Journal of Chromatography, 527 (1990) 189–195
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5155

Note

Determination of paraquat in rat brain using ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography with ultraviolet detection

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(First received August 28th, 1989; revised manuscript received November 9th, 1989)

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) is an extremely effective, non-selective herbicide widely used in many countries of the world. Unfortunately, paraquat is also toxic to humans and animals, and several human deaths have been attributed to paraquat poisoning [1–4].

Paraquat produces mainly lung injury [5] but there have also been reports of a toxic effect on heart [1,6], liver [7] and kidney [8]. More recently, brain damage has been described in some patients who died from paraquat poisoning [9,10].

When directly microinfused into several areas of the rat brain, the herbicide produces neuropathological lesions and behavioural and motor alterations [11,12]. Marked behavioural and neurological disorders are also evoked in rats following systemic administration (5 mg/kg per day subcutaneously for fifteen consecutive days) of paraquat [12]. Evidence exists demonstrating that paraquat induces dose-dependent conditioned taste aversions and weight loss

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0378-4347/90/$03.50

when subcutaneously injected in rats [13]. Barbeau et al. [14] reported that a significant correlation exists between paraquat use and incidence of Parkinson's disease in some regions of Canada. All these observations are consistent with the hypothesis of a direct interaction of paraquat with specific areas of the central nervous system, indicating that the herbicide is able to reach the brain.

In order to ascertain whether paraquat, after systemic administration, is able to cross the blood–brain barrier and to reach specific areas of the rat brain, ion-pair, reversed-phase high-performance liquid chromatography (HPLC) was utilized to measure paraquat brain concentrations in rats subcutaneously injected with several doses of the herbicide. HPLC procedures have been described for the analysis of paraquat in various biological samples, such as serum [15], blood [16,17], urine [18,19], gastric aspirate [18] and liver [16]. The separation of paraquat by isocratic elution was performed on analytical columns packed with octadecylsilica [16,17,19], cation exchanger [15] or with a material prepared by chemically bonding γ-aminopropyltriethoxysilane to alumina [18]. The best peak shapes were obtained on C_{18} reversed-phase columns, using ion-pairing reagents in the mobile phase. Developments in the extraction of cations into organic solvents as ion-pairs allowed a new approach to paraquat extraction from biological samples. Thus, ion-pair extraction into organic solvents was applied to the analysis of paraquat in liver and blood [16], and an ion-pair liquid–solid extraction, using disposable cartridges of octadecylsilica, has been described for the extraction of the herbicide from urine [19], the latter method being rapid, easy to perform and providing high recovery values.

In the present study, the ion-pair liquid–solid extraction, previously described by Gill et al. [19] for the extraction of paraquat from urine, has been employed, with suitable modifications, in the extraction of paraquat from rat brain. An ion-pair, reversed-phase HPLC method with UV detection was developed to measure paraquat concentrations in the brain extracts.

EXPERIMENTAL

Materials

Cetrimide (hexadecyltrimethylammonium bromide), 1-heptanesulphonic acid sodium salt and paraquat dichloride (1,1′-dimethyl-4,4′-bipyridinium dichloride) were purchased from Sigma (St. Louis, MO, U.S.A.). Triethylamine, orthophosphoric acid (Suprapur, 85%), perchloric acid (Suprapur, 70%), hydrochloric acid (Suprapur, 30%) and methanol (HPLC grade) were obtained from Merck (Darmstadt, F.R.G.). Ammonium hydroxide (ACS reagent) was purchased from Aldrich Chemie (Steinheim, F.R.G.) and acetonitrile (HPLC grade) from Carlo Erba (Milan, Italy). 1,1′-Diethyl-4,4′-bi-

pyridinium diiodide, the diethyl analogue of paraquat, was kindly supplied by ICI (Macclesfield, U.K.) and was used as the internal standard. The Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

HPLC apparatus

A high-performance liquid chromatograph (Beckman Instruments, Berkeley, CA, U.S.A.) consisting of two Model 110B pumps with a Model 420 controller was used. Samples were loaded via an Altex Model 210A injection valve fitted with a 20-μl sample loop. A Beckman Model 163 variable-wavelength UV detector monitored the column effluent at 258 nm (flow cell volume 14 μl). Peak heights were quantified using a Shimadzu (Kyoto, Japan) Chromatopac C-R3A data processor. Separation by isocratic elution was performed on a 250 mm$\times$4.6 mm I.D. Altex Ultrasphere ODS column (C_{18} reversed-phase, particle size 5 μm) preceded by a 45 mm$\times$4.6 mm I.D. Beckman Ultrasphere ODS guard column (C_{18} reversed-phase, particle size 5 μm).

Mobile phase

A solution containing the specific equivalent of 7.5 m*M* sodium heptanesulphonate and 0.10 *M* orthophosphoric acid was made up in 0.45-μm filtered doubly distilled water. The pH was adjusted to 3.00 with triethylamine, and the organic modifier, acetonitrile, was added to yield a 10% (v/v) proportion. The mobile phase was then passed under vacuum through a FHUP04700 0.5-μm filter (Millipore, Bedford, MA, U.S.A.). The flow-rate was 1.0 ml/min (10.3 MPa) at ambient temperature.

Preparation of reagent solutions

Alkaline cetrimide was prepared by dissolving cetrimide (500 mg) and ammonium hydroxide (d=0.900; 5 ml) in water (1000 ml).

Alkaline sodium heptanesulphonate was prepared by dissolving sodium heptanesulphonate (2 g) and ammonium hydroxide (d=0.900; 2 ml) in water (100 ml).

To prepare acidic methanol, 30% hydrochloric acid (10 ml) was dissolved in methanol (1000 ml).

The 1,1′-diethyl-4,4′-bipyridinium diiodide solution was 10 μg/ml in water.

Experimental animals

Adult Wistal Morini male rats (180–200 g) were used. The rats were subcutaneously injected with paraquat (1, 2.5 or 5 mg/kg). After 60 min, the animals were sacrificed and the brains rapidly removed, weighed and stored at -20°C until analysis.

Preparation of Sep-Pak C_{18} cartridges

The Sep-Pak C_{18} cartridges were prepared for extraction by passing water (5 ml), methanol (5 ml), water (5 ml), alkaline cetrimide (8 ml), water (5

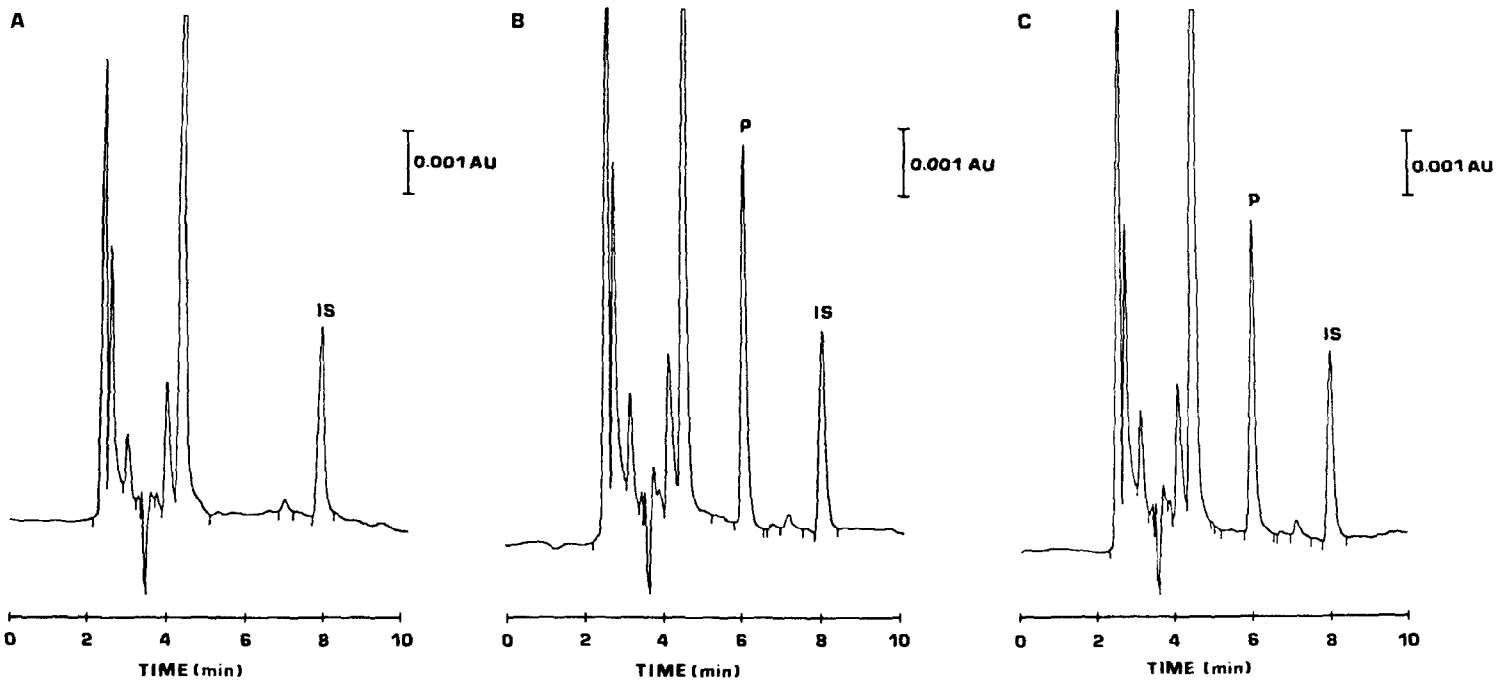

Fig. 1. HPLC elution profiles of extracts from rat brains: (A) blank brain spiked with internal standard (250 ng/g wet brain weight); (B) blank brain spiked with paraquat (240 ng/g wet brain weight) and internal standard (250 ng/g wet brain weight); (C) brain of a rat treated with paraquat (5 mg/kg s.c.) and killed 1 h after herbicide administration. Chromatographic conditions as described in text; 0.008 a.u.f.s. Peaks: P = paraquat; IS = internal standard.

ml), methanol (10 ml), water (5 ml) and alkaline sodium heptanesulphonate (10 ml).

Sample preparation

Frozen whole brains were individually thawed, placed in polypropylene tubes containing 4 ml of 0.10 *M* perchloric acid and the internal standard (250 ng/g wet brain weight) and homogenized with an ultrasonic cell disrupter (Model 250, Branson, Danbury, CT, U.S.A.). Homogenates were centrifuged (15 000 *g*, 15 min) in an Ole Dich 154.RF centrifuge (Ole Dich Instrument makers, Hvidovre, Denmark) and the supernatants were transferred to polypropylene tubes containing 500 μl of ammonium hydroxide. After 30 s of mechanically agitation and subsequent centrifugation (15 000 *g*, 15 min), the supernatants were removed into a 5-ml syringe and passed through the pretreated Sep-Pak C_{18} cartridges. The cartridges were washed with water (5 ml) and methanol (10 ml), and the eluates were discarded. Paraquat and internal standard were then eluted with acidic methanol (5 ml), and the collected eluates were evaporated to dryness at 40°C under a stream of air. The residue was dissolved in mobile phase (200 μl/g wet brain weight) by vortex-mixing for 60 s, removed in a 1-ml syringe and filtered through a 0.45-μm Millex HV-4 filter unit (Millipore) prior to injection onto the chromatographic column. The injection volume was 20 μl.

RESULTS AND DISCUSSION

Calibration was performed by adding known amounts of paraquat to whole brains from untreated animals to give final paraquat concentrations ranging from 15 to 360 ng/g wet brain weight (internal standard 250 ng/g wet weight) and processing the samples as described above. Results were plotted as the ratio of peak heights of paraquat to internal standard versus concentrations of paraquat. The ratio for an unknown sample was converted into a concentration (expressed as ng/g wet brain weight) by use of this calibration curve.

The six-point calibration curve of peak-height ratio versus amount of paraquat added to the samples was linear over the concentration range examined (15–360 ng/g wet weight). Linear regression analysis of the calibration curve data (each point is the mean of four determinations) indicated no significant deviation from linearity ($r=0.9999$).

The inter-assay precision of the method was evaluated as the coefficient of variation (C.V.) for the peak-height ratio at each concentration of paraquat added. The average C.V. was 3.7% (range 1.2–6.5%).

The absolute analytical recovery of paraquat from brain tissue was estimated by comparing peak heights obtained from the injection of extracts prepared from blank brains spiked with known amounts of paraquat (range 15–360 ng/g wet weight) with peak heights obtained from the injection of equiv-

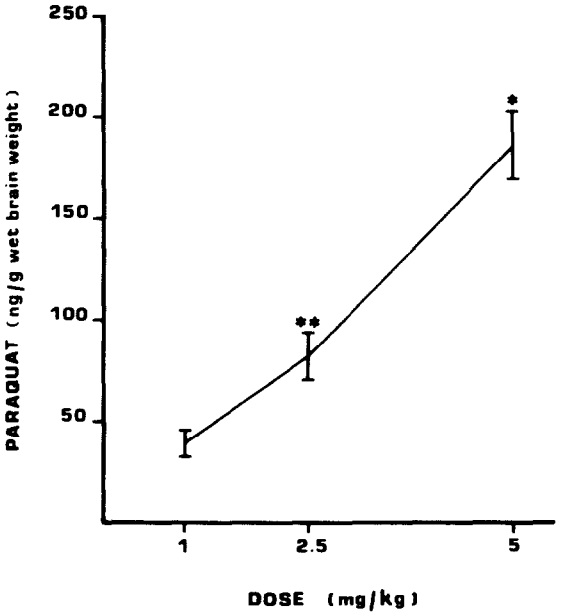

Fig. 2. Dose-related brain paraquat concentrations in rats subcutaneously injected with the herbicide (1, 2.5 and 5 mg/kg) and killed 1 h later. Each point represents the mean ± S.E. of six to eight animals. (*) $p < 0.01$ versus 1 and 2.5 mg/kg; (**) $p < 0.05$ versus 1 mg/kg.

alent aqueous standard solutions (range 0.075–1.8 μg/ml). Recoveries were in the range 88–99%, with an average recovery of 94.3%.

Fig. 1 shows representative chromatograms from two blank brains spiked with (A) internal standard (250 ng/g wet weight) and (B) paraquat (240 ng/g wet weight) and internal standard (250 ng/g wet weight). Fig. 1A shows no peak that could interfere with paraquat analysis. Under the chromatographic conditions specified, paraquat and the internal standard eluted at 6 and 8 min, respectively.

The detection limits above the background of a blank brain were typically less than 15 ng/g wet brain weight, which are well below the concentration levels encountered in the brains of rats treated with paraquat.

Paraquat brain concentrations, measured in rats subcutaneously injected with the herbicide, were related to the administered doses with values (mean ± S.E.) of 39.0 ± 6.61 (1 mg/kg s.c.), 82.0 ± 12.12 (2.5 mg/kg s.c.) and 185.6 ± 17.51 (5 mg/kg s.c.) ng/g wet brain weight (Fig. 2). A chromatogram from the brain of a rat treated with paraquat (5 mg/kg s.c.) is shown in Fig. 1C.

CONCLUSIONS

We have presented here a reliable, easy to perform and specific method for the detection of paraquat in rat brain, using ion-pair, reversed-phase HPLC with UV detection.

Sample preparation involves ion-pair liquid–solid extraction on disposable cartridges of octadecylsilica. The method had been used to measure brain paraquat concentrations in rats subcutaneously injected with several doses of the herbicide. The present preliminary results demonstrate that paraquat, subcutaneously injected in rats, is able, at least with the doses and the route of administration used, to cross the blood–brain barrier.

In previous experiments [11,12] it has been demonstrated that paraquat, directly microinfused (from 100 ng to 10 μg) into different areas of the rat brain, produces similar behavioural and electrocortical alterations and neuropathological lesions. However, a specific neurotoxicity for some neuronal populations, after systemic administration, cannot be excluded on the basis of the effects induced by direct intracerebral infusion, since a particular pharmacokinetic distribution of paraquat in the brain and/or uptake by some specific neurons might account for a neurotoxicological action limited to a specific neuronal population. Thus, further experiments are in progress in our laboratory in order to ascertain whether a selective accumulation of paraquat occurs in some specific areas of the rat brain following acute and chronic systemic treatment.

ACKNOWLEDGEMENTS

We are grateful to ICI (Macclesfield, U.K.) for kind supply of 1,1′-diethyl-4,4′-bipyridinium diiodide. Partial support from the Italian Council of Research (CNR, Rome, Italy) is gratefully acknowledged. Our thanks to Mr. Giovanni Politi for his skilful technical assistance.

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