DETERMINATION OF PARAQUAT IN PLASMA AND URINE BY MICELLAR LIQUID CHROMATOGRAPHY

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Abstract. The study aimed to apply micellar liquid chromatography (MLC) to detect paraquat levels in human plasma and urine samples at a medical decision level for suspected poisoning, which is not achieved by the current ion-pairing LC method. Paraquat in (acid-treated) human plasma and urine samples was analyzed using C18 Eclipse XDB column maintained at 30°C and an aqueous micellar mobile phase (pH 3.0) composed of 50 mM sodium dodecyl sulfate, 15% (v/v) acetonitrile, 0.5% (v/v) diethylamine, and 9% (v/v) n-butanol, with paraquat monitored by measuring absorbance at 258 nm. The limit of detection of plasma paraquat (PPQ) and urine paraquat (UPQ) was 0.02 and 0.05 μg/ml respectively; and the limit of quantification was 0.05 and 0.16 μg/ml respectively. Dynamic range for both PPQ and UPQ was achieved at 0.16-100 μg/ml and recovery of spiked (2-10 μg/ml) PPQ and UPQ samples ranged from 100.4-102.9%. Within run repeatability of PPQ and UPQ, detection was obtained at 1.8 and 3.5% relative standard deviation (RSD) respectively and within laboratory repeatability of 4.16 and 7.31% RSD respectively, with a turn-around time of eight minutes. In conclusion, the MLC method was capable of detecting paraquat at a medical decision level for suspected poisoning, namely, 0.2 and 0.9 μg/ml in plasma and urine respectively.

Keywords: medical decision level, micellar liquid chromatography, paraquat, plasma, urine

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INTRODUCTION

Paraquat (1,1′-dimethyl[4,4′-bipyridine]-1,1′-diium dichloride) (PQ) is a potent contact herbicide used worldwide. The advantages of PQ compared to other herbicides are that it is fast-acting, non-selective, resistant to being washed off within minutes of application, and partially detoxified upon contact with soil (Summers, 1980). This led to the adoption of a no-ploughing agricultural practice (Hood *et al*, 1963). However, owing to its high toxicity, which can result in accidental poisoning, use of PQ in agriculture has been banned in over 67 countries (Stuart *et al*, 2023).

In a systematic review, Boedeker *et al* (2020) reported that nearly 400 million cases occur annually worldwide from unintentional, acute pesticide poisoning. Prior to the official banning of PQ importation into Thailand from 1 June 2020 (Ministry of Industry, 2020) (although farmers are allowed to use remaining stocks), Thailand import of herbicides rose from 68,800 tons in 2008 to 88,800 tons in 2019, but, interestingly, there was a drop in pesticide poisoning from 19 to 13 cases per 100,000 population respectively (Laohaudomchok *et al*, 2021).

PQ exposure to inadequately protected external surfaces of users can result in eye injury, nosebleed, and skin irritation and burn, while absorption can cause severe damage to lungs and kidneys (Neumeister and Isenring, 2011). Rather worrisome, a recent meta-analysis indicated an association between paraquat exposure and Parkinson's disease (Tangamornsuksan *et al*, 2019). There is no antidote to PQ poisoning.

Diagnosis of PQ toxicity depends on detailed interview of past history of exposure, clinical signs and symptoms (Lock and Wilks, 2010). A simple spot test for paraquat in urine involving addition of sodium dithionite (generating a blue color) has a sensitivity of ~10 mg/l (Paraquat diagnostic kit, Syngenta, Basel, Switzerland), whereas a medical decision level for suspected poisoning is 0.9 mg/l in urine and 0.2 mg/l in plasma (Wu, 2006). An ion-pairing liquid chromatography (IPLC) is generally employed for quantification of PQ in human plasma and urine (García-Alvarez-Coque *et al*, 2017). IPLC has a limit of detection of 0.1 mg/l in biological fluids, but the technique requires time-consuming preparation of column, produces inconsistent retention time and non-symmetrical elution peak, and suffers from an inability to completely remove ion-pair reagents from the stationary phase leading to a short column lifespan.

Here, we describe the application of a micellar liquid chromatography (MLC) to directly determine PQ in plasma and urine samples capable of detection at a medical decision level for suspected PQ poisoning. The method has a rapid turnaround time and requires minimum turnover of the reverse phase column compared to IPLC. In Thailand and other countries where paraquat is still used in agricultural techniques, there will always be cases of suspected paraquat toxicity. An accurate assessment of the degree of exposure to this harmful herbicide will allow for quick treatment and prevent long-term damage to tissues and organs.

MATERIALS AND METHODS

Reagents

Paraquat (98% methyl viologen dichloride hydrate) was from Aldrich (St Louis, MO) and sodium dodecyl sulfate (SDS; 97%) was from Tokyo Chemical Industry (Tokyo, Japan). Acetonitrile, diethylamine, n-butanol, orthophosphoric acid, perchloric acid, and water (DI type 1, 18.2 MΩ.cm) were from Merck (Darmstadt, Germany).

Preparation of working solutions

Spiked working solutions of 0.2, 10, 20, 40, 60, 80, and 100 μg/ml PQ were prepared in herbicide-unexposed human plasma and urine. Non-spiked samples were employed as negative controls. All solutions were stored at -20°C until analyzed. The micellar mobile phase was stored at ambient temperature in a fume cabinet until used (within two months).

Sample preparation for MLC analysis

A 500-μl aliquot of spiked plasma or urine working solution was mixed with 200 μl of 70% methanol containing 7% (v/v) perchloric acid. The solution was cooled to ≤-18°C for 10 minutes, then centrifuged at 15,000 *g* for 10 minutes at room temperature (Paixao *et al*, 2002) and the supernatant was removed for subsequent MLC analysis.

MLC protocol

Forty-microliter aliquots of PQ-spiked samples and negative controls were injected via an autosampler into an Agilent 1260 Infinity HPLC instrument (Agilent Technologies, Santa Clara, CA) equipped with an Eclipse XDB C18 column (150 mm x 4.6 mm id, 4.6 μm diameter bead) maintained at 30°C, and eluted with a micellar mobile phase at a flow rate of 1.5 ml/min. Absorbance at 258 nm of PQ was monitored using a photodiode array detector (Agilent Technologies, Santa Clara, CA).

Samples collection

Paraquat-unexposed plasma and urine samples used in the study were from left-over pooled plasma and urine samples from healthy individuals attending routine check-ups at out-patient clinical units. Test plasma and urine samples for quantification of paraquat levels using the optimized MLC protocol were of an anonymous (to the researchers) individual submitted from an outside medical institution. Plasma and urine samples were stored at -20°C until analyzed.

Ethical approval

The research protocols were approved by the Human Research Ethics Committee, Faculty of Medicine Ramathibodi Hospital, Mahidol University (approval No. COA. MURA2022/261). No prior written consent from a subject is required as a sample submitted for assay at the Toxicology Unit, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University is coded indicating only the analyte(s) requiring identification/ quantification.

RESULTS

Choice of stationary phase

An Eclipse XDB C18 (octadecyl silica) column (Agilent Technologies, Santa Clara, CA) was chosen for its stability over a pH range of 2-9 and working temperature of 30-40°C.

Micellar mobile phase composition, pH and temperature

The (optimized) mobile phase composition was 50 mM SDS in water (DI type 1) pH 3.0 (adjusted with orthophosphoric acid), 1% (v/v) acetonitrile, 0.5% (v/v) diethylamine, and 9% (v/v) n-butanol. The retention time of plasma (PPQ) and urine (U)PQ was 5.97 and 5.98 minutes respectively (Fig 1).

MLC analytical performance

The limit of detection (LOD), limit of quantitation (LOQ) and dynamic range were determined according to EURACHEM guidelines (Magnusson and Örnemark, 2014), and accuracy and precision according to CLSI EP15-A3 guidelines (CLSI, 2014). LOD of PPQ and UPQ was 0.02 and 0.05 μg/ml, LOQ was 0.05 and 0.16 μg/ml, percent recovery (spiked 2.0-10.0 μg/ml) was 100.6-102.6 and 100.4-102.9%, precision of detection was 4.2 and 7.3% relative standard deviation (RSD), and within laboratory repeatability was 4.16 and 7.31% coefficient of variation (CV) respectively (Table 1). Linear dynamic ranges of both PPQ and UPQ detection were 0.16-100 μg/ml (r^2 = 0.99) (Fig 2). Sensitivity and

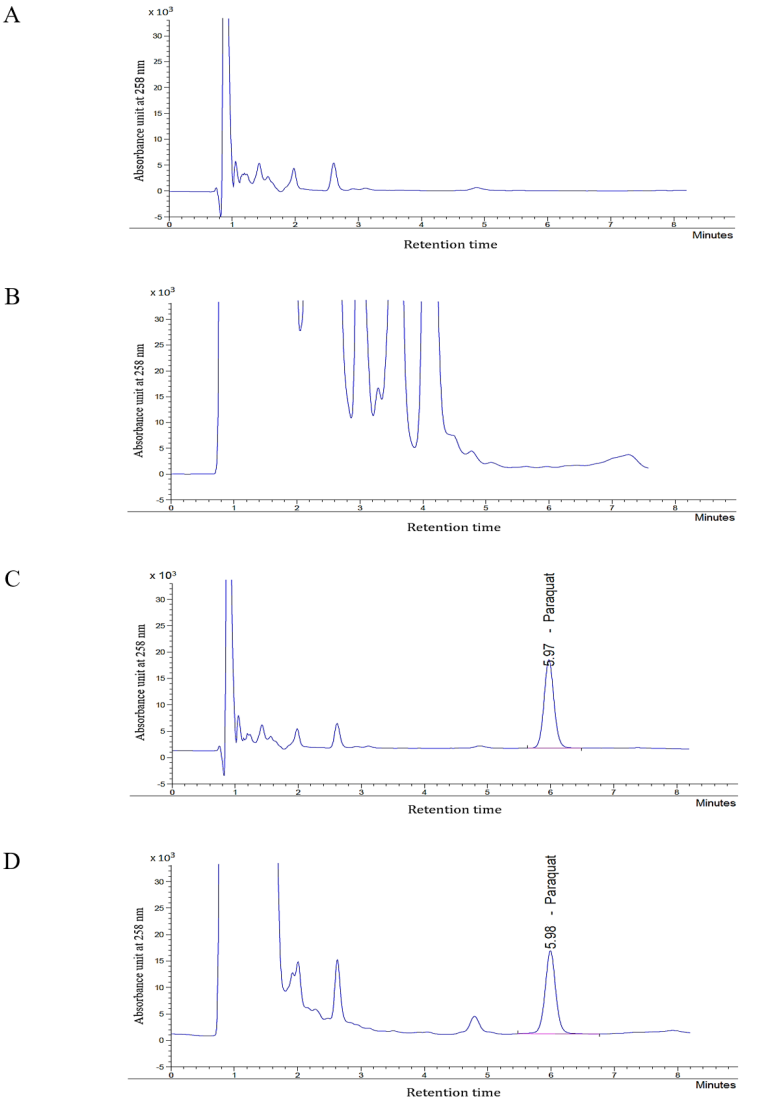

Fig 1 - Micellar liquid chromatograms of (A) blank plasma, (B) blank urine, (C) paraquat-spiked plasma, and (D) paraquat-spiked urine

Micellar liquid chromatography was performed using an Agilent 1260 Infinity HPLC instrument (Agilent Technologies, Santa Clara, CA) equipped with an Eclipse XDB C18 column (150 mm x 4.6 mm id; 4.6 µm diameter bead) maintained at 30°C. Micellar mobile phase contained 50 mM sodium dodecyl sulfate, 15% acetonitrile, 0.5% diethylamine, and 9% n-butanol, pH 3.0. Paraquat was monitored by measuring absorbance at 258 nm.

id: internal diameter; mm: millimeter; µm: micrometer; nm: nanometer.

Table 1

Limit of detection (LOD) and limit of quantitation (LOQ) of spiked plasma and urine paraquat by micellar liquid chromatography

Parameter	Plasma paraquat[a] (μg/ml)	Urine paraquat[b] (μg/ml)
Mean concentration of paraquat detected	0.20	0.20
SD (n = 10)	0.005	0.020
LOD (3 x SD)	0.01	0.06
LOQ (10 x SD)	0.05	0.20

[a]Plasma spiked with 0.20 μg/ml paraquat; [b]Urine spiked with 0.20 μg/ml paraquat

μg/ml: microgram per milliliter; SD: standard deviation

specificity of the MLC detection method were not determined as, currently, there is no "gold standard" for quantification of PPQ and UPQ.

Analysis of test samples

Plasma and urine samples submitted from an outside hospital of an anonymous individual suspected of accidental paraquat poisoning but with negative results previously obtained using an in-house IPLC method (Paixao *et al*, 2002) were analyzed by the MLC method using the optimized conditions, resulting in the detection of PPQ and UPQ of 0.23 and 0.90 μg/ml respectively (Fig 3), values at the limit of minimum levels required for a medical decision of paraquat toxicity, namely, 0.2 μg/ml in plasma and 0.9 μg/ml in urine (Wu, 2006).

DISCUSSION

Quantitative determination of PQ is performed employing several types of chromatography, *viz* ion-exchange chromatography (IEC), IPC and gas chromatography (GC) (García-Alvarez-Coque, 2017). However, each technique suffers from various weaknesses, such as those described above for IPC, the most often used technique; IEC requires the use of expensive columns and GC involves time-consuming extraction of analytes (García-Alvarez-Coque, 2017).

Demand for "green chemistry" has spurred the development of MLC, which uses an aqueous micellar solution, composed of a surfactant in a solution of acetonitrile as the mobile phase (Armstrong and Nome, 1981). The advantages of MLC

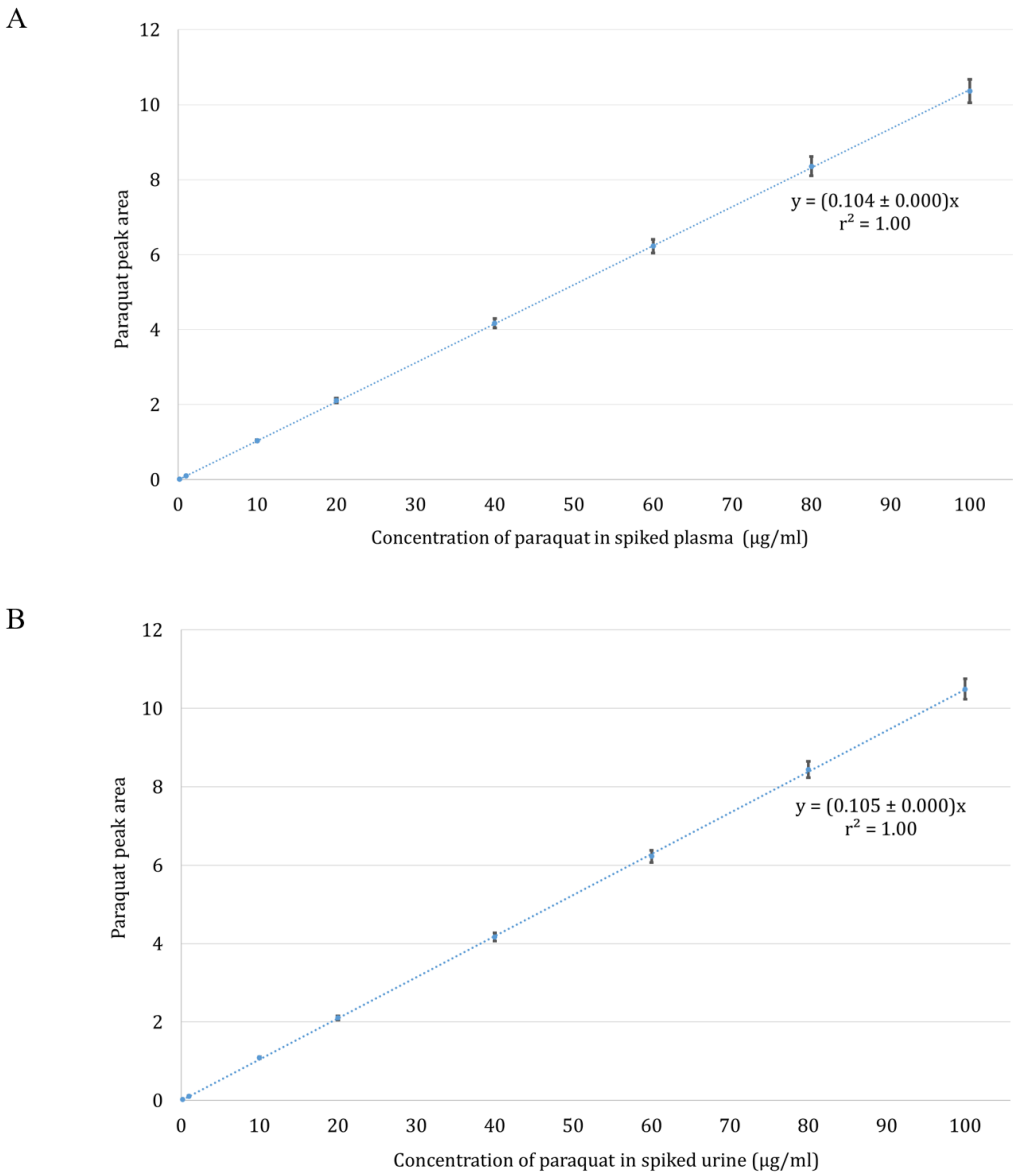

Fig 2 - Micellar liquid chromatography calibration curve for paraquat in plasma (A) and urine (B)

Chromatography was carried out as described in legend to Fig 1 using plasma and urine both spiked with paraquat at 0.2-100 µg/ml. Each determination was carried out in triplicate.

µg/ml: microgram per milliliter; r^2: coefficient of determination.

Table 2

Home environmental factors associated with falls on binary logistic regression analysis

Parameter	Spiked plasma paraquat (μg/ml)			Spiked urine paraquat (μg/ml)		
	2.0	5.0	10.0	2.0	5.0	10.0
Mean (μg/ml)	2.0	5.0	10.3	2.0	5.1	10.2
Grubbs' limit (μg/ml)	1.8-2.3	4.7-5.4	9.1-11.4	1.6-2.4	4.4-5.9	8.9-11.5
Mean value within Grubb's limit	Pass	Pass	Pass	Pass	Pass	Pass
n_0	5	5	5	5	5	5
$S_B = \sqrt{(MS_B - MS_R)/n_0}$	0.08	0.10	0.36	0.14	0.22	0.22
$S_R = \sqrt{MS_R}$	0.04	0.05	0.12	0.06	0.11	0.36
$S_{WL} = \sqrt{\frac{MS_B - MS_R}{n_0} + MS_R}$	0.09	0.11	0.38	0.15	0.25	0.42
$SEM = \sqrt{\frac{1}{nRep}\left[S^2_{WL} - \left(\frac{nRep-1}{nRep}\right)S^2_R\right]}$	0.035	0.045	0.165	0.061	0.101	0.123
Verification interval (μg/ml)	1.9-2.1	4.9-5.1	9.5-10.5	1.8-2.2	4.7-5.3	9.7-10.3
Mean value within verification interval	Pass	Pass	Pass	Pass	Pass	Pass
% Recovery	102.3	100.6	102.6	100.4	102.9	102.0

Table 2 (cont)

Parameter	Spiked plasma paraquat (μg/ml)			Spiked urine paraquat (μg/ml)		
	2.0	5.0	10.0	2.0	5.0	10.0
Acceptance criteria						
USFDA[a], ICH[b] (±15%, ±20% at LOQ; ±3% for drug analysis)	Pass	Pass	Pass	Pass	Pass	Pass
SWGTOX[c] (±20%, ±10% is acceptable)	Pass	Pass	Pass	Pass	Pass	Pass
% CV_R	1.81	1.07	1.18	2.85	2.23	3.50
% CV_{WL}	4.16	2.22	3.74	7.31	4.82	4.13
Acceptance criteria						
USFDA[a], ICH[b] (≤15% CV, ≤20% CV at LOQ)	Pass	Pass	Pass	Pass	Pass	Pass
SWGTOX[c] (≤20% CV)	Pass	Pass	Pass	Pass	Pass	Pass

[a]USFDA (2018); [b]ICH (2022); [c]SWGTOX (2013)

CV: coefficient of variance; CV_R: repeatability standard deviation; CV_{WL}: within-laboratory standard deviation; LOQ: limit of quantitation; MS_B: mean square of between-run; MS_R: mean square of repeatability; μg/ml: microgram per milliliter; *nRep*: number of replicates per run; n_0: the average number of results per run; S_B: estimate for repeatability; S_R: estimate for between-run; S_{WL}: estimate for within-laboratory imprecision; *SEM*: standard error of mean

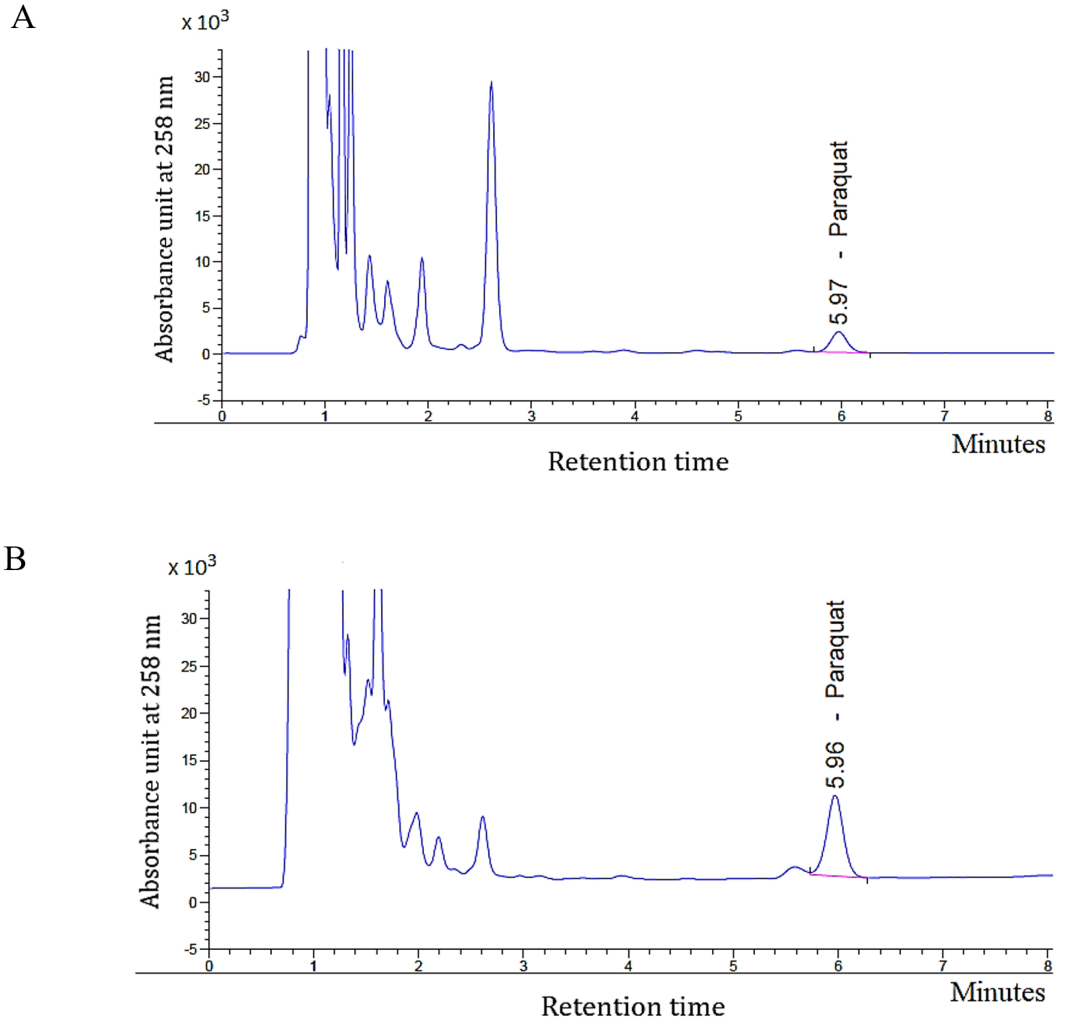

Fig 3 - Micellar liquid chromatograms of (A) plasma and (B) urine samples from a patient suspected of paraquat poisoning

Chromatography was carried out as described in legend to Fig 1.

nm: nanometer

compared to IEC and IPC are the improved ability to separate mixtures of charged and neutral analytes and the capability to directly inject (following protein precipitation) plasma and other physiological fluids into an LC instrument. However, a major weakness of MLC is its reduced efficiency caused by the micelles, but this can be improved by the addition of a judicious small amount of an organic solvent to improve peak resolution (Esteve-Romero *et al*, 2016).

Surfactants used in MCL can be divided into three categories: anionic (*eg*, SDS), uncharged (*eg*, Brij-35) and cationic (*eg*, cetyl trimethylammonium bromide). SDS was chosen as the surfactant here because it has a relatively low critical micelle concentration (5 mM) and being an anionic micelle can bind with cationic PQ. Diethylamine was employed as an end-capping agent, and n-butanol was chosen as the organic component to adjust retention time (Ruiz-Angel *et al*, 2002). Acidic pH was to maintain PQ and diethylamine in the cationic state. The binding of 50-200 mM SDS to five Hypersil stationary matrices is in the order: unmodified silica > cyanopropyl > methyl > octyl > octadecyl silica (Berthod and Gaecia-Alvarez-Coque, 2000); hence, an octadecyl silica column (Eclipse XDB C18; Agilent Technologies, Santa Clara, CA) was chosen for its stability over a pH range of 2-9 and working temperature of 30-40°C. Optimization of mobile phase parameters was achieved as follows: keeping all parameters fixed at a particular value except for one parameter that was varied until an optimal MLC condition was achieved, *ie*, rapid elution time and symmetrical peak shape; and with the latter parameter kept at its optimal value, the process was then sequentially repeated until all parameters were optimized. All analytical performances of MLC in the detection of PPQ and UPQ were within acceptable limits according to both European (Magnusson and Örnemark, 2014) and US (CLSI, 2014) guidelines, to ensure that the protocol procedures can be performed in as many laboratories as possible worldwide.

The technique was applied to PPQ and UPQ samples of a Thai individual admitted with suspected PQ poisoning. Standard clinical criteria for diagnosis of suspected PQ poisoning are the patient's history of exposure to the herbicide, laboratory findings of systemic toxicity and a positive urine dithionite test. Although an in-house IPLC quantitative assay for PQ (Paixao

et al, 2002) was negative for the individual suspected of PQ toxicity, the optimized in-house MLC assay described in the current study was able to determine PPQ and UPQ at a medical decision level for suspected poisoning, *ie*, 0.2 and 0.9 μg/ml in plasma and urine respectively (Wu, 2006).

The limitation of the study was the inability to determine the sensitivity and specificity of the in-house MLC technique for detecting PPQ and UPQ owing to the lack of a "gold standard" for comparison.

In conclusion, optimal conditions were established for micellar liquid chromatography analysis of paraquat in human plasma and urine at levels required for a medical decision on paraquat toxicity. The technique requires a standard reverse-phase liquid chromatography system operating in an isocratic elution mode and equipped with a UV-visible spectrophotometer; the optimized protocol provides improved sensitivity and a short turnaround time. This operating system can be readily performed in a hospital laboratory equipped with a standard high-performance liquid chromatography system, located in the Asian Pacific region (and elsewhere in the world) where there is a risk of accidental paraquat poisoning arising from the application of this herbicide in agricultural practice.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

Armstrong DW, Nome F. Partitioning behavior of solutes eluted with micellar mobile phases in liquid chromatography. *Anal Chem* 1981; 53: 1662-6.

Berthod A, Gaecia-Alvarez-Conque C, editors. Micellar liquid chromatography. 1st ed. Boca Raton, FL: CRC Press; 2000.

Boedeker W, Watts M, Clausing P, Marquez E. The global distribution of acute unintentional pesticide poisoning: estimations based on a systematic review. *BMC Public Health* 2020; 20: 1875.

Clinical and Laboratory Standards

Institute (CLSI). User verification of precision and estimation of bias; Approved Guideline - Third edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2014.

Esteve-Romero J, Albiol-Chiva J, Peris-Vicente J. A review on development of analytical methods to determine monitorable drugs in serum and urine by micellar liquid chromatography using direct injection. *Anal Chim Acta* 2016; 926: 1-16.

García-Alvarez-Coque MC, Torres-Lapasio JR, Navarro-Huerta JA. Secondary equilibria in reversed-phase liquid chromatography. In: Fanali S, Haddad P, Poole CF, Riekkola ML, eds. Liquid chromatography: fundamentals and instrumentation. 2nd ed. Amsterdam, the Netherlands: Elsevier; 2017. p. 125-46.

Hood AEM, Jameson HR, Cotterell R. Destruction of pastures by herbicides such as paraquat as a substitute for ploughing. *Nature* 1963; 197: 748.

International Council for Harmonization of Technical Requirements for Pharmaceutical for Human Use (ICH). Validation of analytical procedure Q2(R2) draft version, 2022 [cited 2023 Feb 01]. Available from: URL: https://database.ich.org/sites/default/files/ICH_Q2-R2_Document_Step2_Guideline_2022_0324.pdf

Laohaudomchok W, Nankongnab N, Siriruttanapruk S, *et al*. Pesticide use in Thailand: current situation, health risks, and gaps in research and policy. *Hum Ecol Risk Assess* 2021; 27: 1147-69.

Lock EA, Wilks MF. Paraquat. In: Krieger RI, ed. Hayes' Handbook of pesticide toxicology. 3rd ed. Amsterdam, the Netherlands: Elsevier; 2010. p. 1767-823.

Magnusson B, Örnemark U, editors. Eurachem Guide: The fitness for purpose of analytical methods – a laboratory guide to method validation and related topics (2nd ed), 2014 [cited 2023 Feb 01]. Available from: URL: https://www.eurachem.org/images/stories/Guides/pdf/MV_guide_2nd_ed_EN.pdf

Ministry of Industry. Notification of Ministry of Industry: List of hazardous substances (No. 6), 2020 [cited 2023 Feb 01]. Available from: URL: https://www.jetro.go.jp/ext_images/thailand/pdf/2020ENMOINotiListHazNo6BE2563.pdf

Neumeister L, Isenring R. Paraquat: unacceptable health risks for users (3rd ed), 2011 [cited 2023

Feb 01]. Available from: URL: https://www.publiceye.ch/fileadmin/doc/Pestizide/2011_Paraquat_Unacceptable_Health_Risk_Report.pdf

Paixao P, Costa P, Bugalho T, Fidalgo C, Pereira LM. Simple method for determination of paraquat in plasma and serum of human patients by high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002; 775: 109-13.

Ruiz-Angel MJ, Caballero RD, Simo-Alfonso EF, Garcia-Alvarez-Coque MC. Micellar liquid chromatography: suitable technique for screening analysis. *J Chromatogr A* 2002; 947: 31-45.

Scientific Working Group for Forensic Toxicology (SWGTOX). Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. *J Anal Toxicol* 2013; 37: 452-74.

Stuart AM, Merfield CN, Horgan FG, *et al*. Agriculture without paraquat is feasible without loss of productivity - lessons learned from phasing out a highly hazardous herbicide. *Environ Sci Pollut Res Int* 2023; 30: 16984-7008.

Summers LA. The Bipyridinium Herbicides. New York, NY: Academic Press; 1980.

Tangamornsuksan W, Lohitnavy O, Sruamsiri R, *et al*. Paraquat exposure and Parkinson's disease: a systematic review and meta-analysis. *Arch Environ Occup Health* 2019; 74: 225-38.

United States Food and Drug Administration (USFDA). Bioanalytical Method Validation: guidance for industry, 2018 [cited 2023 Feb 01]. Available from: URL: https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf

Wu AHB. Tietz clinical guide to laboratory tests. 4th ed. Philadelphia, PA: Saunders; 2006.