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DETERMINATION OF THE HERBICIDES PARAQUAT AND DIQUAT IN BLOOD AND URINE BY GAS CHROMATOGRAPHY

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SUMMARY

A gas chromatographic method is described for determining paraquat (I) and diquat (II) in human blood and urine. I or II was readily precipitated as its reineckate complex (III and IV) by addition of Reinecke reagent, although blood required deproteination with 3.4% perchloric acid or 10% trichloroacetic acid. The precipitation is completed in 1 h at room temperature. III and IV were easily reduced by treatment with a mixture of sodium borohydride and nickel(II) chloride to afford corresponding perhydrogenated products, 1,1′-dimethyl-4,4′-bipiperidine (V) from III and *trans*- and *cis*-perhydrodipyrido[1,2-*a*:2′,1′-*c*] pyrazine (VI) from IV. The perhydrogenated products were determined by gas–liquid chromatography (GLC) without interference from the original components of the blood and urine.

The GLC method (5% potassium hydroxide solution with 5% Apiezon L on Chromosorb W AW DMCS) is suitable for simultaneous determination with hydrogen flame-ionization detection in the range 1–70 μg/ml (0.7–50.7 μg/ml as I ion; 0.5–35.6 μg/ml as II ion) of I and II in blood and urine. The method could be applicable to the determination of the chemicals in *postmortem* tissue and as pollutants in soils.

INTRODUCTION

Accidental and suicidal poisonings with paraquat (I) and diquat (II), which are effective contact herbicides, have increased each year in many countries since they exhibit, at chronic threshold doses, remarkable selective toxicity for the lung[1] (I) and for the eye lens[1] (II); I is especially lethal. Consequently, much attention has been devoted to studies of methods for their determination in human body fluids and *postmortem* tissue. In previous papers, we reported that herbicide preparations based on N-alkylbipyridylium derivatives[2–4], such as I[2,3], II[2,3] and morphamquat[4], can be determined by gas–liquid chromatography (GLC) of their reduction products obtained by treatment with sodium borohydride alone, and with a mixture of sodium borohydride and a transition metal salt, *e.g.*, nickel(II) chloride.

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In addition to GLC[2–7], analyses have been carried out by spectrophotometry[8–17], spectrofluorimetry[18], polarography[19,20], thin-layer chromatography[21,22], high-performance liquid chromatography (HPLC)[23–25] and off-line combination of isotachophoresis and mass spectrometry (MS)[26]. Among these methods, spectrophotometry[12–26], polarography[20], HPLC[24] and GLC[7,12,15] have been utilized in assays of body fluids.

When using spectrophotometry, the separation and purification of I and II from body fluids prior to quantitative analysis can be performed by column chromatography with an ion-exchange resin[12–14], by selective extraction with an ion-associated reagent[15] or with Sep-Pak C_{18} cartridges[16]. These procedures are time consuming and troublesome. Differential pulse polarography[20] was employed for the direct determination of I in serum and urine, but no the assay of II was carried out. HPLC[24] with UV spectrophotometric detection was used for the direct analysis of these chemicals in urine, but in spite of its good sensitivity it was inadequate for assays in other body fluids, *e.g.*, blood.

The direct application of our previously reported GLC method[2] to body fluids has been unsatisfactory because of the appearance of side peaks arising from usual components of body fluids and the requirement for a large amount of reductant, although I in human plasma was determined by a modification[7] of our method. However, II could not be determined[7].

This paper describes the GLC determination of I and II in human blood and urine using reineckate complexes and their perhydrogenated products in a sodium borohydride–nickel(II) chloride reduction system.

EXPERIMENTAL

Apparatus

GLC was carried out with a glass column (2 m × 0.3 cm I.D.) on a Hitachi Model 073 gas chromatograph equipped with a hydrogen flame-ionization detector (HFID). GLC–MS was conducted with a JEOL JMS-300 instrument equipped with a glass column (1 m × 0.2 cm I.D.) packed with 3% SE-30, and 5% potassium hydroxide plus 5% PEG 20M at 150°C and a pressure of helium of 1.0 kg/cm^2. The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current of 50 μA, and a temperature of the ion source of 170°C.

Materials

I (1,1′-dimethyl-4,4′-bipyridylium dichloride) and II (1,1′-ethylene-2,2′-bipyridylium dibromide), purified by recrystallization from the commercial preparations, were used.

Blood and urine were collected from a healthy volunteer.

Reagents

All chemicals were of analytical-reagent grade. Fresh diethyl ether was used in the extraction of reduction products.

Preparation of the reineckate complexes (III and IV) of I and II in blood and urine

Blood (1 ml) containing 0.5–70 μg/ml of I or II was treated with 4 ml of 3.4% perchloric acid or 1.5 ml of 10% trichloroacetic acid solution and the mixture was cen-

trifuged for 10 min at 1200 *g*. The supernatant and washings, which were obtained by washing the protein precipitate with the deproteination agent, were combined and used as the sample solution described below. Urine (1 ml) containing 0.5–70 μg/ml of I or II was used directly for the examination, if necessary after centrifugation.

To the sample solution was added 1 ml of 1.0% reinecke salt solution. If I or II was present in large amounts, the mixture become turbid at once. The mixture was allowed to stand for 1 h at room temperature, then centrifuged at 1200 g. The precipitate (reineckate complexes III and IV) was washed with 1 ml of 1.0% reinecke salt solution.

Reduction of reineckates (III and IV) with sodium borohydride and nickel(II) chloride

The precipitate (III and IV) was dissolved in 2 ml of 0.3 *M* hydrochloric acid in a steam bath, then 1 ml of 0.2 *M* nickel(II) chloride solution and 0.5 ml of toluene as defoaming agent were added. To the solution was carefully added 1 ml of 2.6 *M* sodium borohydride solution at 15°C. A black precipitate (nickel boride) was immediately formed with evolution of hydrogen. With continuous evolution of hydrogen, the mixture was allowed to stand for 1 h at room temperature, then extracted four times with 2-ml volumes of diethyl ether. The combined organic layer was dried over anhydrous potassium carbonate, acidified with a few drops of trifluoroacetic acid and evaporated to dryness under reduced pressure.

Procedure A

In the assay of 10–70 μg/ml of I and II, the residue was dissolved in 200 μl of ethyl acetate and a small amount of potassium carbonate was added. A 1-μl volume of the ethyl acetate solution was injected into the gas chromatograph. GLC was performed on a glass column (2 m × 0.3 cm I.D.) packed with 5% potassium hydroxide plus 5% PEG 20M on Chromosorb W AW DMCS (60–80 mesh) at 175°C with a nitrogen flow-rate of 30 ml/min (injection temperature 250°C, attenuation 1 × 8). The internal standard was 0.006% dibenzyl. A calibration graph (Fig. 3) was constructed by the use of aqueous solutions (1 ml) of different concentrations (I, 10, 30 and 50 μg/ml; II, 10, 40 and 70 μg/ml) of I and II, the above procedure (A) being applied.

Procedure B

In the assay of 1–70 μg/ml of I and II, the residue was dissolved in 50 μl of ethyl acetate and a small amount of potassium carbonate was added. A 1-μl volume of the ethyl acetate solution was injected into the gas chromatograph. GLC was performed on a glass column (2 m × 0.3 cm I.D.) packed with 5% potassium hydroxide plus 5% Apiezon L on Chromosorb W AW DMCS (60–80 mesh) at 205°C with a nitrogen flow-rate of 30 ml/min (injection temperature 250°C, attenuation 1 × 8). The internal standard was 0.004% xanthene. A calibration graph (Fig. 5) was constructed by the use of aqueous solutions (1 ml) of different concentrations (I, 1, 5, 10 and 12 μg/ml; II, 1, 5, 10 and 20 μg/ml) of I and II, the above procedure (B) being applied.

Elemental analysis of III and IV

I (128 mg; 0.5 mmol) or II (172 mg; 0.5 mmol) was dissolved in water (20 ml). To the slution were added 75 ml (2.0 mmol) of 1.0% reinecke salt solution at room temperature with stirring. The reineckate complex (III or IV) was liberated immedi-

ately as a precipitate and the mixture was allowed to stand for 1 h. The precipitate (III, pink; IV, yellow) was obtained in 95–97% yield and washed thoroughly with water because of difficulty in recrystallizing of the compound. Elemental analysis: calculated for $C_{20}H_{26}N_{14}S_8Cr_2 \cdot H_2O$ (III), C 28.56, H 3.36, N 23.32%; found, C 28.92, H 3.14, N 23.82%. Calculated for $C_{20}H_{24}N_{14}S_8Cr_2 \cdot H_2O$ (IV), C 28.63, H 3.12, N 23.37%; found, C 28.75, H 2.90, N 23.88%.

Influence of reaction time and temperature on the preparation of the reineckates (III and IV) of I and II

An aqueous solution (1 ml) of I (50 μg; 0.19 μmol) or II (50 μg; 0.16 μmol) was treated with 1 ml of 1.0% reinecke salt solution at various temperatures (5, 15 and 28°C) and various times (15 and 30 min, 1, 2 and 3 h). The reineckates thus obtained were perhydrogenated with sodium borohydride–nickel (II) chloride and the resulting reduction products were examined by GLC following procedure A.

RESULTS AND DISCUSSION

During studies on the separation and purification of I and II from body fluids such as blood and urine, we found that reinecke salt (ammonium tetrathiocyanodiammonochromate), generally used as a precipitant for primery and secondary amines, is also an excellent precipitant for the quaternary ammonium salts I and II. The treatment of I or II (aqueous solution, 0.5–70 μg/ml) with 1% reinecke reagent readily gave the reineckate complex (III or IV) as a pink or yellow precipitate. The precipitation of I and II was complete in the range 1–70 μg/ml (0.7—50.7 μg/ml as I ion; 0.5–35.6 μg/ml as II ion) of I or II aqueous solution, as confirmed by GLC, decribed below. Fig. 1 shows that the formation of III and IV is complete in 1 h at various temperatures (5, 15 and 28°C).

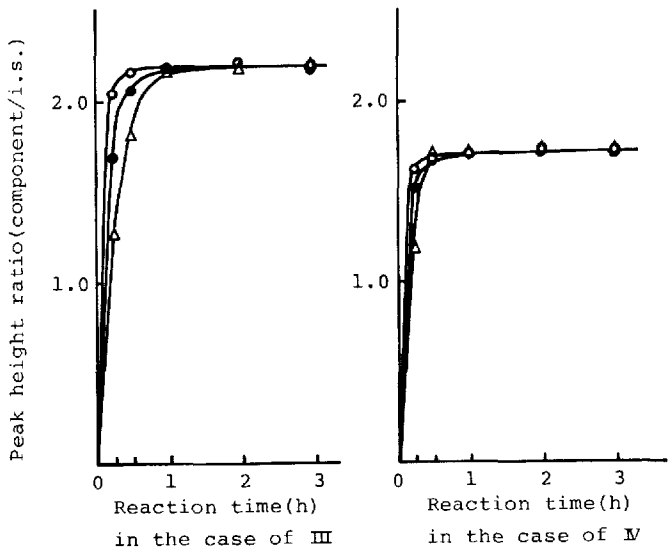


Fig. 1. Reaction time of reinecke salt with I or II. The content of I or II in the reineckate complex (III or IV) was determined by GLC (procedure A). Internal standard (i.s.): dibenzyl. Temperature: ○, 28°C; ●, 15°C; △, 5°C.

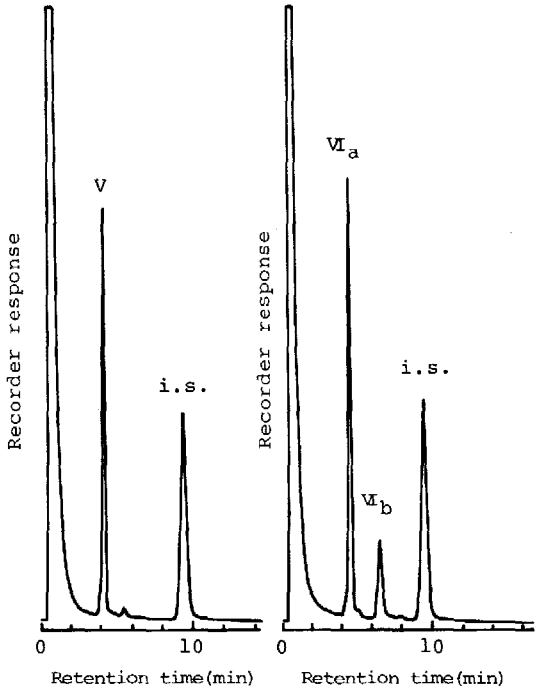


Fig. 2. Gas chromatogram of the perhydrogenated products derived from III and IV (procedure A). Internal standard (i.s.): dibenzyl.

The reineckate complexes (III and IV) were demonstrated by elemental analysis to be composed of paraquat or diquat ion and reinecke ion $[Cr(NH_3)_2(SCN)_4]^-$ in the molar ratio of 1:2.

Direct addition of the reinecke reagent to blood containing I or II precipitated the reineckate (III and IV) together with the usual components of blood. The presence of contaminants in the reineckate precipitates was undesirable for the subsequent reduction (sodium borohydride–nickel(II) chloride system) of III and IV, because of the formation of incompletely reduced products together with the perhydrogenated products. When the reinecke reagent was added to the supernatant of blood (containing 0.5–70 μg/ml of I or II) deproteinized with 3.4% perchloric acid or 10% trichloroacetic acid, respectively, the reineckates (III and IV) precipitated readily in 1 h. With urine, the precipitation of the reineckates (III and IV) occurred smoothly without the perchloric or trichloroacetic acid treatment.

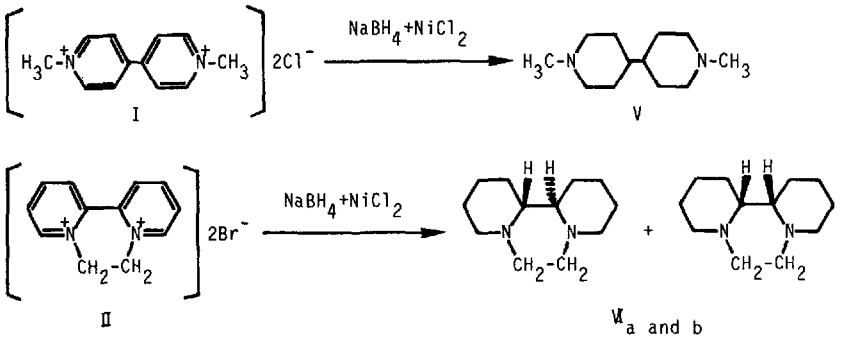
The reineckates (III and IV) of I and II thus obtained were dissolved in 0.3 *M* hydrochloric acid and treated with sodium borohydride and nickel (II) chloride to give perhydrogenated products (V and VI, respectively). The reduction products were chromatographed in a glass column with 5% potassium hydroxide plus 5% PEG 20M on Chromosorb W AW DMCS at 175°C. No side peaks arising from blood and urine were observed on GLC. As shown in Fig. 2, the reduction products are readily detected as a single symmetrical peak (V) for III, and two symmetrical peaks (VI_a and VI_b) for IV, as in the direct reduction of I and II. The assay of II was carried out on the basis of the main peak (VI_a) for IV.

When an equal amount of nickel(II)chloride [0.5 ml of 0.02 *M* nickel(II) chloride, 1.0 ml of 2.6 *M* sodium borohydride] in the case of the reduction of quaternary ammonium salts[3,4,27–29] was used in dilute acidic solutions (e.g., 1 ml of 0.3 *M* hydrochloric acid) of III or IV, undesirable peaks due to by-products resulting from incomplete reduction of I and II appeared. The perhydrogenation was complete with a large excess of nickel(II) chloride, such as a combination of 1 ml of 0.2 *M* nickel(II) chloride and 1 ml of 2.6 *M* sodium borohydride, in the range 0.5–70 μg/ml of I and II. However, in this reduction system, neutral solutions of the reineckates (III and IV) gave incomplete reduction products as by-products.

Such resistance to complete reduction of the reineckate complexes (III and IV) could be explained by the following findings. Thiocyanate existing in the reineckates was reduced with sodium borohydride and nickel(II) chloride to yield sulphide ion. In aqueous acidic solution (which gradually became alkaline with excess of sodium borohydride), most of the sulphide ion was immediately released as hydrogen sulphide gas, identified by basic lead acetate test paper, with hydrogen. The sulphide ion remaining in neutral and alkaline solution acted as a catalyst inhibitor (nickel boride).

The reduction products were identified as 1,1′-dimethyl-4,4′-bipiperidine (V) and as *trans*- and *cis*-perhydrodipyrido[1,2-*a*:2′,1′-*c*]pyrazine (VI_a and VI_b) by comparing the GLC retention times and the mass spectra with those of authentic samples[3]. Studies on the structures and absolute configurations of VI_a and VI_b will be reported elsewhere.

The reaction scheme is as follows:



The determination of I and II in human blood and urine under isothermal conditions (5% potassium hydroxide plus 5% PEG 20M column; procedure A) with an HFID was performed by the peak-height ratio method using dibenzyl as internal standard. A linear relationship existed between the concentration in the range 10–70 μg/ml of I and II, as shown in Fig. 3.

For microscale analysis at levels below 10 μg/ml of I and II, the reduction products (V, VI_a and VI_b) should be dissolved in the minimum volume of organic solvent (*e.g.*, 50 μl of ethyl acetate) in order to obtain satisfactory peaks on the gas chromatogram. In the above case, a side peak [retention time (t_R) = 4.5 min] arising from impurities in the reducing agent appeared as a shoulder on peaks of V (t_R = 4.4 min) and VI_a (t_R = 4.8 min), and interfered with the microscale assay. However, using a glass column as in procedure B, packed with 5% potassium hydroxide plus 5% Apiezon L on Chromosorb W AW DMCS, the side peak was observed as two peaks (a, t_R =

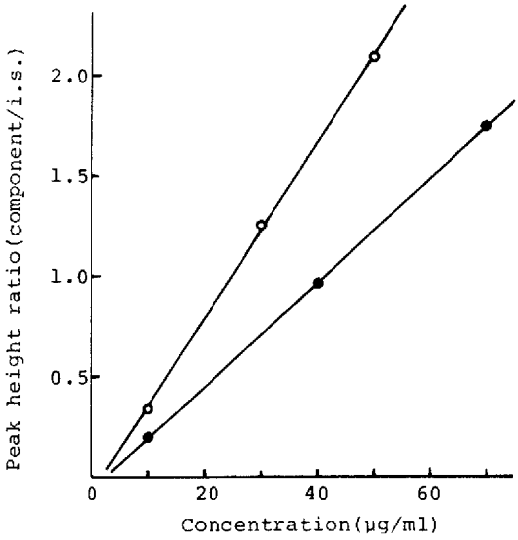


Fig. 3. Calibration graph for (○) I and (●) II (procedure A). Internal standard (i.s.): dibenzyl.

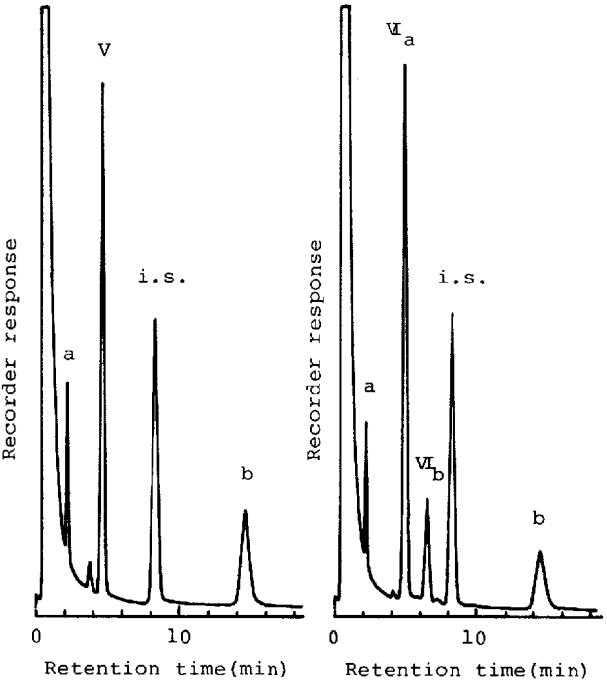


Fig. 4. Gas chromatogram of the perhydrogenated products (V, VI_a and VI_b) derived from III and IV (procedure B). Internal standard (i.s.): xanthene.

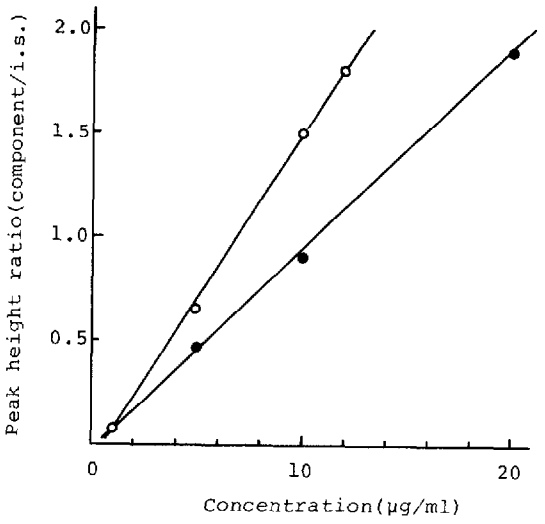


Fig. 5. Calibration graphs for (○) I and (●) II (procedure B). Internal standard (i.s.): xanthene.

2.1 min; b, t_R = 13.2 min). The peaks of V, VI_a and VI_b (t_R = 4.4, 4.8 and 6.3 min, respectively) could be separated satisfactorily from the side peaks, as shown in Fig. 4. Thus, the use of this column permitted determinations in the range 1–70 μg/ml (0.7–50.7 μg/ml as I ion; 0.5–35.6 μg/ml as II ion) of I and II. Under these conditions, no peaks derived from endogenous compounds in blood and urine were detected.

The calibration graphs (Fig. 5) for I and II showed good linearity using xanthene as the internal standard (peak-height ratio method). The limits of detection with the HFID were 0.5 μg (0.36 μg as I ion) for I and 1.0 μg (0.51 μg as II ion) for II.

The results of the analyses of I and II in blood and urine are given in Table I.

I and II in a mixture were satisfactorily separated from each other on the glass column in procedure B, as shown in Fig. 6, hence the GLC procedure is adequate for the simultaneous determination of I and II in blood and urine.

TABLE I

RECOVERY OF I AND II IN BLOOD AND URINE

Herbicide	*Amount added (μg)*	*Recovery (%) (mean ± S.D.; n=5)*	
		*Blood**	Urine
Paraquat (I)	1	57.7 ± 8.5	67.2 ± 7.5
	5	91.2 ± 3.6	87.0 ± 3.9
	10**	88.9 ± 5.1	91.6 ± 5.1
	30**	81.7 ± 8.1	97.2 ± 3.7
	50**	81.1 ± 4.7	97.2 ± 6.8
Diquat (II)	1	50.3 ± 10.2	52.6 ± 16.5
	5	91.0 ± 2.7	95.8 ± 7.8
	10**	80.0 ± 4.2	83.3 ± 12.6
	40**	83.0 ± 13.0	83.1 ± 5.9
	70**	89.8 ± 9.3	85.8 ± 4.0

* Deproteinized with 10% trichloroacetic acid.

** Performed by procedure A (results obtained by procedure B were similar).

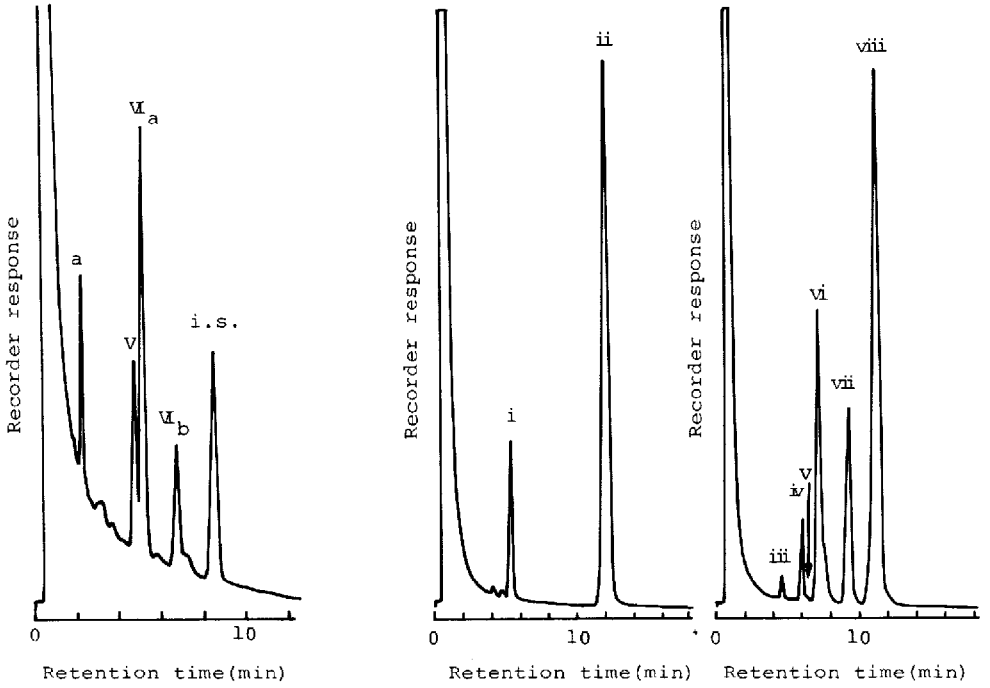


Fig. 6. Gas chromatogram of the mixture of the perhydrogenated products (V, VI_a and VI_b) derived from III and IV (procedure B). Internal standard (i.s.): xanthene.

Fig. 7. Gas chromatogram of the reduction products derived from III and IV with sodium borohydride (procedure A). III: two peaks [i, *m/e* 194(M^+:$C_{12}H_{22}N_2^+$, containing one double bond); ii, *m/e* 192 (M^+:$C_{12}H_{20}N_2^+$, 2 double bonds)]. IV: six peaks [iii and v, *m/e* 194 (M^+:$C_{12}H_{22}N_2^+$); iv and vi, *m/e* 192 (M^+:$C_{12}H_{20}N_2^+$, one double bond); vii and viii, *m/e* 190 (M^+:$C_{12}H_{18}N_2^+$, two double bonds)].

The original compounds in the side peaks formed on addition of sodium borohydride to nickel(II) chloride solution were readily dissolved in diethyl ether, and GLC–MS (column, SE-30) showed parent ion peaks at *m/e* 97 for the first peak (a, t_R = 2.1 min) and at *m/e* 149 for the second peak (b, t_R = 13.2 min). Those compounds have still not been identified, but might be complexes of borane and the alkylamine used for recrystallization of sodium borohydride*. Further investigations on the structures of the compounds are in progress.

The reineckate complexes (III and IV; 1–70 μg/ml as I or II) in acidic aqueous solution were also reduced with excess of sodium borohydride alone (1 ml of 2.6 *M* sodium borohydride) to afford incomplete reduction products in a similar manner to that of direct reduction of I and II with sodium borohydride as reported previously[2]. The incomplete reduction products were excellently chromatographed on the column in procedure A** to give two peaks for III and six peaks for IV, as shown in Fig. 7. On the basis of the main peaks (III, peak ii; IV, peak viii), the determination of I and II was possible (internal standard: dibenzyl), but the response of the peaks was low compared with that of the perhydrogenated products (V, VI_a and VI_b). The reduction method with sodium borohydride alone is useful for qualitative analysis because of the appearance of characteristic peaks for I and II in a constant ratio.

* Special grade of a number of commercial products was examined.

** The resolution of the column in procedure B for the incomplete reduction products was not better than that with the column in procedure A, as two peaks (i and ii) of III emerged as one peak with a shoulder, and six peaks (iii–viii) of IV appeared as three peaks on the alkaline Apiezon L column.

CONCLUSION

The herbicides I and II in human blood and urine are readily precipitated as the reineckate complexes (III and IV) by addition of reinecke reagent, although blood required deproteination with 3.4% perchloric acid or 10% trichloroacetic acid. The reineckates (III and IV) of I and II thus obtained were easily reduced by treatment with a mixture of sodium borohydride and nickel(II) chloride to afford the corresponding perhydrogenated products, 1,1′-dimethyl-4,4′-bipiperidine (V) from III and *trans*- and *cis*-perhydrodipyrido[1,2-*a*:2′,1′-*c*]pyrazine (VI) from IV. The perhydrogenated compounds (V, VI_a and VI_b) were determined by GLC without interference from the original components of blood and urine.

A glass column packed with 5% potassium hydroxide plus 5% Apiezon L on Chromosorb W AW DMCS is recommended for the assay in the range 1–70 μg/ml (0.7–50.7 μg/ml as I ion; 0.5–35.6 μg/ml as II ion) of I and II, as the peaks of the reduction products (V, VI_a and VI_b) were separated sufficiently from the side peaks arising from the contaminant in the reducing agent.

Further, this procedure is applicable to the simultaneous determination of I and II in cases of poisoning with a mixed preparation of the chemicals and when I or II is contaminated with the other product.

The GLC method is suitable for routine determinations in forensic applications, as it is very simple and much less time consuming than ion-exchange column chromatography. The GLC response of I and II with an HFID would be increased if a nitrogen-selective flame-ionization detector were used.

This method could be also used for the determination of I or II in *postmortem* tissue and as environmental pollutants in soils.

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