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Development and Application of Immunoassay for Paraquat: Radioimmunoassay

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ABSTRACT: A sensitive radioimmunoassay for paraquat is reported. Anti-paraquat antisera were produced by repeated immunization in rabbits with 1-methyl,1′-hexanoic acid-4,4′-bipyridinium (MHBP) coupled to bovine serum albumin (BSA). Less than 0.5 ng of paraquat dichloride was detectable by this assay system. These antisera were strongly cross-reactive with the bipyridyl ring and methyl group in either the 1- or 1′-position of paraquat. The determination of paraquat in tissues of paraquat-poisoned cadavers was also carried out.

KEYWORDS: toxicology, paraquat, radioimmunoassay, tissues (biology)

Paraquat (1,1′-dimethyl-4,4′-bipyridinium) is widely used as a herbicide. While there is no evidence of deleterious side effects in normal use, accidental or deliberate ingestion of paraquat can be lethal [*1,2*]. In the field of forensic toxicology, the validation of the method for the identification and quantification of paraquat in specimens is required. A radioimmunoassay (RIA) has been well known to be a sensitive and reliable method to answer this requirement. In this paper we describe the development of RIA for paraquat and its forensic science application.

Materials and Methods

Chemicals

Paraquat dichloride (1,1′-dimethyl-4,4′-bipyridinium dichloride) and 6-bromohexanoic acid were purchased from Aldrich Chemical Co., Milwaukee, WI. Monoquat chloride (1-methyl-4,4′-bipyridinium chloride), diethyl paraquat diiodide (1,1′-diethyl-4,4′-bipyridinium diiodide), diquat dibromide (1,1′-ethylene-2,2′-bipyridinium dibromide), and morfamquat dichloride (1,1′-bis [3,5-dimetyl-morpholino-carboxymethyl]-4,4′-bipyridinium

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dichloride) were generous gifts from Plant Protection Division, ICI, PLC., England. Reduced paraquat was synthesized by the method as described by Draffan et al. [3]. MPTP chloride (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine chloride) and MPP chloride (1-methyl-4-phenyl-pyridine chloride) were purchased from Research Biomedicals Inc., Wayland, MA. [^{14}C]Methyl iodide (specific activity, 58 mCi/mmol) and (methyl[^{3}H]) paraquat dichloride (specific activity, 2.5 Ci/mmol) were obtained from Amersham International plc., England. Sep-Pak C_{18} cartridges were supplied by Waters, Division of Millipore Corp., Bedford, MA. All other reagents were obtained from Nakarai Chemical, Ltd., Kyoto, Japan.

Colorimetry of Paraquat in Serum, Urine, and Tissue

The concentration of paraquat in serum, urine, and tissue collected at autopsy was measured by a colorimetric method using a Sep-Pak C_{18} cartridge as described elsewhere [4]. A level at least as low as 2 μg of paraquat dichloride was detectable by this assay system. Blanks in specimens by this method were negligible because of selective extraction of paraquat [4].

Preparation of Tissue Samples

Organs fixed in 10% formalin fixative from seven cadavers who died from paraquat poisoning were used for this study (Table 1). One gram of brain, liver, kidney, heart muscle, and lung was minced with scissors and homogenized with ten times its volume of 0.1 *N* hydrochloric acid using a universal homogenizer [5]. The whole homogenate was centrifugated at 10 000 rpm for 20 min at 4°C using a Hitachi RP-20-5 refrigerated centrifuge; an aliquot of the supernatant (0.01 to 0.2 mL) was used for this assay. This aqueous portion of each specimen was first evaporated under a stream of nitrogen gas to remove the formalin content which is known to deteriorate the antibody protein, and the residue was redissolved with 0.1 mL of 0.01 *M* phosphate-buffered saline (pH 7.4, PBS).

Preparation of Immunogen

Labeled 1-methyl-4,4′-bipyridinium iodide was prepared by the method of Fatori and Hunter with a slight modification [6]. Ten grams of 4,4-bipyridyl and 9.1 grams of methyl

TABLE 1—*Fatal paraquat poisoning cases and their paraquat concentrations in sera and urines measured by the colorimetry.*

						Colorimetry	
Case	Sex	Age	Source	Amount	Survival	Serum	Urine
1	M	50	Gramoxone bottle	450 mL	less than 10 h	NT[a]	NT
2	M	59	bottled drinks	unknown	31 h	ND[b]	ND
3	M	38	Gramoxone bottle	100 mL	36 h	5.2[c]	4.5
4	M	48	bottled drinks	unknown	42 h	5.9	12.1
5	F	59	Gramoxone bottle	unknown	86 h	4.3	NT
6	M	37	Gramoxone bottle	10 mL	4 d	trace	trace
7	M	50	bottled drinks	unknown	12 d	NT	NT

[a]Not tested.
[b]Not detected.
[c]μg/mL.

iodide containing [^{14}C]methyl iodide (0.1 mCi) were reacted in 100 mL of dry chloroform and stirred overnight at room temperature. After stirring, 1-methyl-4,4′-bipyridinium iodide was collected by filtration, washed with dry chloroform, and then stored in a vacuum desiccator over silica gel.

1-Methyl, 1′-hexanoic acid-4,4′-bipyridinium (MHBP) as a paraquat hapten was synthesized according to the method of Niewola et al. [*7*] with a slight modification and coupled to bovine serum albumin (BSA) (Fig. 1). The degree of conjugation was calculated to be about 20 mol of hapten per BSA (molecular weight 68 000) on the basis of the radioactivity.

Immunization

A 0.5-mL volume of paraquat-BSA solution in saline which contains 1.0 mg of protein was emulsified with an equal volume of complete Freund's adjuvant. Male albino rabbits, weighing 2.5 to 3.0 kg, were injected subcutaneously as described previously [*8*]. They received 1.0 mL of the emulsion once every two weeks for four months. Blood was collected from a carotid artery ten days after the final injection and allowed to clot at 4°C. The serum was separated by centrifugation and served as the source of antibodies.

RIA Procedure

For the dilution of antiserum and reagents, PBS was used. For the preparation of the standard curve for RIA and for the determination of each sample, each assay tube (0.5 mL)

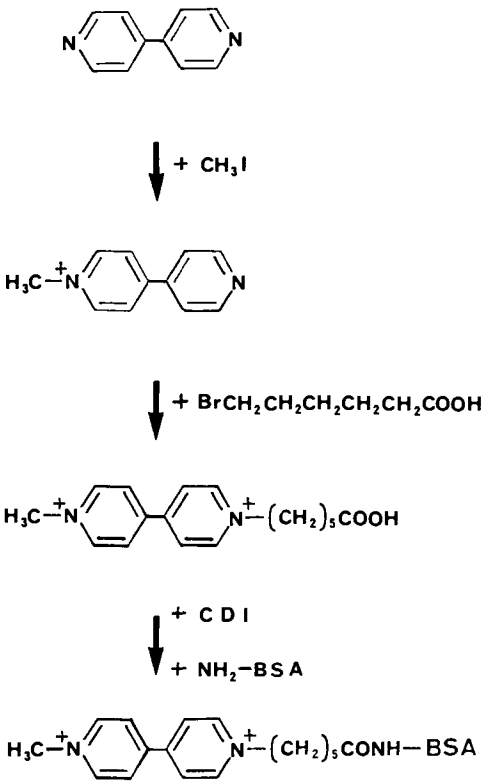


FIG. 1—*Reaction scheme for preparation of immunogen.*

contained as follows: 0.1 mL of [^{3}H]paraquat (about 5000 dpm); 0.1 mL of 1% bovine serum gamma globulin; 0.1 mL of PBS containing unlabeled paraquat dichloride, other reagents, or samples; 0.1 mL of diluted antiserum (1 : 100); and 0.1 mL of PBS. These assay mixtures were incubated for 2 h at room temperature. After incubation, antiserum-bound [^{3}H]paraquat was separated from the free by the salting-out method as described previously [*8*] and was measured in a liquid scintillation spectrometer.

Results and Discussion

In the standard curve procedure, the antiserum-bound [^{3}H]paraquat with increasing amounts of unlabeled paraquat was decreased competitively and linearly up to 32 ng of paraquat in Fig. 2. In the absence of nonradioactive paraquat, the antiserum of the 1 : 100 dilution could bind about 50% of [^{3}H]-labeled paraquat by this system. As shown in Fig. 2, a level at least as low as 0.5 ng of paraquat could be detected, and amounts of paraquat dichloride causing a 50% inhibition were 3.8 ng. The intraassay coefficient of variation for ten duplicate determinations of samples containing 6-ng paraquat dichloride/0.1 mL was 7.9%, and its interassay coefficient of variation was 12.8%. Blank-fixed tissue specimens could not inhibit the binding of antiserum to [^{3}H]-labeled paraquat.

The specificity of anti-paraquat antibody was evaluated by the cross-reactivity studies with paraquat, bipyridyl derivatives, reduced paraquat, MPTP, and MPP (Tables 2 and 3). Monoquat and diethyl paraquat showed relatively significant cross-reactivities with this antibody. Diquat, morfamquat, MPTP, and reduced paraquat could not bind the antibody, and MPP as a similar congener to monoquat was little recognized by the antibody. These findings suggest that both a bipyridyl ring and a methyl group of either 1- or 1′-position of paraquat are strongly recognized by this specific antibody.

The concentrations of paraquat in sera and specimens collected at autopsy, determined by colorimetry using Sep-Pak C_{18} cartridges [*4*], are listed in Table 1. Of seven paraquat-poisoning cases, the paraquat level of Case 3, collected at autopsy and not fixed in the formalin fixative, was also determined by colorimetry; the level of paraquat of liver, kidney, heart

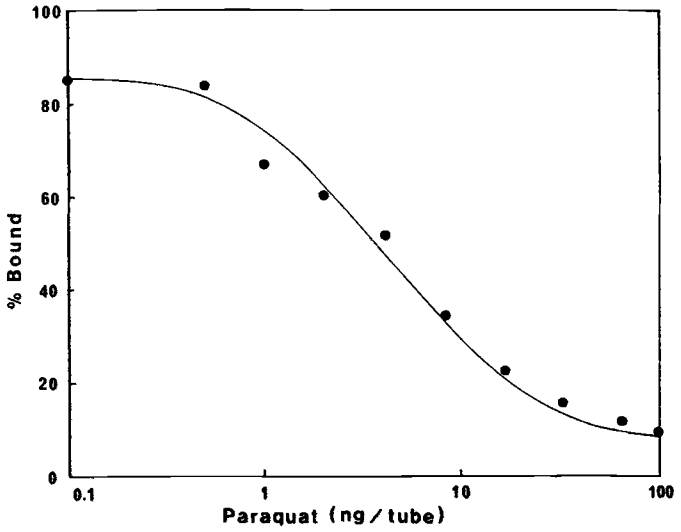


FIG. 2—*Inhibition of binding of antiserum to [^{3}H] paraquat by paraquat dichloride.*

TABLE 2—*Cross-reactivity of antiserum with bipyridyl derivatives and reduced paraquat.*

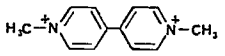
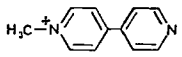
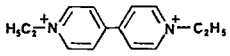
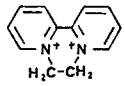
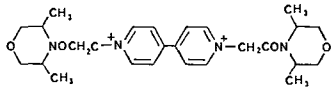
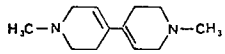
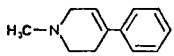
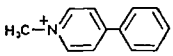
Generic Name	Structure	Cross-Reactivity, %
Paraquat		100
Monoquat		16.5
Diethyl-paraquat		5.59
Diquat		0.0038>
Morfamquat		0.0038>
Reduced paraquat		0.38>

TABLE 3—*Cross-reactivity of antiserum with similar analogs to paraquat.*

Generic Name	Structure	Cross-Reactivity, %
MPTP		0.0038>
MPP^+		0.76

muscle, and lung was 10.8-, 22.6-, 4.7-, and 11.1-μg/g wet weight, respectively. On the other hand, Table 4 shows the concentrations of paraquat in tissues of the seven poisoning cases fixed during two years which were determined by the present RIA. From these results, most paraquat levels in fixed tissues were much lower than those in unfixed ones as expected, especially in the lung tissue. This indicates that during fixation the paraquat incorporated into the tissue is easily released into the fixative although this release seems to depend upon the condition of fixation. The RIA demonstrated here seems to be enough to determine these low levels of paraquat even in the fixed tissues because of its high sensitivity. The extraction procedure of paraquat is very easy compared to that for instrumental analyses, such as gas chromatography and high pressure liquid chromatography [*9, 10*].

In this paper we demonstrated the development of a sensitive and reliable RIA for paraquat and applied this RIA to the determination of paraquat in cadaveric specimens. The RIA reported herein will be advantageous in forensic science and clinical fields.

TABLE 4—*Concentrations of paraquat in tissues among seven paraquat-poisoned cadavers measured by the present RIA.*

Case	Brain	Liver	Kidney	Heart Muscle	Lung
1	21[a]	8.4	5.6	12	10
2	0.31	0.48	0.23	0.33	0.40
3	0.19	0.24	0.68	0.62	0.20
4	0.15	0.40	0.16	0.30	0.24
5	1.3	0.54	0.25	0.29	0.29
6	0.47	0.30	2.0	0.17	0.34
7	0.32	0.25	0.31	0.09	0.13

[a]μg/g wet weight.

Acknowledgment

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