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Determination of paraquat in raw and formalin-fixed tissues by electron spin resonance (ESR) spectroscopy

K. Minakata[1], O. Suzuki[1], T. Morita[2], and M. Asano[1]

[1]Department of Legal Medicine, Hamamatsu University, School of Medicine, Hamamatsu, 431-31 Japan
[2]Department of Pathology I, Hamamatsu University, School of Medicine, Hamamatsu, 431-31, Japan

Summary. ESR method was applied to determine paraquat levels in fresh and formalin-fixed tissues. Paraquat was converted to paraquat radical by adding sodium dithionite to tissue homogenates and detected by ESR. Paraquat levels of more than 0.2 μg/ml homogenate could be quantified with 0.1 ml of the homogenate. The use of manganese ions for standardization of paraquat signal enabled much more accurate ESR measurements because this ion was quite stable and its signal did not overlap that of paraquat. Even with tissues fixed in formalin, tissues paraquat levels were measurable after removing formalin from the tissue extract. This fact was verified by studying two cases; the tissues were kept in formalin for 1.5 years in case 1 and for 6.5 years in case 2. In both cases, the paraquat contents in tissues were 0.02–0.08 μg/g. In this way ESR is one of the most suitable methods in determining low levels of paraquat in tissues even after they were preserved in formalin for a long time.

Key words: Paraquat, ESR – Tissues, formalin-fixed tissues – Manganese ion, paraquat radical

Zusammenfassung. Das Elektronen-Spin-Resonanz-Verfahren (ESR) wurde zur Bestimmung von Paraquatkonzentrationen in frischen und formalinfixierten Geweben herangezogen. Die Bildung des Paraquat-Radikals aus Paraquat erfolgt mit Hilfe eines Zusatzes von Natriumdithionit. Mit dem beschriebenen ESR-Verfahren können Paraquatkonzentrationen von über 0.2 μg/ml beim Einsatz von 0.1 ml Homogenisat bestimmt werden. Durch die Verwendung von Manganionen als Eichsubstanzen bei der ESR-Messung konnte eine bessere Standardisierung erzielt werden. Dieses Ion ist hinsichtlich der Signale recht stabil und interferiert nicht mit dem Paraquat-Signalen. Selbst im Falle formalinfixierter Gewebeschnitte gelang die quantitative Bestimmung des Paraquat, wenn zuvor das Formalin entfernt wurde. Die Methode

Offprint requests to: K. Minakata

wurde auf zwei Fälle angewandt: Bei Fall 1 betrug die Formalinverweildauer 1,5 Jahre, bei Fall 2 6,5 Jahre. In beiden Fällen lagen die Paraquatkonzentrationen in den Geweben zwischen 0,02 und 0,08 µg/g. Es konnte gezeigt werden, daß die ESR-Methode außerordentlich geeignet ist, um selbst in formalinfixierten Geweben nach langer Expositionszeit auch niedrige Paraquatkonzentrationen zu bestimmen.

Schlüsselwörter: Paraquat, ESR – Gewebe, formalinfixierte Gewebe – Manganionen, Paraquatradikal

Introduction

ESR method is sensitive and rapid for detecting paraquat in serum, urine, and drinks [1, 2]. It does not require removal of protein in contrast with methods of HPLC, GC, and spectrophotometry [3–9]. In our previous paper [1], we demonstrated that ESR signals of paraquat could be detected with high sensitivity only by reducing paraquat to its radical with sodium dithionite at high pH. In the present study we improved the previous method by introducing manganese ions (Mn^{++} in MgO) as a standard of paraquat signal intensity, and applied this method to raw tissue homogenates and tissues fixed in formalin. Simultaneous observation of ESR spectra of paraquat radical and Mn^{++} in each glass capillary enabled a convenient standardization of paraquat.

To facilitate formation of the paraquat radical, tissues were homogenized before mixing with sodium dithionite. For tissues stored in formalin, extraction of paraquat was performed by tenderization of tissues by adding $NaHCO_3$ prior to homogenization. Removal of formalin from paraquat extract was made by its elution through a Sep-Pak C18 ion exchange column [3, 10].

By this method, we were able to detect paraquat in formalin-fixed tissues preserved for 1.5 and 6.5 years.

Materials and methods

Mn^{++} in MgO was obtained from JEOL Ltd., Tokyo (Japan) and paraquat dichloride obtained from Sigma, St. Louis, MO (USA). Other chemicals used were of analytical grade. Sep-Pak C18 cartridges were obtained from Water Associate Inc., Milford (USA). Glass capillaries with an inner diameter of 1 mm used for ESR measurements were also taken for hematocrit measurements.

Fresh tissues used for spiking the paraquat were obtained at autopsy in our laboratory. Formalin-fixed tissues of case 1 kept for 1.5 years were obtained from the Enshu Hospital, Hamamatsu, and tissues of case 2 also fixed in formalin for 6.5 years were preserved in our laboratory. In case 1, 5 g of each organ and in case 2, 2.5 g of each were used.

Instrumentation

A JEOL JES-FE2XG ESR spectrometer was used with a modulation width of 0.8 gauss and a microwave power of 5 mW. When the paraquat content was more than 2 µg/ml, the spectrometer setting was 3283 gauss, sweep range 100 gauss, sweep time 16 min and response time 1 s, respectively. Two Mn^{++} signals ($g = 2.0340$ and $g = 1.9810$) were measured as references. When the paraquat content was less than 2 µg/ml, the spectrometer setting was 3307 gauss,

sweep range 50 gauss, sweep time 30 min and response time 3 s, respectively. One Mn^{++} signal ($g = 1.9810$) was measured as a reference.

Measurements of paraquat in raw tissues

The intensity of the reference signal of Mn^{++} was calibrated by standard paraquat solutions as follows. To each 90 µl of the standard paraquat solution (0.2–10 µg/ml) in 0.1 *M* sodium phosphate buffer, pH 9, 10 µl of sodium dithionite solution (2%) was added to produce paraquat radicals. The solution was put into a capillary and sealed with parafilm or clay. Mn^{++} in MgO was put into another capillary. Both capillaries were placed in an X-band ESR cell. The signals of paraquat radical and Mn^{++} obtained simultaneously were compared with each other to determine the content of Mn^{++}. In this way, the capillaries containing different amounts of Mn^{++} were calibrated by the known amounts of paraquat (Fig. 1).

ESR spectroscopy is applicable to the measurements of radicals in a solid state. The formation of the paraquat radicals in a tissue was, however, hardly proceeded because of difficulty in the mixing of dithionite ions will paraquat molecules in a solid tissue. To remove this problem, homogenization of a tissue was necessary prior to the mixing with sodium dithionite and ESR measurement. To determine the paraquat content spiked in a tissue, a spiked tissue was homogenized in four volumes of 0.1 *M* sodium phosphate buffer pH 11 by use of a Polytron homogenizer (Kinematica, Luzern, Switzerland). If necessary,the homogenate was centrifuged for 1 min at 1000 rpm to remove large solid fragments which prevent the homogenate from entering into a capillary. Then, 90 µl of the homogenate was treated in the same way as desribed above. The signal of paraquat radical and that of Mn^{++} were compared to determine the paraquat content in the homogenate (Fig. 1) and the accuracy of the ESR measurement was examined using the spiked tissues.

Measurements of paraquat in tissues fixed in formalin

In these tissues, formalin was found to inhibit paraquat radical formation but not to decompose a paraquat molecule, as described in the next section. To extract paraquat from the for-

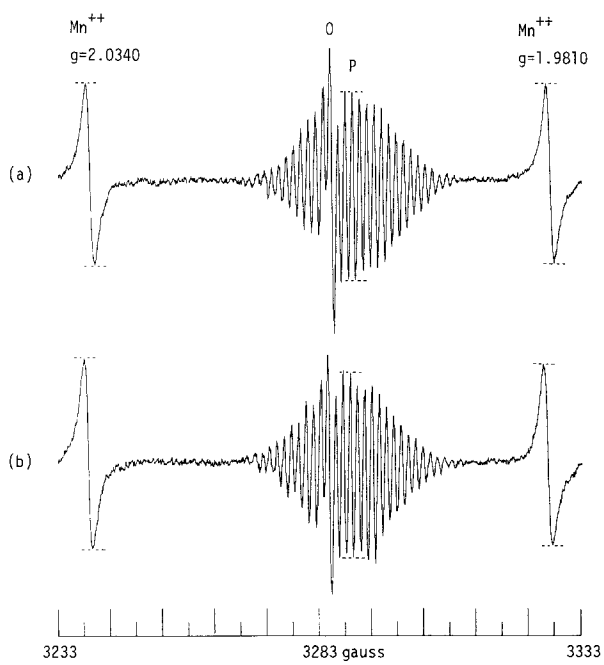

Fig. 1a, b. ESR spectra of paraquat radical and Mn^{++}. (**a**) Two Mn^{++} peaks at $g = 2.0340$ and 1.9810 are calibrated by the central peak of paraquat radical denoted by *P*. The concentration of paraquat is 10 µg/ml. The symbol O is an unidentified signal induced by the reaction of sodium dithionite with the buffer solution. (**b**) Paraquat radical in lung homogenate is quantified to be 10 µg/ml with the calibrated Mn^{++} peaks. The calculated value agrees with the spiked amount (10 µg/ml)

malin-fixed tissues, tenderization of these tissues was primarily required before homogenization. Therefore, the following two processes were necessary before ESR measurements, i.e., extraction of paraquat from tissues (steps 1–5), and removal of formalin with a Sep-Pak C18 cartridge (steps 6–9).

1. Add 10 ml of saturated $NaHCO_3$ solution to 5 g of a minced tissue and homogenize it with a polytron homogenizer.
2. Incubate the homogenate at 37°C for 18 h.
3. Homogenize again and centrifuge it at 15,000 *g* for 10 min, and get the supernatant.
4. Wash the precipitate twice by adding 10 ml water and repeat step 3.
5. Combine the three supernatants obtained by steps 3 and 4.
6. Adjust the pH of the supernatant to 13 with NaOH.
7. Pass the solution twice through an activated Sep-Pak C18 cartridge to adsorb paraquat.
8. Wash the cartridge three times with 2 ml water.
9. Elute the paraquat with 2 ml of 0.1 *N* HCl solution.

ESR measurement of the solution was made according to the procedure described in the previous section. When the concentration of paraquat was less than 0.2 μg/ml, the solution was concentrated in vacuo prior to the ESR measurement.

Results

Figure 1a and b indicate an ESR spectrum of Mn^{++} calibrated with the standard paraquat solution and that of paraquat in lung homogenate, respectively. In the homogenates of the kidney, spleen or lung, the signal intensity of the paraquat which had been added to the organs coincided with that of the reference paraquat signal with the same concentration in the buffer solution at the first stage less than 10 min, but decreased to 90% within 1 h. In the homogenate of the liver, however, the initial paraquat level was 95% and decreased to 70% within 1 h. The rate of decrease of the paraquat signal in the liver homogenate was unchanged with increasing the pH from 9 to 13, or increasing the final content of sodium dithionite from 0.2% to 3.0%. The homogenates themselves of the liver, spleen, lung, and kidney did not show any ESR signals which interfered with those of paraquat radical. Therefore, paraquat in the homogenate could be quantified without any pretreatment in spite of the fact that the tissue homogenates contained larger amounts of oxidase, reductase, oxidant, or reductant than plasma or urine.

For tissues fixed in formalin, formalin inhibited paraquat radical formation with sodium dithionite, but formalin did not decompose the paraquat molecules. This fact was confirmed by mixing paraquat with 10% formalin for 2 months. Figure 2 indicates the relation between the concentration of formalin and the formation of paraquat radical measured by ESR. When the concentration of formalin was above 10^{-3}, the radical was not produced. This tendency was more prominent for trichloroacetic acid although the pH of all these solutions were adjusted to above 9.5. Perchloric acid, methanol, ethanol, and acetone had no effect on the radical formation up to 10% concentration. It was, therefore, necessary to remove formalin when formalin-fixed tissues were dealt with.

The tissue fixed in formalin was very tough and most of the paraquat was not extracted into aqueous phase even after severe homogenization with a polytron

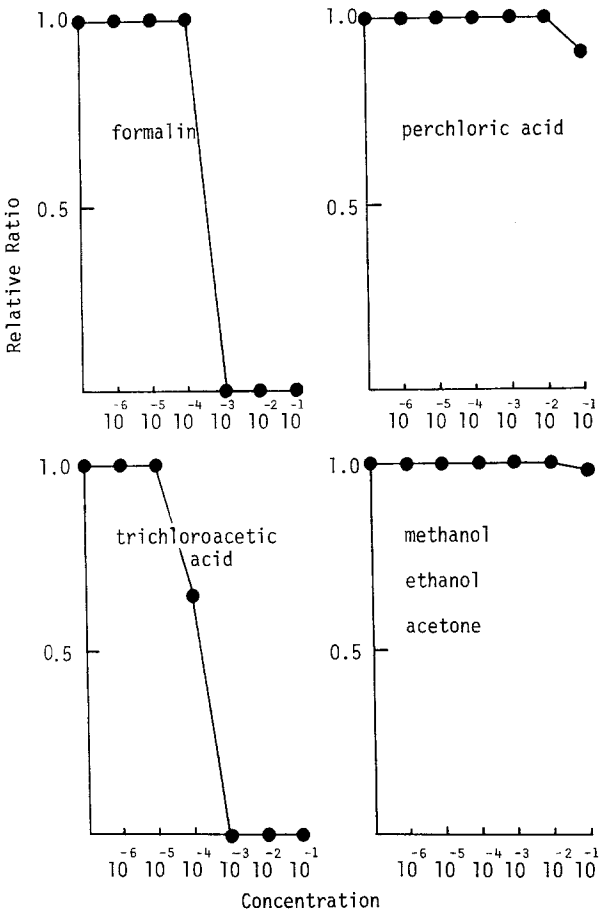

Fig. 2. Effects of protein-denaturing agents on the paraquat radical formation. The pH of all the solutions are adjusted to above 9.5

homogenizer. The extraction efficiency of paraquat was much better when the tissue was incubated in the presence of $NaHCO_3$ at 37°C for 18 h because the tissue was softened remarkably. The effect of incubation in $NaHCO_3$ solution is shown in Figure 3. The specimen used was the lung fixed in formalin in case 2. The usefulness of the $NaHCO_3$ solution was also confirmed with tissues of the liver, spleen, and kidney. In this figure, signal (a) corresponds to the sample extracted with $NaHCO_3$ at first and with water twice and (b) extracted with water three times. The signal (c) represents the signal of the lowest concentration for the detection, 0.2 μg/ml.

We describe here briefly the clinical data on the two cases studied.

Case 1

A woman aged 74 was admitted to a clinic because she had vomited greenish liquid several times and had abdominal pain, dyspnea, and dysuria. On day 6, she was transferred to the Ensyu hospital because of icterus, continuation of the above mentioned symptoms, ulcer of the tongue, and lung rales. On day 7, the conditions deteriorated and she died. In this case,

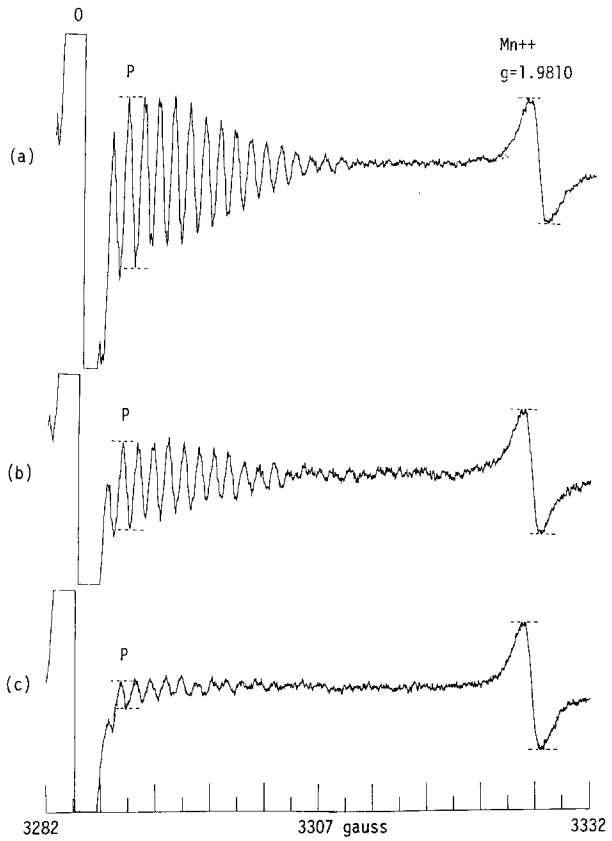

Fig. 3a–c. ESR spectra of paraquat radical extracted from lung in case 2. **a)** Paraquat extracted from 2.5 g of lung in case 2 with saturated $NaHCO_3$ solution once and with water twice. The ESR spectrum was measured after its concentration. (**b**) Paraquat extraction from 2.5 g of lung in case 2 only with water three times. The ESR spectrum was measured after its concentration. (**c**) ESR spectrum of paraquat solution of the lowest concentration (0.2 μg/ml) for its detection

Table 1. Paraquat levels in two cases

	Lung μg/g	Spleen μg/g	Kidney μg/g	Liver μg/g	Formalin solution μg/ml
Case 1					
$NaHCO_3$ extract	0.07	0.08	0.04	0.02	0.006
Water extract	0.008	0.009	0.008	0.007	
Case 2					
$NaHCO_3$ extract	0.06	0.04	0.03	0.02	0.03
Water extract	0.03	0.03	0.02	0.02	

paraquat was not examined until her death because she did not confess the ingestion of paraquat.

Autopsy findings. The autopsy was performed 1 h after her death. the esophagus and trachea were erosive and ulcerated. The hemorrhages were observed in both lungs, liver, and abdominal cavity. The color test using liver homogenate did not show the presence of paraquat. The histological findings, however, suggested the paraquat poisoning [11, 12]. To clarify the cause of her death, the tissues in formalin was sent to us 1.5 years after her death.

Chemical examination. Large blocks of tissues weighing more than 50 g each were preserved in 10% formalin. Each 5 g of liver, kidney, spleen lung and 30 ml of formalin solution were subjected to analyses. The extracted solutions were concentrated because the contents of paraquat were less than 0.2 μg/ml. Paraquat was quantified using a calibrated Mn^{++} signal at $g = 1.9810$. The paraquat contents were shown in Table 1.

Case 2

A man aged 37 was admitted to a hospital with a history of having taken paraquat solution sold as a brand of Gramoxone, a herbicide. The paraquat was detected in his urine and vomit at that time. Although peritoneal dialysis was performed, he died 3.5 days after ingestion. Autopsy was performed at our laboratory 16 h after his death.

Autopsy findings. The victim showed general icterus. The mucosa of esophagus was erosive and hemorrhagic. The lungs were heavy (right 1050 g, left 920 g), congested, edematous, and hemorrhagic. The kidney showed a tubular necrosis. The gastric mucosa was congested and contained hemorrhagic spots.

Chemical examination. The paraquat could not be detected by GC/MS at that time, although 10 g of raw liver and kidney had been used. After 6.5 years we tried again to detect paraquat in these tissues. Paraquat was detected in both tissues and formalin solution by the present ESR method. Figure 3 indicates the ESR spectra of paraquat in the lung with the calibrated Mn^{++}. The paraquat contents in several organs in case 2 are summarized in Table 1.

Discussion

The quantification of paraquat by ESR using calibrated Mn^{++} has not been devised in the previous report [1]. Simultaneous measurment of paraquat and Mn^{++} is a convenient method which does not require the separate measurement of standard paraquat solutions. Mn^{++} in MgO is stable at room temperature and can be used at any time. Its signal does not overlap that of paraquat. Therefore, the use of Mn^{++} is very useful for accurate determination of paraquat and could be applied to quantification of any other radicals which do not overlap the signal of Mn^{++}.

Our paper is the first trial for measurements of paraquat in raw tissue homogenates. This method, however, was inapplicable when paraquat concentration in the homogenate was below 0.2 μg/ml. In this situation, extraction and concentration of paraquat was necessary; for this purpose, the use of Sep-Pak C18 cartridges [3, 10] was the best because of simplicity and rapidity. Perchloric acid seems superior to trichloroacetic acid, if necessary, to remove protein from raw tissue homogenate because the former does not inhibit the paraquat radical formation, whereas the latter does [9] as shown in Fig. 2.

From tissues fixed in formalin it is very hard and difficult to extract paraquat with high recovery. The levels of paraquat extracted only with water for four kinds of organs were almost the same as the level of the external formalin solution in both cases (Table 1). The paraquat level extracted from tissues softened with $NaHCO_3$ was 3 to 10 times higher than that of the external formalin solution in case 1. Therefore, in this case most of paraquat seems to be retained inside the blocks of tissues. There were not many differences between paraquat

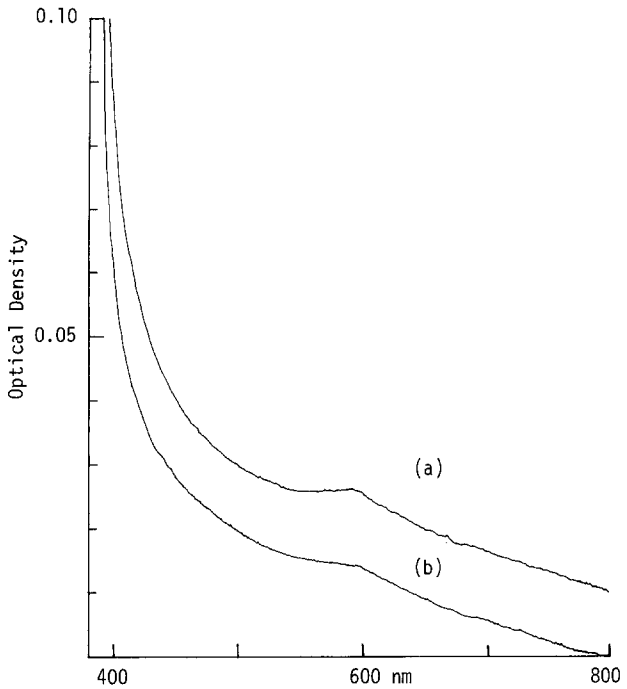

Fig. 4a, b. Absorption spectra of paraquat radical extracted from lung in case 2. **(a)** Paraquat extracted as described in Fig. 3a. **(b)** Paraquat extracted as described in Fig. 3b

levels measured with and without $NaHCO_3$ in case 2 because the tissues had been cut into small pieces and washed once. In both cases, paraquat levels in tissues may be much lower than 1 μg/ml, which is the lowest limit of the detection of color due to paraquat in clear solution.

The purification of the sample is necessary in GC, HPLC, and the spectrophotometric method [3–9]. Figure 4 indicates the absorption spectra of paraquat in lung, the same specimen that was used in ESR measurement desribed in Fig. 3a and b. As shown in this figure, the impurity which affects the optical measurement is contained considerably even after the removal of protein and formalin using a Sep-Pak C18 cartridge. In the ESR method, these impurities do not interfere with the measurement as shown in Fig. 1b in tissue homogenate and in Fig. 3a and b in formalin-fixed tissues.

The intensity of the ESR signal gradually decreases to about 90% within 1 h in homogenates of the lung, spleen, and kidney, and a little faster in homogenate of the liver. The latter specimen shows a slight decrease just after the reaction with dithionite. The reason for this is unknown, although it might be due to some reactive components contained in these tissues.

The paraquat levels in tissues are usually very low even with its fatal intake [11]. When the paraquat level is low, the relative ratio of impurity may increase. In such an occasion, ESR is one of the most suitable methods because it is not influenced by most impurities unless the impurities react with paraquat. Furthermore, ESR is a sensitive method which can detect paraquat more than 0.02 μg present in a block of an organ; it is 15 times lower than the detection limit of the

photometric assay. The present method which enables detection of paraquat in formalin-fixed tissues is quite useful for the re-examination for paraquat poisoning in old case, in which only formalin-fixed organs are available.

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