Schimadzu, Duisburg, Germany). The separations were achieved on an analytical chromatographic column (Discovery HS F5, 250 mm × 4 mm, 5 μm, Supelco, Prague, Czech Republic) at 40 °C. A flow rate of 1.0 mL/min was found to be optimal for the analyses. The UV detector was set to the dual wavelength mode. The first channel worked at 275 nm, the

second channel monitored a wavelength of either 244 nm (for analysis of Dp44mT) or 260 nm (PKIH). An injection volume of 30 μL was used in these analyses. Dp44mT and its synthetic precursors were separated using a mixture of 2 mM EDTA and acetonitrile (60:40; v/v) as the mobile phase. Appropriate separation of PKIH and its synthetic precursors were achieved using a mobile phase composed of phosphate buffer (0.01 M Na_2HPO_4, 2 mM EDTA, adjusted to pH 3 using 25% H_3PO_4), methanol and acetonitrile in a ratio of 65:21:14 (v/v/v).

2.3. Preparation of stock solutions

The stock solutions (500 μg/mL) of Dp44mT, PKIH and 2,2′-dipyridylketone were prepared by dissolution of these agents in methanol. In order to obtain the stock solutions (500 μg/mL) of 4,4-dimethyl-3-thiosemicarbazide and isoniazid, appropriate amounts of the substances were dissolved in DMSO and water, respectively.

2.4. Analytical method validation

2.4.1. Limit of detection (LOD), lower limit of quantification (LOQ)

The LOD and LOQ of synthetic precursors were determined as the lowest concentrations of the analytes that provided signal-to-noise ratios of 3:1 and 10:1, respectively [38].

2.4.2. Linearity

The linearity of the methods was confirmed using standard solutions at six different concentrations of analytes within the range of 15–150 μg/mL and 5–100 μg/mL for the chelators and the synthetic precursors, respectively. These standard solutions were prepared by appropriate dilution of stock solutions with acetonitrile (Dp44mT) or a mixture of methanol and 2 mM EDTA in a ratio 50:50 (PKIH). Each solution was analyzed in triplicate. Mean peak area values were plotted against the corresponding concentrations of analytes. The linearity of this plot was evaluated using least-squares linear regression analysis.

2.4.3. Precision and accuracy

Intra-day precision was determined as the R.S.D. calculated from the analyses of six individually prepared standard solutions at three concentrations (16, 60 and 150 μg/mL) and (5, 50 and 100 μg/mL) for the chelators and synthetic precursors, respectively. The same experiment was repeated the next day to determine inter-day precision. The accuracy was calculated as a recovery of these analyses.

2.5. Stress tests

The inherent chemical stability of the chelators as well as the stability-indicating power of the methods were evaluated by the analyses of the samples exposed to basic chemical and physical stress conditions. According to ICH recommendations, the degradation behaviour of these chelators was studied under acidic, alkaline, neutral and oxidative conditions as well as heat and UV radiation [28,39].

2.5.1. Acidic, alkaline, neutral conditions and oxidative stress

For purposes of the stability study, the stock solutions of both chelators (2 mg/mL) were prepared by dissolving an appropriate amount of Dp44mT and PKIH in methanol. Then 1 mL of the stock solutions was mixed with the same volume of each degradation media (1 M HCl, 1 M NaOH, water or 3% hydrogen peroxide) to achieve the test solutions. The test solutions were maintained in a water bath at 50 °C for 240 min. After this time period, 0.2 mL of each test solution was mixed with either 0.1 mL of the acid or alkali or 0.1 mL of phosphate buffer at pH 7.0 (neutral and oxidative degradation). Finally, all samples were diluted with 0.1 mL of phosphate buffer (pH 7.0) followed by 1.6 mL of acetonitrile (Dp44mT) or methanol (PKIH) and then analyzed in triplicate. The control samples composed of methanol and the degradation media (1:1, v/v) were analysed as well.

2.5.2. Dry heat and UV degradation study

Approximately 10 mg of both substances were laid out uniformly under a UV lamp set at 254 nm or maintained in an oven at 80 °C. After 24 h, an appropriate amount of each substance was dissolved in acetonitrile (Dp44mT) or a mixture of methanol and 2 mM EDTA in a ratio 1:1 (PKIH) and analyzed employing the HPLC methods.

3. Results and discussion

3.1. HPLC method development

In order to achieve sufficient separations of each chelator from its synthetic precursors, different chromatographic conditions were tested. In initial experiments, a standard HPLC column (250 mm × 4 mm, Merck, Germany) packed with LiChrospher 100 RP-C18 (5 μm) as a stationary phase was selected for separations. However, analyses using the chelators suffered from the unacceptable peaks shapes and the column was then changed to a Discovery HSF5 (250 mm × 4 mm, 5 μm; Supelco, Prague, Czech Republic). This stationary phase allowed maintenance of the peak shapes within an acceptable range (Table 1). Mixtures composed of water or phosphate

Table 1
The tailing factors of the chelators Dp44mT and PKIH and their respective precursor compounds using a Discovery HSF5 column

Compound ($n = 3$)[a]	Tailing factor
Dp44mT	1.187
4,4-Dimethyl-3-thiosemicarbazide	1.262
2,2'-Dipyridyl ketone	1.229
PKIH	1.643
Isoniazid	1.361
2,2'-Dipyridyl ketone	1.552

Dp44mT and its precursors were separated using 2 mM EDTA and acetonitrile (60:40; v/v) as the mobile phase. In contrast, separation of PKIH and its precursors were achieved using a mobile phase of phosphate buffer (0.01 M Na_2HPO_4, 2 mM EDTA, pH 3), methanol and acetonitrile in a ratio of 65:21:14 (v/v/v).

[a] n: number of determinations.

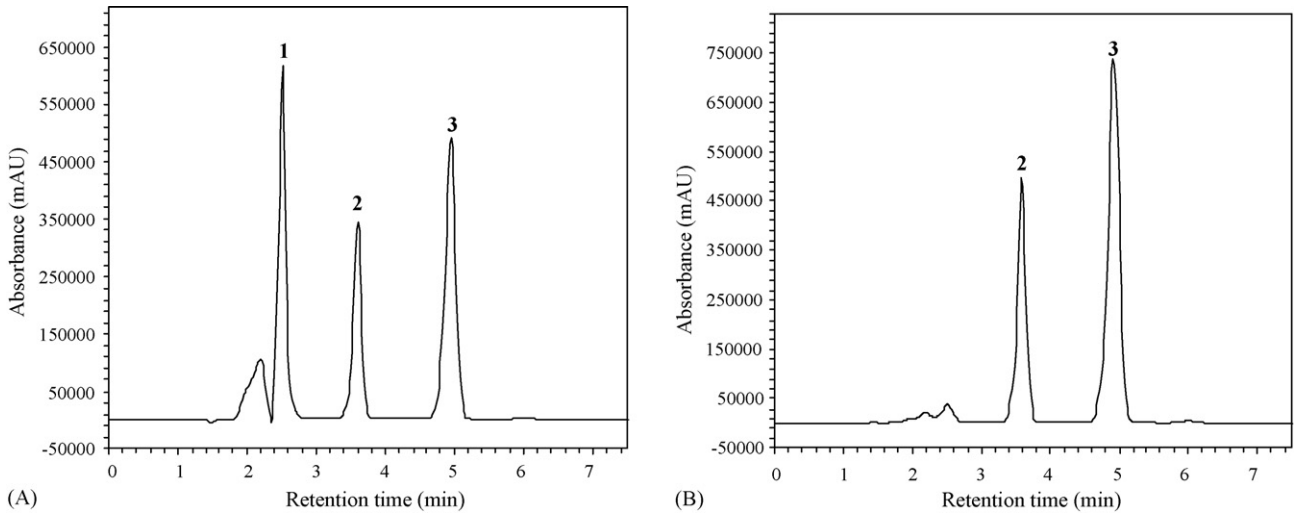

Fig. 2. The representative chromatograms of the separation of Dp44mT (*3*) and its synthetic precursors: 4,4-dimethyl-3-thiosemicarbazide (1) and 2,2′-dipyridyl ketone (2) detected at 244 (A) and 275 nm (B). Dp44mT and its precursors were analysed using 2 mM EDTA and acetonitrile (60:40; v/v) as the mobile phase.

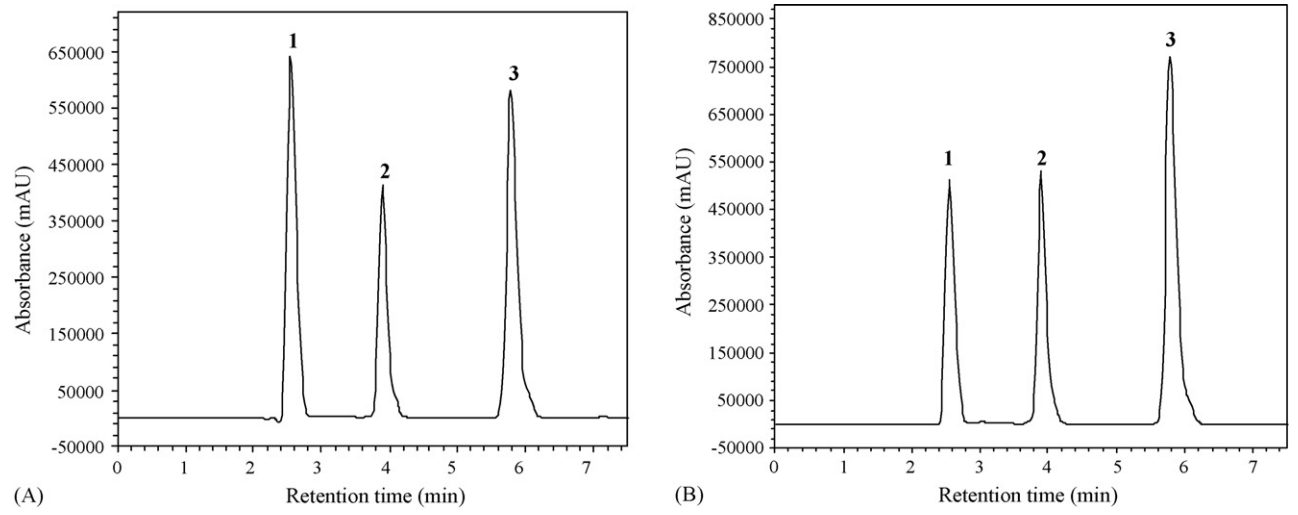

Fig. 3. The chromatograms of the separation of PKIH (3) and corresponding synthetic precursors: isoniazid (1) and 2,2′-dipyridyl ketone (2), detected at 260 (A) and 275 nm (B). PKIH and its precursors were analysed using a mobile phase of phosphate buffer (0.01 M Na_2HPO_4, 2 mM EDTA, pH 3), methanol and acetonitrile in a ratio of 65:21:14 (v/v/v).

buffers and organic solvent (methanol or acetonitrile) in different ratios were tested as mobile phases. A small amount of EDTA was added to the aqueous part of the mobile phase to prevent the formation of complexes between chelator and iron or other metals within the chromatographic system. Moreover, this additive slightly improved the peak shapes of the chelators. Whereas the mobile phase consisting of a mixture of EDTA and acetonitrile was capable of separating Dp44mT and the corresponding

Table 2
Regression data of the calibration curves related to the analysis of each chelator and their respective synthetic precursors

Compound	Linear regression	Correlation coefficient, *R*	UV detection wavelength (nm)
Dp44mT	$y = 0.0000117x + 1.8266762$	0.9999781	275
4,4-Dimethyl-3-thiosemicarbazide	$y = 0.0000059x - 1.5414245$	0.9999019	244
2,2′-Dipyridyl ketone	$y = 0.0000109x + 0.0529990$	0.9999901	275
PKIH	$y = 0.0000107x - 0.1329433$	0.9999507	275
Isoniazid	$y = 0.0000177x - 0.255984$	0.9998515	260
2,2′-Dipyridyl ketone	$y = 0.0000117x - 0.0181084$	0.9999823	275

The linearity of the methods was confirmed with in the ranges 15–150 μg/mL and 5–100 μg/mL for chelators and synthetic precursors, respectively. Dp44mT and its precursors were analysed using 2 mM EDTA and acetonitrile (60:40; v/v) as the mobile phase. PKIH and its precursors were analysed using a mobile phase of phosphate buffer (0.01 M Na_2HPO_4, 2 mM EDTA, pH 3), methanol and acetonitrile in a ratio of 65:21:14 (v/v/v).

Table 3
Intra-day precision calculated from the analysis of six separately prepared standard solutions at three concentrations of chelators and synthetic precursors

	Concentration (μg/mL)			Recovery % ($n=6$)	±S.D.; R.S.D.
	Added	Found	±S.D.; R.S.D.*		
Dp44mT	152.12	153.97	±0.55; 0.36	101.22	±0.36; 0.36
	60.848	61.11	±0.37; 0.61	100.43	±0.61; 0.61
	15.212	15.90	±0.17; 1.08	104.54	±1.13; 1.08
4,4-Dimethyl-3-thiosemicarbazide	100.80	100.70	±1.33; 1.33	99.90	±1.32; 1.33
	50.40	50.71	±0.66; 1.30	100.62	±1.31; 1.30
	5.04	5.08	±0.07; 1.30	100.71	±1.31; 1.30
2,2′-Dipyridyl ketone	99.04	99.17	±0.37; 0.37	100.14	±0.37; 0.37
	49.52	48.83	±0.30; 0.61	98.60	±0.60; 0.61
	4.95	5.08	±0.05; 1.05	102.52	±1.08; 1.05
PKIH	155.00	155.20	±0.73; 0.47	100.13	±0.47; 0.47
	62.00	61.72	±0.38; 0.62	99.55	±0.62; 0.62
	15.50	15.45	±0.15; 0.94	99.71	±0.94; 0.94
Isoniazid	103.00	102.65	±0.44; 0.43	99.66	±0.43; 0.43
	51.50	99.66	±0.98; 1.94	99.08	±1.94; 1.94
	5.15	5.26	±0.05; 1.02	102.04	±1.04; 1.02
2,2′-Dipyridyl ketone	101.60	100.48	±0.49; 0.49	98.90	±0.48; 0.49
	50.80	50.80	±0.35; 0.69	100.01	±0.69; 0.69
	5.08	5.07	±0.03; 0.58	99.75	±0.58; 0.58

synthetic precursors, in the case of PKIH, the mobile phase had to be more complex. In order to obtain sufficient separation of PKIH and its synthetic precursors, phosphate buffer adjusted to pH 3 had to be employed. Furthermore, acetonitrile was partially substituted for methanol. The dual wavelength mode was used to achieve sensitive detection of the chelator and synthetic precursors. The representative chromatograms of the separations of the chelators and the corresponding synthetic precursors are shown in the Figs. 2 and 3.

3.2. Linearity, precision and accuracy, LOD and LOQ

The correlation coefficients ($R>0.999$) of the calibration plots proved good linearity for both methods. Table 2 shows regression data for all chelators as well as their synthetic precursors. The intra and inter-day precision and accuracy are displayed as Tables 3 and 4, respectively. The limits of detection and quantification for the synthetic precursors were found to be 1.5 and 5 μg/mL (2,2′-dipyridylketone), 1.3 and 4.5 μg/mL

Table 4
Inter-day precision calculated from the analysis of six separately prepared standard solutions at three concentrations of chelators and synthetic precursors

	Concentration (μg/mL)			Recovery % ($n=6$)	±S.D.; R.S.D.
	Added	Found	±S.D.; R.S.D.*		
Dp44mT	150.96	152.39	±0.60; 0.39	100.95	±0.40; 0.39
	60.38	61.47	±0.63; 1.03	101.81	±1.05; 1.03
	15.10	15.37	±0.16; 1.05	101.79	±1.07; 1.05
4,4-Dimethyl-3-thiosemicarbazide	99.59	97.79	±1.59; 1.62	98.20	±1.60; 1.62
	49.79	50.07	±0.49; 0.97	100.56	±0.98; 0.97
	4.98	5.02	±0.10; 2.03	100.79	±2.04; 2.03
2,2′-Dipyridyl ketone	99.04	98.32	±0.37; 0.38	99.27	±0.38; 0.38
	49.52	48.73	±0.68; 1.40	98.41	±1.38; 1.40
	4.95	5.02	±0.04; 0.70	101.42	±0.71; 0.70
PKIH	142.00	141.37	±0.89; 0.63	99.55	±0.63; 0.63
	56.80	56.70	±0.49; 0.86	99.82	±0.85; 0.86
	14.20	14.04	±0.15; 1.09	98.90	±1.08; 1.09
Isoniazid	94.00	92.44	±0.80; 0.87	98.34	±0.85; 0.87
	47.00	46.84	±0.70; 1.50	99.66	±1.49; 1.50
	4.70	4.79	±0.07; 1.46	101.86	±1.49; 1.46
2,2′-Dipyridyl ketone	104.00	104.92	±0.80; 0.77	100.89	±0.77; 0.77
	52.00	52.43	±0.61; 1.17	100.83	±1.18; 1.17
	5.20	5.24	±0.05; 1.04	100.67	±1.05; 1.04

(4,4-dimethyl-3-thiosemicarbazide) and 1.5 and 4.8 μg/mL for isoniazid.

3.3. Forced degradation

3.3.1. Dp44mT

After a 4 h incubation of Dp44mT to acidic (pH 0.5) the peak area of the chelator fell to 77% of the initial amount. It was observed that under these conditions, Dp44mT decomposed into two degradation products which were detected at the retention times of 2.58 and 3.58 min, respectively (Fig. 4A). Since their retention times as well as UV spectra determined through photodiode array (PDA) analysis were similar with those obtained from the analysis of the corresponding precursors, hydrolysis of the thiosemicarbazone bond can be considered as the main degradation pathway under acidic conditions. In fact, acid-catalysed hydrolysis across the hydrazone linkage has been reported for other structurally related hydrazone ligands such as pyridoxal isonicotinoyl hydrazone and its analogs [40].

In contrast to acid-catalyzed degradation, Dp44mT was more stable in alkaline medium (pH 13) where after a 4 h of incubation, the peak area of the chelator decreased to only 92% of its initial value. Furthermore, an additional 4 h of incubation resulted in little further degradation. A small peak was detected on the chromatogram at a retention time of 3.58 min (Fig. 4B) which was consistent with 2,2′-dipyridylketone (one of the synthetic precursors of Dp44mT). The experiments carried out in neutral medium showed that Dp44mT was highly stable under these conditions (Fig. 4C), with virtually no degradation being observed.

In the sample exposed to hydrogen peroxide, one new large peak (RT 4.06 min) was detected, while the peak of parent Dp44mT (RT 4.91 min) disappeared (Fig. 4D). According to the literature, this finding may be explained by hydrogen peroxide-induced conversion of the thiocarbonyl to carbonyl group of

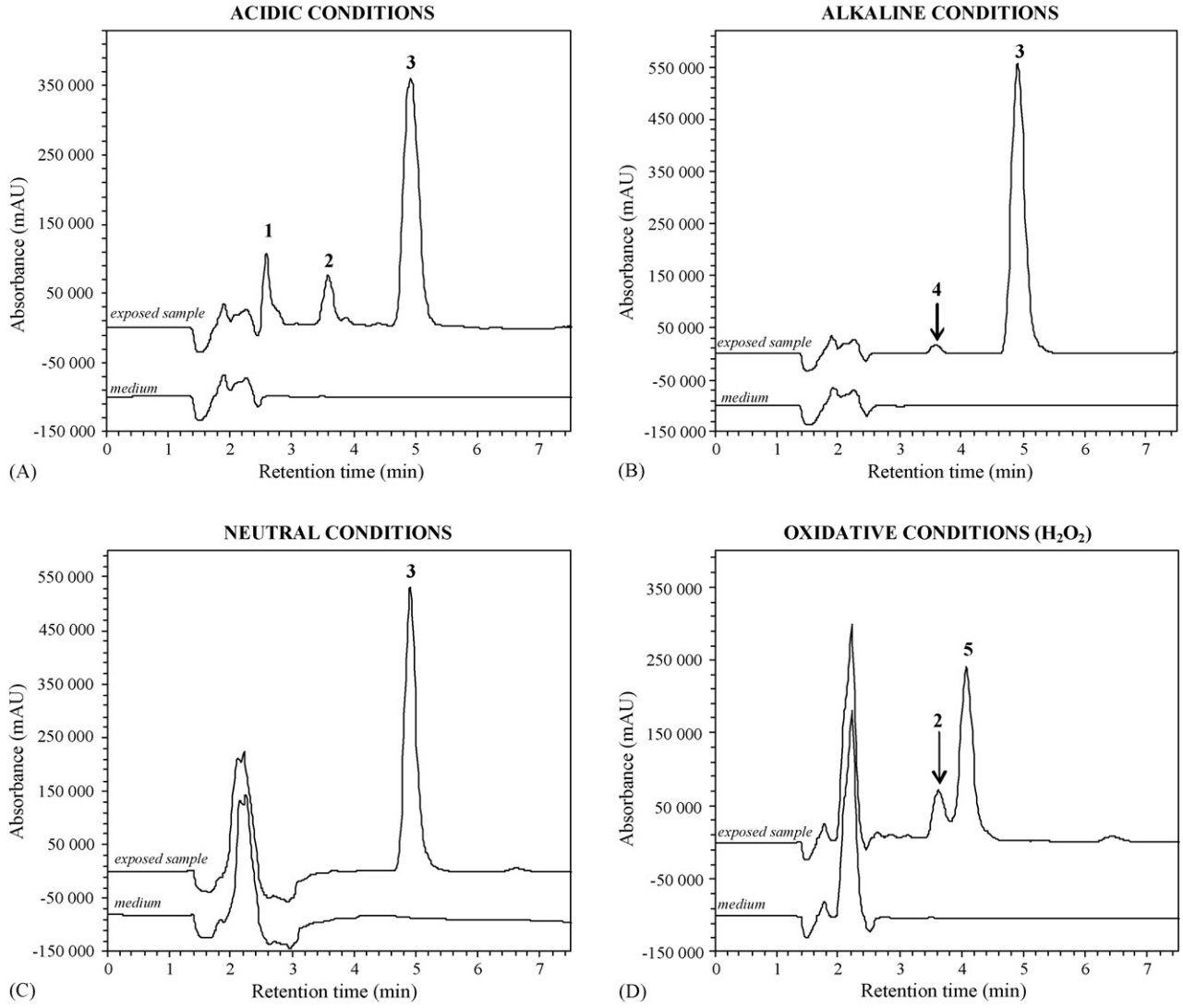

Fig. 4. The chromatograms showing acid (A), alkaline (B), neutral (C) and oxidative (D) degradation of Dp44mT (3) (detected at 244 nm). The samples were analysed using 2 mM EDTA and acetonitrile (60:40; v/v) as the mobile phase. The degradation products: 4,4-dimethyl-3-thiosemicarbazide (1), 2,2′-dipyridyl ketone (2) and unknown degradants (4 and 5).

the chelator [41]. Besides the main oxidative product, the synthetic precursor 2,2′-dipyridylketone was also detected in the exposed samples (Fig. 4D). However, due to the intensive peak (at RT 4.06 min) arising from treatment with hydrogen peroxide, 4,4,dimethyl-3-thiosemicarbazone (the other synthetic precursor) could not be detected. The presence of the synthetic precursor in the exposed sample revealed that hydrogen peroxide could also result in hydrolysis of the thiosemicarbazone bond. Experiments focusing on photo and thermal stability of solid Dp44mT showed that this substance was not markedly susceptible to degradation under the tested conditions (data not shown).

According to PDA analyses, neither peak of the degradation products which appeared in these analyses interfered with the peak corresponding to the parent Dp44mT chelator. Thus, these results confirmed the validity of the HPLC method not only for purity but also for routine stability evaluation of this chelator. Furthermore, these stability experiments indicated that Dp44mT is virtually stable in neutral media and can be also considered to be relatively stable under alkaline conditions. In the current study, only partial degradation of Dp44mT was observed when the compound was exposed to acidic conditions. According to these findings, Dp44mT did not seem to be markedly susceptible to hydrolysis. Thus, respecting general precautions, hydrolysis need not be a major problem from the viewpoint of short-term stability in aqueous pharmaceutical vehicles. On the contrary, Dp44mT was found to be very sensitive to the oxidising agent, hydrogen peroxide. This observation is important from the view point of selection of the handling and storage conditions as well as composition of application media and pharmaceutical delivery.

3.3.2. PKIH

PDA analysis as well as the agreement of the retention times showed that after a 4 h incubation, the acid degradation of PKIH (pH 0.5) lead to a decrease in the peak area of the parent chelator to 23% of its initial value (Fig. 5A). At the same time, the formation of both synthetic precursors (isoniazid: – RT 2.59 min; 2,2′-dipyridyl ketone: RT 3.89 min) occurred (Fig. 5A). These

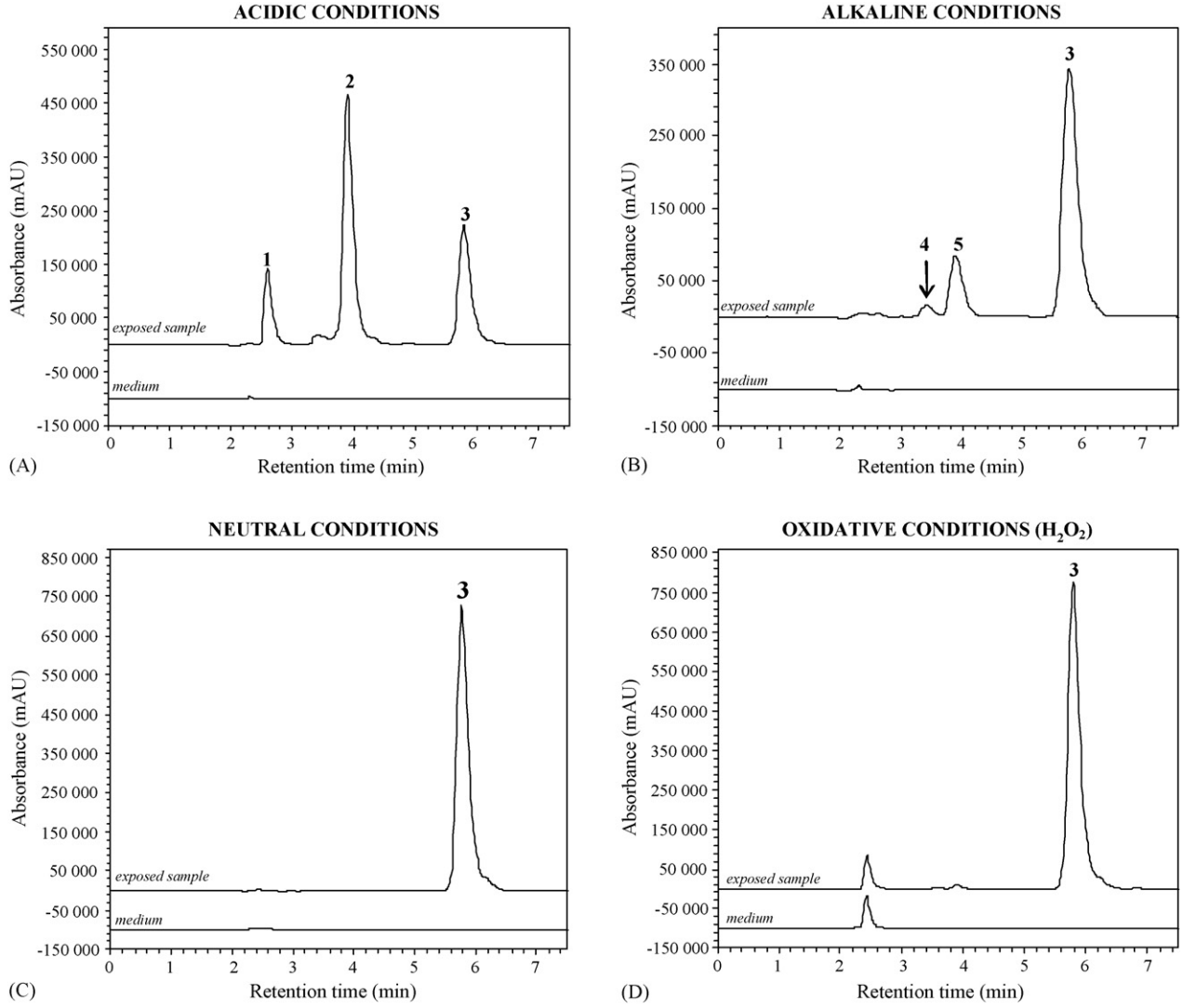

Fig. 5. The chromatograms showing acid (A), alkaline (B), neutral (C) and oxidative (D) degradation of PKIH (3) (detected at 275 nm). The samples were analysed using a mobile phase of phosphate buffer (0.01 M Na_2HPO_4, 2 mM EDTA, pH 3), methanol and acetonitrile in a ratio of 65:21:14 (v/v/v). The degradation products: isoniazid (1), 2,2′-dipyridyl ketone (2), isonicotinic acid (4) and unknown (5).

observations suggested hydrolysis of the parent chelator into its respective precursors across the thiosemicarbazone bond. However, in the case of alkaline hydrolysis (pH 13), the situation was quite different, since more than 73% of the initial amount of the chelator was found at the end of this experiment (Fig. 5A). Two degradation products were detected (RT 3.21 and 3.95 min) in the samples exposed to alkaline conditions (Fig. 5B), but PDA analysis did not confirm their similarity with the synthetic precursors. These findings led us to consider another possible degradation pathway, namely, splitting of the hydrazide bond. Considering this case, isonicotinic acid should be present in the exposed sample. Since the retention time as well as the PDA analysis confirmed the similarity of one degradation product (RT 3.21 min) to isonicotinic acid, the splitting of the hydrazide bond could be considered as main degradation pathway under alkaline conditions. Stability experiments carried out in neutral medium indicated that this chelator is quite stable under this condition (Fig. 5C). Oxidative stress tests using H_2O_2 demonstrated that PKIH is stable under these stress conditions (Fig. 5D). Photo and thermal stability experiments demonstrated that PKIH was stable (data not shown).

Since peak purity (PDA analysis) did not find any interference of degradation products with the analysis of PKIH, the method presented herein is suitable and convenient for stability evaluation. Despite the fact that PKIH appears to be somewhat more susceptible to acid and base-catalyzed hydrolysis than Dp44mT, in neutral medium this chelator was quite stable which is clearly relevant for its use in physiological systems. Interestingly, in comparison with the lead compound of aroylhydrazone iron chelators (PIH) [42], PKIH seems to be markedly more stable. Improved hydrolytic stability of both chelators under the conditions of this study is definitely favourable from the pharmaceutical point of view.

4. Conclusions

In this study, precise and accurate HPLC methods suitable for purity and stability evaluation of two novel iron chelators with potent anti-cancer activity are described. These methods were validated according to ICH recommendations. The stability indicating-power of these methods was confirmed by the analysis of the samples exposed to selected chemical stress conditions (acid, alkaline, neutral and oxidative stress, influence of heat and UV radiation). The stability experiments revealed that Dp44mT is relatively resistant against hydrolysis, but the susceptibility to oxidative degradation may be an issue, which deserves to be taken into account in further pharmaceutical and pharmacological development. On the other hand, PKIH was found to be resistant against oxidation, but somewhat more sensitive to hydrolysis, particularly to acidic conditions. Since both chelators were stable in neutral medium, their stability in common solvents and application media of physiological pH should not be a critical issue. Furthermore, Dp44mT and PKIH were resistant against the influence of both UV light and heat. This study provides validated analytical methodologies which support the development of these promising drug candidates. Moreover, information about the inherent stability of the compounds could be utilized in further investigations focused on these novel iron chelators.

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