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An HPLC method for the simultaneous determination of neurotoxic dipyridyl isomers in human plasma

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Abstract

Several studies on dipyridyl isomers have suggested that they are neurotoxic and that chronic exposure to these compounds could be a potential human health hazard. A reversed phase HPLC method was developed for the simultaneous quantitation of 2,2′-dipyridyl and its four positional isomers, 2,3′-, 2,4′-, 3,4′- and 4,4′-dipyridyl in human plasma. Plasma samples were basified, extracted with 1-chlorobutane, evaporated, the residue reconstituted in mobile phase, and an aliquot part was analyzed by HPLC. Chromatographic separations were performed on a C_{18} reversed phase Sunfire™ column eluted with a mobile phase composed of potassium phosphate (pH 3.5; 25 mM)–acetonitrile (80:20, v/v). Isomers were separated with good resolution, and quantification was determined utilizing an internal standard of quinoxaline. The method has been validated over a range from 30 to 2000 ng/ml with correlation coefficients higher than 0.995. Extraction recoveries for the dipyridyl isomers averaged from 65 to 92%. Limit of detection and limit of quantitation for the dipyridyl isomers ranged from 15 to 70 ng/ml and 30 to 90 ng/ml, respectively. The inter- and intra-day variation did not exceed 7% with an accuracy range of 96–102%. The described analytical method was successfully utilized for the determination of dipyridyl isomers in human plasma and suggested the need for more routine monitoring of tobacco smokers and other individuals who are involuntarily exposed to environmental source of dipyridyl isomers.

Keywords: Dipyridyl isomers; Environmental exposure; Tobacco smoke; Plasma; HPLC

1. Introduction

Dipyridyl isomers exist in six positional isomeric forms, namely as a group of compounds sharing two connecting pyridine rings, i.e., 2,2′-, 2,3′-, 2,4′-, 3,3′-, 3,4′- and 4,4′-dipyridyl. The structures of these isomeric compounds are shown in Fig. 1. The neurotoxicity of dipyridyl isomers and other related compounds has been recently reviewed [1]. All dipyridyl isomers except 3,4′-dipyridyl have been detected as natural products and/or degradation products of materials found in the environment. The two major environmental sources of dipyridyl compounds are pyrolytic degradation of tobacco products and degradation of the herbicide paraquat and diquat. The 2,2′-, 2,3′-, 2,4′-, 3,3′-, and 4,4′-dipyridyl isomers have been found in tobacco and tobacco smoke [1]. The 2,2′-, 2,4′- and 4,4′-dipyridyls have been found as impurities in paraquat and in diquat, two herbicides that are frequently used in the United States and other countries for weed control [2].

Studies on the toxicological effects of dipyridyls in humans are limited. Some articles have summarized the toxicities of the two herbicides paraquat [3] and diquat [4]. Paraquat's neurotoxic effects have been attributed mainly to impurities and additives, including 2,2′-, 2,3′-, 2,4′-, 3,3′-, and 4,4′-dipyridyls. High potential mutagenicity was observed for 2,2′-, 2,3′-, 2,4′-, and 3,3′-dipyridyl [5]. An interview survey of 242 workers who had been exposed to 4,4′-dipyridyl revealed that 55% of workers had developed cancerous skin lesions of varying severity [6]. Another study showed that 4,4′-dipyridyl may cause carcinogenesis through a suppression of TNF-α production and secretion mechanism [7].

The potential of dipyridyls to cause metabolic enzyme induction or inhibition could contribute to their toxicity. For example,

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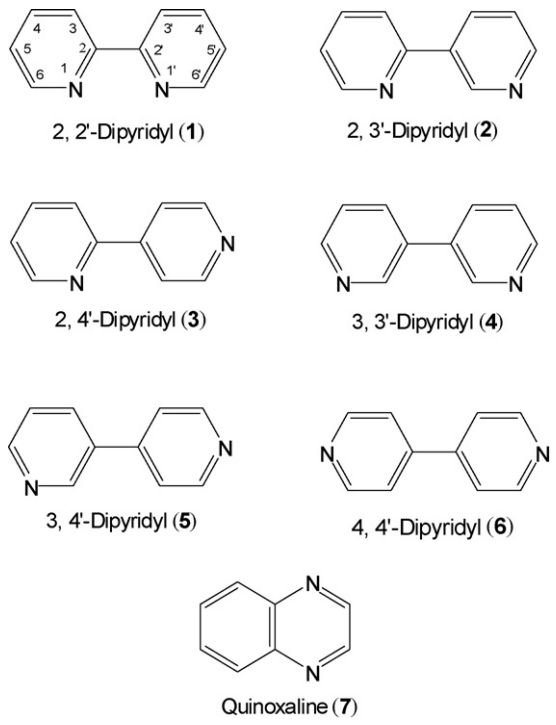

Fig. 1. Chemical structures of dipyridyl isomers (**1**)–(**6**) and the internal standard, quinoxaline (**7**).

2,2′-, 2,4′- and 4,4′-dipyridyl isomers have been reported to induce hepatic microsomal cytochrome levels in rats [8], and both 2,2′- and 2,4′-dipyridyls have been reported to inhibit human liver aldehyde dehydrogenase [9]. Several toxic effects of dipyridyls have been reported in animal models. An acute oral LD_{50} of 256 mg/kg was determined for 2,2′-dipyridyl in rats [10], and high doses of 2,2′- and 4,4′-dipyridyls have been shown to be neurotoxic in Sherman rats [10,11]. The reported data on the neurotoxic properties of dipyridyls suggest the need for further studies on their potential hazard to human health.

The 2,2′-dipyridyl isomer is a potent, low-polarity iron (Fe^{2+}) chelator, and can form complexes that reduce the late phase vasospasm in a primate model of subarachnoid hemorrhage [12–14]. This powerful and distinctive iron (Fe^{2+}) chelating property of 2,2′-dipyridyl can easily distinguish it from the other dipyridyl isomers.

Surprisingly, there are no previous analytical reports on the simultaneous separation and quantitation of dipyridyl isomers in human plasma, and few studies have reported on the detection and identification of dipyridyls from natural sources. A study was carried out to isolate 2,2′-dipyridyl from overheated, brewed coffee using a gas chromatography–mass spectroscopy (GC/MS) analysis [15]. Another study, using a mass spectrometric method, was able to isolate and identify 2,2′-dipyridyl in a hexane extract of human feces [16]. Other work has described the isolation 2,2′-dipyridyl from the high boiling point fraction of cigarette smoke condensate [17], and from the thermal degradation products of nicotine in a combustion tube [18]. It is worth noting that these methods did not distinguish the 2,2′-dipryridyl from other isomeric dipyridyls which may also be found in the environment. The development of multi-isomer bioanalytical methods is usually difficult and challenging, due to the fact that isomeric compounds usually have very similar chemical and physical properties and have to be simultaneously extracted and selectively analyzed.

The aim of the present study was the development and the optimization of a simple, rapid, and sensitive HPLC method for the simultaneous detection and quantitation of five dipyridyl isomers in human plasma. The method was designed to be used to identify the extent of human exposure to the environmental pollutant 2,2′-dipryridyl and other dipyridyl isomers. The development, performance, and clinical application of this assay are described herein.

2. Experimental

2.1. Chemicals and reagents

Reference standards of the dipyridyl isomers used in this study, i.e. 2,2′-, 2,3′-, 2,4′-, 3,3′- and 4,4′-dipyridyl (Fig. 1, structures **1**–**5**) and quinoxaline (Fig. 1, structure **7**) were all obtained from Sigma–Aldrich (Milwaukee, WI). 3,4′-dipyridyl (Fig. 1, structure **6**) was synthesized in our laboratories utilizing the published method of Ishikura et al. [19]. Sodium hydroxide, potassium phosphate monobasic, ferrous sulfate, 1-chlorobutane, and acetonitrile (HPLC grade) were all obtained from Fisher Scientific (Pittsburg, PA). Drug-free plasma was supplied by Innovative Research Inc. (Southfield, MI). High-purity water (18 MΩ) was obtained from a NanoPure Diamond purification system supplied by Barnstead International (Woodhaven, MI).

2.2. HPLC system

Analyses were conducted on an Agilent 1100 series chromatographic system (Agilent Technologies, Palo Alto, CA) comprised an isocratic pump (model G1310A), degasser (model G1322A), automated sampler (model G1313A) and a VWD UV detector (model G1314A). Data analysis, including peak recording and integration, was performed utilizing Agilent ChemStation software (version 4.2).

Separation of isomeric dipyridyl was achieved with a C18 Sunfire™ (4.6 mm i.d., 300 mm long; 5 μm particle size) column (Waters Milford, MA) protected by a SecurityGuard™ in-line filter frit (Phenomenex®, Torrance, CA). The mobile phase consisted of 25 mM potassium dihydrogen phosphate (KH_2PO_4), pH 3.5 adjusted with 85% phosphoric acid (H_3PO_4), and acetonitrile (80:20, v/v). The mobile phase was prepared daily, filtered through a 0.45 μm filter (Millipore, Bedford, MA) and degassed before use. The HPLC system was run at ambient temperature with a flow-rate of 1.5 ml/min, and all eluted compounds were monitored at a wavelength of 240 nm. The total chromatographic run time was 10 min.

2.3. Preparation of standards and controls

Primary stock solutions of each of the analytes were prepared by dissolving in HPLC mobile phase to give a 1 mg/ml solution.

A stock solution of the internal standard (IS) quinoxaline at 5 μg/ml was also prepared in mobile phase. All solutions were stored at 4 °C. Calibration standards were prepared by spiking 0.5 ml of blank plasma with 50, 100, 200 or 300 μl aliquots of analyte solutions. The total volume was then adjusted to 1 ml by the addition of mobile phase. The quality control (QC) samples were prepared using blank plasma containing each of the dipyridyl isomers.

2.4. Sample preparation

Blood samples from volunteers were collected in EDTA-containing tubes and immediately centrifuged for 15 min at 3000 rpm and 4 °C. The plasma was then collected and frozen at −70 °C until analyzed. A 0.5 ml volume of the plasma sample was gently vortex-mixed for 1 min with 20 μl 0.1 M NaOH, 10 μl of internal standard and 0.75 ml of extraction solvent (1-chlorobutane). The mixture was then centrifuged at 8000 rpm for 5 min at ambient temperature. The organic layer was quantitatively collected in a clean glass tube and evaporated to dryness under a gentle stream of nitrogen. Finally, the residue was re-dissolved in 50 μl mobile phase, transferred to a silanized autosampler vial, and a 20 μl aliquot part was injected into the HPLC system.

2.5. Calibration curves

The calibration curves were prepared over the range of 25–2000 ng/ml and consisted of nine concentration points (2000, 1500, 1000, 750, 500, 250, 125, 50, and 25 ng/ml) of each of the dipyridyl isomers in plasma. Plasma samples were quantified using the integrated peak area ratios between the dipyridyl compounds and that of IS. Peak area ratios were plotted against concentrations and linear regression equations were used to determine slopes and correlation coefficients for all analytes.

2.6. Method validation

The limit of detection (LOD) was determined as lowest concentration giving a response of three times the average of baseline. The limit of quantitation (LOQ) was determined as the lowest concentration analyzed with accuracy within ±20% and a precision <20%. The linearity of the response was studied with standard solutions prepared in blank matrix extract; the concentration range studied was from 25 to 2000 ng/ml. The choice of the best method of extraction was based on the recovery, the purity of the extracts, and the number of dipyridyl isomers successfully extracted. Extraction efficiencies were determined in triplicates by comparing the peak area of standards spiked with 500 ng/ml of each of the five dipyridyl isomers. The samples were extracted according to the described procedure and recovery was calculated as the mean and R.S.D.%.

The precision and accuracy of the method were obtained by comparing predicted concentration to actual concentration of dipyridyl isomers in blank plasma. Inter- and intra-day variation was calculated by triplicate analysis of spiked plasma at 500 ng/ml concentration of dipyridyl analytes, and expressed as the relative standard deviation percentage (R.S.D.%). Acceptance criteria for precision were set at ≤10%.

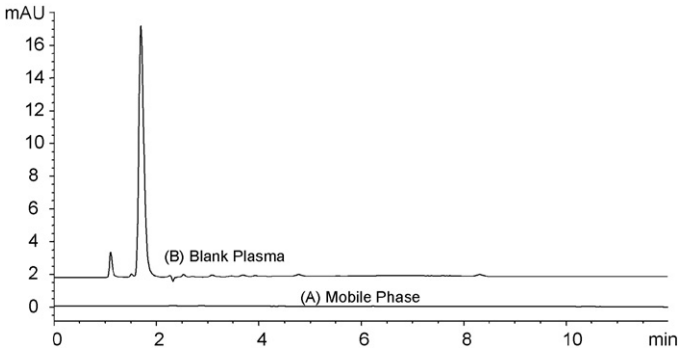

Fig. 2. Chromatograms of mobile phase (A) and extracted blank plasma (B) Samples.

3. Results and discussion

3.1. Selectivity

Potential interference from endogenous substances was investigated. Blank plasma samples were analyzed for peaks interfering with the detection of the dipyridyl isomers or the IS sample by the described procedure. No endogenous substances in plasma that could interfere in these analyses were identified. Representative chromatogram of blank mobile phase and extracted blank plasma were shown in Fig. 2.

3.2. Optimization of chromatographic conditions

Several attempts were performed in order to obtain the best compromise separation-resolution of all the studied dipyridyl isomers, in the shortest time. The Sunfire analytical column and the mobile phase used for this method afforded a well-defined separation between the dipyridyl analytes, internal standard and endogenous components. A typical chromatogram is shown in Fig. 3, and afforded retention times for 4,4′-, 3,3′-, 2,4′-, 2,3′-, 2,2′-dipyridyl isomers and quinoxaline of 2.5, 3.1, 3.5, 4.9, 7.2 and 8.5 min, respectively. Increasing the ratio of phosphate buffer to acetonitrile from 80:20 to 90:10 afforded better resolution by shifting the retention times of the analytes further from

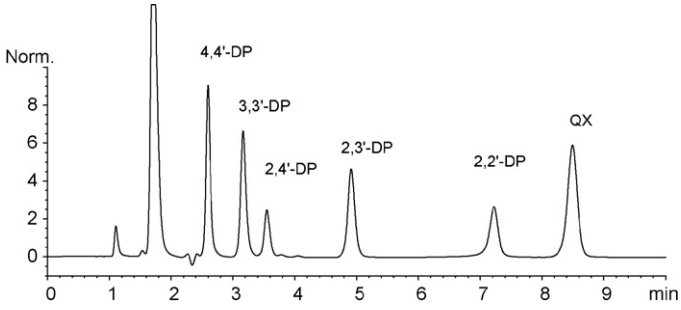

Fig. 3. Representative chromatogram of extracted plasma sample containing 500 ng/ml of each of the dipyridyl isomers and the internal standard; peaks shown (left to right) are 4,4′-, 3,3′-, 2,4′-, 2,3′-, 2,2′-dipyridyl and quinoxaline (QX), respectively.

Table 1
Linearity limit of detection (LOD), and limit of quantification (LOQ) for the isomeric dipyridyls

Compound	LOD (ng/ml)	LOQ (ng/ml)	Regression equation	Linearity r^2
4,4′-DP	15	30	$Y = 0.0018x - 0.0404$	0.9982
3,3′-DP	25	35	$Y = 0.0016x + 0.0967$	0.9958
2,4′-DP	50	65	$Y = 0.0011x - 0.0343$	0.9981
2,3′-DP	40	55	$Y = 0.0022x - 0.0851$	0.9970
2,2′-DP	70	90	$Y = 0.0012x - 0.0765$	0.9974

the solvent front, but this process lowered the peak response of the analytes. Increasing the mobile phase pH from 3.5 to 4 also increased the retention time of all peaks between 2 and 5 min, but decreased the response for the 2,2′-dipyridyl isomer, while increasing the overall run times by up to 5 min. Quinoxaline was selected as an internal standard (IS) because of its structural similarity to the dipyridyl isomers, and good recovery efficiency (94%) as compared to dipyridyl isomers.

Several attempts were made to include 3,4′-dipyridyl isomer in the above analytical method. However, these were unsuccessful due to peak overlap of this isomer with the 4,4′-dipyridyl peak which elutes near the solvent front. The best achievable separation (0.2 min resolution) was obtained by altering the mobile phase composition ratio to (80:20, v/v) 25 mM KH_2PO_4: 0.01% TFA. The addition of $FeSO_4$ to a solution containing all 5 dipyridyl isomers resulted in a dramatic shift in the retention time of only the 2,2′-dipyridyl peak, from 7.2 to 1.4 min. All other dipyridyl isomer retention times remained unchanged.

3.3. Linearity

The calibration curves were based on triplicate analyses of each dipyridyl concentration. A linear correlation was found over the entire studied range 30–2000 ng/ml with correlation coefficients $\geq$0.995 for the dipyridyl isomers.

Integrated peak area ratios between each of the dipyridyl compounds and the IS were calculated and slopes, intercepts and correlation coefficients were determined; these are summarized in Table 1. LOD and LOQ for the studied compounds are also summarized in Table 1.

3.4. Recovery, accuracy, and precision

The recoveries of the dipyridyl isomers from the extraction procedure were studied by spiking the plasma matrix with three different concentrations of each isomer; i.e. 1000, 500, and 250 ng/ml ($n = 3$). Average recoveries ranged from 65 to 93% and were found to be independent of the concentration, Table 2.

The data on precision and accuracy are presented in Table 2. The precision of the method was demonstrated by repetitive analysis. The data from inter- and intra-day precision analysis were calculated as relative standard deviations (R.S.D.s) of three QC samples in relation to the standard curve equation value at a concentration of 500 ng/ml. Assay results in plasma showed acceptable precision, affording R.S.D.% ranges from 1.8 to 6.2%. The accuracy of the determinations was also acceptable; accuracy of all tested samples ranged from 96.8 to 102% (see Table 2).

3.5. Stability of dipyridyl isomers in plasma

Because it is often necessary to store plasma samples for extended time before analysis, the stability of the dipyridyl isomers in plasma was examined by analyzing spiked quality control samples at 250 and 500 ng/ml concentrations stored at −20 °C for varying periods of time. No instability of dipyridyl isomers in spiked samples were observed over three freeze-thaw cycles or during storage at -20 °C for 45 days; all areas under the curve (AUC) of the dipyridyl isomer peaks were unchanged (AUC was ±3.5%). Dipyridyl isomer QC samples showed no significant degradation after 24 h at room temperature (2% deviation of the spiked value). Standard stock solutions of dipyridyl isomers were also found to be stable (AUC was ±3%) for at least 3 days at 4 °C. These results indicate that dipyridyl isomers are stable during storage and sample preparation.

3.6. Application to human volunteers

The developed method has been applied to the detection and quantitation of dipyridyl isomers in plasma from a group

Table 2
Recovery precision and accuracy for dipyridyl isomers at 500 ng/ml concentration

Compound	Recovery mean[a] ± R.S.D. (%)	Inter-day		Intra-day[b]	
		Precision (%)	Accuracy R.S.D. (%)	Precision (%)	Accuracy R.S.D. (%)
4,4′-DP	84 ± 5.6	2.2	102.0	2.5	99.3
3,3′-DP	75 ± 2.8	1.8	98.1	2.8	98.3
2,4′-DP	65 ± 7.4	2.5	97.5	3.5	97.2
2,3′-DP	93 ± 5.4	0.9	99.1	3.1	101.4
2,2′-DP	87 ± 9.3	5.1	96.7	6.2	96.8

[a] Three replicates for each dipyridyl isomer.
[b] Concentration calculated on each of 3 consecutive days.

Table 3
Quantitation of dipyridyl isomers in human plasma from 10 volunteers

Sample no.	Smoker (yes/no)	2,2′-DP[a]	2,4′-DP[b]
1, 4, 6, 7, 8, 9, 10	No	–	–
2	No	65.2[c]	94.3
3	Yes	78.1	120.3
5	Yes	90.2	127.4

[a] 2,2′-Dipyridyl isomer levels measured by HPLC as described in Section 2.
[b] 2,4′-Dipyridyl isomer levels measured by HPLC as described in Section 2.
[c] Values in ng/ml.

of 10 volunteers, who signed a consent form and provided blood samples. The plasma samples were analyzed according to the extraction procedure previously described. 2,2′- and 2,4′-dipyridyl isomers were both detected in three of the ten samples, affording 2,2′-dipyridyl levels in the range 65.2–90.2 ng/ml and 2,4′-dipyridyl levels in the range 90.3–127.4 ng/ml, these results are summarized in Table 3.

Two of the three volunteers whose plasma contained the 2,2′- and 2,4′-dipyridyl isomers (sample no. 3 and 5) were found to be smoker, based upon there total continine in plasma values which ranged from 20.1 to 209 ng/ml (measured utilizing the published procedure of Gosheh et al. [20]). The third individual (sample no. 2) was classified as a non-smoker, but had a total continine in plasma concentration of 0.4 ng/ml, and was tentatively identified as a passive smoker.

The detection of dipyridyl isomers in the plasma of these human volunteers suggests that exposure of these individuals to tobacco smoke may explain the presence of these xenobiotics in the plasma samples.

In the same analytical runs of the plasma samples from which 2,2′- and 2,4′-dipyridyl were detected and quantified, the addition of $FeCl_2$ caused a shift in the retention time of the 2,2′-dipyridyl peak (from 7.1 to 1.7 min) confirming the identity of the dipyridyl isomer (data not shown). It was also possible to detect small amounts of 2,2′- and 4,4′-dipyridyl isomers; however, these were well below quantitation levels. Although the analytical method was not able to completely separate the 3,4′- and 4,4′-dipyridyl isomers, since the former dipyridyl isomer has not been identified as an environmental contaminant, the concentration of the 4,4′-isomer at 2.6 min can be quantified with high probability.

4. Conclusions

For the first time, a simple and sensitive HPLC method for the simultaneous detection and quantitation of five dipyridyl isomers in human plasma has been successfully developed with acceptable precision, accuracy, linearity and stability. The applicability of this method has been successfully demonstrated by the detection of nanogram concentrations of 2,2′- and 2,4′-dipyridyl in plasma samples of human volunteers who have been exposed to tobacco smoke. The present study suggests the need for more routine monitoring of human exposure to neurotoxic dipyridyl isomers in the environment, and especially in individuals who are smokers. The method can also be utilized to support studies on pharmacokinetic and toxicology studies of dipyridyl isomers in animals.

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