A Detection Scheme for Paraquat Poisoning: Validation and a Five-Year Experience in Australia

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Abstract

We report the validation of a quantitative method for paraquat in plasma and urine using high-performance liquid chromatography (HPLC) with ultraviolet detection (260 nm). Furthermore, we illustrate the use of this method in the clinic (over five years), in conjunction with a qualitative urine paraquat screen. Urine or plasma sample (1 mL) preparation was performed in duplicate using C18 solid-phase extraction. Chromatographic separation was achieved on a Zorbax RX-Silica column (250 x 4.6-mm i.d.). The mobile phase consisted of 96% sodium chloride (5 g/L) and 4% acetonitrile (pH 2.2) pumped at 1.0 mL/min. Using a single-point calibration (1.0 mg/L), the method was found to be linear from 0.1 to 5.0 mg/L. The accuracy and imprecision of the method, over the linear range and for plasma and urine, were 94.7–104.9% and < 12.2%, respectively. The limit of quantitation for both matrices was 0.1 mg/L. The absolute recovery of paraquat from plasma and urine was 79.9 ± 5.3% and 88.2 ± 5.3%, respectively. From January 1995 to February 2000, 47 qualitative urine paraquat screens were requested throughout Australia. Nine screens were positive, and eight were confirmed to have paraquat present by our HPLC method. One sample was not analyzed by HPLC because the patient died prior to analysis. Thus, no false-positive results were reported for the qualitative urine screen. An additional 11 samples were referred for patients with positive screens from other sites for HPLC confirmation. The presence of paraquat was confirmed in nine of these samples. In conclusion, a qualitative urine screen combined with our validated HPLC confirmation is an effective protocol for assessing suspected cases of paraquat poisoning.

Introduction

Paraquat (1,1'-dimethyl-4,4'-bipyridyl) is a contact herbicide that is marketed worldwide (Figure 1). The production of free radicals by single electron reduction during photosynthesis is responsible for the herbicidal action of paraquat. The oxygen free radicals are responsible for toxicity in humans by producing injury to intracellular membranes and organelles and eventually cell death (1).

Serious toxicity is usually associated with ingestion of paraquat. Ingestions of more than 15 mL of the 20% concentrate (> 40 mg paraquat/kg) usually results in death within a few hours to days. Corrosive effects occur in the mouth, pharynx, esophagus, and stomach. Multigorgan failure is responsible in patients who die early, where manifestations can include cardiogenic shock, hemorrhagic pulmonary edema or adult respiratory distress syndrome, cerebral edema, and renal failure (2). Ingestion of 20–40 mg paraquat/kg usually leads to death from respiratory failure within a few weeks. The lung is the primary target organ because of paraquat uptake and accumulation in alveolar epithelial cells. Initially, there is alveolitis that progress to fibrosis and results in death from hypoxia (3).

Although there are limited treatment options for paraquat poisoning, several studies have reported methods for determining prognosis after paraquat exposure by paraquat concentration versus time after ingestion nomograms (4–7). To establish likelihood of survival, the specimen collection time relative to ingestion must be known, and accurate measurement of paraquat in blood or urine is required.

With suspected paraquat poisoning, it has been advocated that a rapid qualitative test be performed initially, followed by a confirmatory test (8). The qualitative test relies on the reduction of paraquat by sodium dithionite under basic conditions that produces a blue color if paraquat is present (9,10).

Several techniques have been reported for the quantitation of paraquat in human biological fluids including radioimmunoassay (11), spectrophotometry (12–14), gas chromatography (15), high-performance liquid chromatography (HPLC) with ultraviolet detection (16–21), and capillary electrophoresis (22). In this

Figure 1. The chemical structure of the paraquat ion.
study, we report the validation of an HPLC method using ultra-violet spectrophotometric detection for the quantitation of paraquat in plasma and urine. Furthermore, we describe our experience, over five years, using this HPLC method in conjunction with a qualitative urine screen to detect paraquat exposure in the clinical setting.

Materials and Methods

Chemicals and reagents

All chemicals used in this work were analytical-reagent grade unless specified. HPLC-grade methanol was purchased from EM Science (Gibbstown, NJ). Reagent-grade deionized water was obtained from a Milli-Q water purification system (Millipore, Milford, MA). Paraquat, 1-heptane-sulfonic acid sodium chloride (Sigma Chemical Co., St. Louis, MO), sodium dithionite, sodium hydrogen carbonate, sodium chloride (BDH Chemicals, Bankside Scientific Co., Brisbane, QLD, Australia), and cetrimide (Aldrich Chemical Co., Sydney, NSW, Australia) were purchased from their respective suppliers. Paraquat stock solutions were prepared in deionized water and stored at -20°C until required for use. The active constituent glyphosate, present as the isopropylamine salt, was obtained from Zero Weedspray® (Arthur Yates & Co. Ltd., Sydney, NSW, Australia). Dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and 2-(2,4,5-trichlorophenoxy) propionic acid (Silvex; 2,4,5-TP) were obtained as a herbicide methyl ester mix (Supelco, Bellefonte, PA).

Quantitative urine and plasma HPLC analysis

HPLC operating conditions. The HPLC system consisted of a model 510 pump, model 712 WISP autosampler, and a model 484 tunable absorbance detector (Waters, Milford, MA). Data acquisition and reduction were performed on a personal computer with Maxima software (Waters). The HPLC column was a Zorbax RX-Silica column (250 mm x 4.6 mm i.d., 5 μm, Activon, Sydney, NSW, Australia) maintained at ambient temperature. The mobile phase consisted of 96% sodium chloride (5 g/L) and 4% acetonitrile (pH 2.2). The system operated at 1.0 mL/min and the column eluent was monitored at 260 nm. Quantitation was based on the peak height of paraquat with a single-point calibrator (1.0 mg/L, that is, linear through zero) used with every batch of patient samples.

The HPLC system used for paraquat analysis was used for a variety of low-volume and infrequent assays performed in our laboratory. When required for use, the system, which was stored in 100% methanol, was flushed with 10% (v/v) methanol in water followed by the appropriate mobile phase. In order to prevent salt precipitation from the paraquat mobile phase, the HPLC system was flushed with 10% (v/v) methanol in water immediately upon completion of paraquat analysis.

Sample preparation. Duplicate standards, controls, and patient samples (1 mL) were mixed with concentrated ammonia (0.2 mL) in 12-mL polypropylene centrifuge tubes. Solid-phase C18 extraction cartridges (Waters) were pretreated sequentially with water (5 mL), methanol (5 mL), acidic methanol (10 mL), water (5 mL), 0.5% (v/v) alkaline cetrimide in water (5 mL), water (5 mL), methanol (10 mL), water (5 mL), and 2% (v/v) alkaline sodium heptanesulfonate in water (10 mL). The sample mixtures (1 mL) were added accurately to the preconditioned cartridges. The loaded cartridges were washed with water (5 mL) and methanol (5 mL). Paraquat was eluted with 1% (v/v) hydrochloric acid in methanol (2 mL) and the eluent evaporated to dryness under a stream of air (70°C). The residue was reconstituted in mobile phase (0.5 mL), and an aliquot (0.1 mL) was injected onto the HPLC system.

Validation studies. The specificity of the method was investigated by analyzing 10 plasma and urine samples from independent subjects. Further, to evaluate the potential for exogenous interferences, diquat; glyphosate; 2,4-D; 2,4,5-T; and 2,4,5-TP were supplemented into urine and the mobile phase was subjected to the extraction procedure, and subjected to HPLC for analysis. The analytical recovery and imprecision of the method for both plasma and samples was tested by analyzing three controls (0.1, 0.2, and 5.0 mg/L) in quadruplicate on four days. The absolute recovery of paraquat was determined by comparing peak heights of extracted controls with equivalent pure solutions in mobile phase (n = 16).

Table I. The Analytical Recovery, Imprecision, and Absolute Recovery of Plasma and Urine Paraquat Quality-Control Samples Analyzed during Validation of the HPLC Method (n = 16)

<table>
<thead>
<tr>
<th>Concentration (μg/L)</th>
<th>Mean recovery (%)</th>
<th>Imprecision (%)</th>
<th>Absolute recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>99.2</td>
<td>12.2</td>
<td>80.8</td>
</tr>
<tr>
<td>0.2</td>
<td>101.8</td>
<td>9.9</td>
<td>79.3</td>
</tr>
<tr>
<td>5.0</td>
<td>102.3</td>
<td>8.8</td>
<td>79.6</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>104.9</td>
<td>5.7</td>
<td>92.9</td>
</tr>
<tr>
<td>0.2</td>
<td>102.5</td>
<td>4.2</td>
<td>88.5</td>
</tr>
<tr>
<td>5.0</td>
<td>94.7</td>
<td>3.0</td>
<td>83.0</td>
</tr>
</tbody>
</table>

* Analytical recovery = mean concentration/quality control concentration * 100%.
* Imprecision = standard deviation/mean concentration * 100%.
* Absolute recovery = peak height of extracted control/peak height of equivalent pure solutions in mobile phase * 100%.

Qualitative urine spot test

Blank, standard (3 mg/L), and patient urine samples (5 mL) were treated with sodium hydrogen carbonate (1 g) in 25-mL plastic specimen containers. Sodium dithionite (0.5 g) was added to the mixture and mixed gently. Samples were viewed approximately 5 min after the addition of the sodium dithionite. The appearance of a blue color was considered a positive result for paraquat. The blank and standard urine acted as negative and positive controls, respectively. In order to evaluate potential exogenous interferences, diquat; glyphosate; 2,4-D; 2,4,5-T; and 2,4,5-TP were supplemented into urine and the samples subjected to the qualitative screen.
Results and Discussion

Validation of HPLC method

The chromatographic conditions used in this study resulted in a retention time of 6.5 min for paraquat and a total chromatographic run time of 8 min. The method was found to have no significant interference at the retention time of paraquat for extracted plasma (n = 10) or urine (n = 10) samples obtained from individual subjects. A representative chromatogram of blank urine and plasma is shown in Figures 2A and 3A, respectively. The blank urine chromatograph (Figure 2A) shows a large peak at 3 min, which was found in 20% of the independent subjects screened; however, good separation from paraquat was evident as shown in Figures 2B and 2C.

Potential exogenous interferences in the qualitative and quantitative methods were assessed. Diquat was investigated as a possible interference for paraquat measurement as some commercial preparations contain both compounds. The urine spot test detected diquat (2 mg/L) which suggests a potential for false-positive results. However, because diquat is used in combination with paraquat, a false-positive indication would be unlikely, but the intensity may be falsely high. Paraquat confirmation can be determined by HPLC, as diquat and paraquat supplemented into urine and plasma was found to be chromatographically resolved (Figures 2B and 3B) with a capacity factor, k', of 1.30 and 2.15, respectively. Glyphosate, the active constituent in many domestic herbicides (3.6 mg/L) and other commercial herbicides 2,4-D, 2,4,5-T, and 2,4,5-TP (500 mg/L) were not detected by either the urine screen or by chromatographic analysis.

The solid-phase extraction procedure described in this paper was modified from a previous study of Gill and co-workers (18). Specifically, Gill et al. (18) propose that these compounds exhibit irreversible adsorption with the silica matrix of the ODS-silica cartridge. However, the addition of the cetrimide prior to sodium heptanesulfonate treatment allowed cetrimide to strongly adhere to the silica sites without being displaced by the quaternary herbicides. The cartridge pre-treatment with sodium heptanesulfonate essentially creates an ion-exchange column. The proposed mechanism is that the hydrophobic heptanesulfonate ions adsorb to the ODS-silica surface to produce a material that acts as a cation exchanger. Therefore, upon addition of the alkanized patient sample the quaternary herbicides are retained on the cartridge as ion pairs while allowing some endogenous material to be removed. The chromatographic conditions described in this study were adapted from Chinchila and Walters (23), who reported the analysis of paraquat and diquat in crops using a silica column and an aqueous mobile phase. The resultant combination of ion-pair extraction using C_{18} solid-phase extraction cartridges
and ion-pair chromatography resulted in excellent specificity for paraquat.

Using the single-point calibration (1.0 mg/L), the method was found to be linear up to 5.0 mg/L. The analytical recovery and imprecision of the method, over the range 0.1 to 5.0 mg/L for plasma and urine, was 94.7% to 104.9% and ≤ 12.2%, respectively (Table I). The limit of quantification for both matrices was 0.1 mg/L. The absolute recovery of paraquat from plasma and urine, at the quality-control concentrations, was 79.9 ± 5.3% and 88.2 ± 5.3%, respectively (Table I).

Although an internal standard was not used for this method, according to the guidelines of Shah et al. (24), the method reported had acceptable analytical recovery and imprecision over the range 0.1 to 5.0 mg/L. The use of a single-point calibration allows for higher throughput of samples compared with a full 5-to-8-point calibration curve (19,21). The limit of quantification, 0.1 mg/L, compared favorably with other reported HPLC methods (16–21) and in most cases provides sufficient sensitivity for prognosis (4).

As an illustration of the practical application of the method, chromatograms of extracted urine and plasma samples obtained from a patient who had ingested paraquat concentrate, are shown in Figures 2C and 3C, respectively. Because the upper limit of quantification was 5 mg/L, dilution of the patient samples were required. In the example shown here, samples were diluted 1:500 (urine) and 1:100 (plasma) with their respective blank matrix. We routinely perform serial dilutions on patient samples (1:10 and 1:100) to ensure that the result lies within our reported analytical range of 0.1 to 5 mg/L.

A five-year experience

From January 1995 to February 2000, 47 urine paraquat qualitative spot tests were referred to our laboratory from hospitals throughout Australia. Nine of the 47 were reported as positive for the presence of paraquat. The presence of paraquat was confirmed by our HPLC method in eight of these samples (plasma, urine or both). One sample was not analyzed by HPLC as the patient died prior to analysis. This patient had an intense blue color in the urine screen suggesting a massive ingestion of paraquat. Thus, no false-positive results were reported for the qualitative urine screen.

To facilitate a more rapid turnaround time in reporting urine screens, many hospitals throughout Australia have established urine screen procedures in their respective emergency or pathology departments. We had an additional 11 referrals of positive urine screens from various sites and HPLC confirmed the presence of paraquat in nine. The two remaining samples had plasma paraquat concentrations of < 0.1 mg/L. However, a negative plasma result (< 0.1 mg/L) does not necessarily reflect a false positive for the urine screen. The urine-to-plasma ratio of paraquat can be high. In our patient cohort who had both quantifiable urine and plasma paraquat determinations (n = 7), the mean ratio was 100:1 (range 1.8:1 to 287:1). In addition, two patients with urine paraquat concentrations of 3.5 and 4.1 mg/L, respectively, had plasma paraquat concentrations of < 0.1 mg/L. These results are consistent with the study by Proudfoot et al. (4) who reported rapid absorption of paraquat with peak plasma concentrations 2 h after ingestion followed by a rapid decline of plasma paraquat associated with tissue distribution and renal clearance.

The analytical performance of the HPLC method over the five years is summarized in Table II. The analytical recovery and imprecision was within acceptable performance criteria (24). Six different scientists analyzed these 17 batches over the 5-year period with acceptable analytical performance, thus illustrating the robustness of this method. Furthermore, no batches failed specifications during this time even with the infrequent use of this methodology.

Although it can be argued that a rapid urine qualitative test is quite specific to diagnose paraquat ingestion, given the potential gravity of the diagnosis, confirmation seems essential, particularly when the quantitative method is a reliable guide to prognosis. In cases of imminent death or certain survival, any drastic treatment measures are withheld. However, in cases with borderline paraquat concentrations the prognosis is cloudy and many clinicians advocate aggressive management.

<table>
<thead>
<tr>
<th>Table II. The Analytical Performance of the Paraquat HPLC Method in Routine Use Over Five Years</th>
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</thead>
<tbody>
<tr>
<td>Weighed-in concentration (mg/L)</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Plasma (n=17)</td>
</tr>
<tr>
<td>0.2</td>
</tr>
<tr>
<td>5.0</td>
</tr>
<tr>
<td>Urine (n = 10)</td>
</tr>
<tr>
<td>0.2</td>
</tr>
<tr>
<td>5.0</td>
</tr>
</tbody>
</table>

* Analytical recovery = mean concentration/quality control concentration * 100%.

* Imprecision = standard deviation/mean concentration * 100%.

Conclusions

The local availability of a urine spot test for the initial analysis of potential paraquat poisoning is essential. We have reported in this paper an HPLC method that is specific, accurate, precise, sensitive and robust for paraquat quantitation in urine and plasma. The qualitative urine screen combined with HPLC confirmation can be effectively used to assess paraquat poisoning.

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References


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