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Fluorometric Determination of 4-Hydroxyifosfamide in Blood and Urine

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A sensitive fluorometric method using 3-aminophenol was developed to measure 4-hydroxyifosfamide, the active metabolite of the antitumor agent ifosfamide, in blood and urine.

Serum and urine samples were mixed well with four volumes of citric acid–phosphate buffer (pH 3.5) to stabilize the 4-hydroxyifosfamide. After extraction with dichloromethane, the extract containing 4-hydroxyifosfamide was evaporated to dryness under reduced pressure. The residue was dissolved in distilled water, and the reagent solution of 3-aminophenol in 2 N hydrochloric acid was added. After the reaction of 4-hydroxyifosfamide with 3-aminophenol, the fluorescence intensity based on the formation of 7-hydroxyquinoline in the reaction mixture was measured by fluorometry. A linear response was obtained in this assay over the ranges of 1—10 and 5—50 nmol/ml in serum and urine, respectively. Ifosfamide and related compounds such as carboxyifosfamide and ifosfamide mustard did not interfere with the assay.

The present method was used to determine the amounts of 4-hydroxyifosfamide in blood plasma and urine samples from rats treated with ifosfamide.

Keywords——4-hydroxyifosfamide; fluorometry; 3-aminophenol; 7-hydroxyquinoline; ifosfamide; rat blood plasma; rat urine

Ifosfamide, which belongs to the class of oxazaphosphorine-alkylating antitumor agents, is inactive *in vitro* and is activated *in vivo* to a cytotoxic species. 4-Hydroxyifosfamide is the primary active metabolite which exists in equilibrium with its tautomer aldoifosfamide under physiological conditions[1] and consequently is very unstable in biological materials.[2] Fluorometric methods for measuring 4-hydroxyifosfamide in biological samples have been described[2,3] but their accuracy and precision are poor because of the instability of the active metabolite.

By examining the reaction conditions with 3-aminophenol in detail, we developed an improved method which is simple, rapid, and accurate enough to determine 4-hydroxyifosfamide levels in blood and urine. This report describes the stabilization of 4-hydroxyifosfamide in the weak acid region of pH 3—4.5 by using citric acid–phosphate buffer and the application of this finding to the determination of 4-hydroxyifosfamide in blood and urine.

Experimental

Apparatus——Fluorescence excitation and emission spectra and their intensities were measured with a Hitachi MPF-4 spectrofluorometer using quartz cells of 5 × 5 mm optical pathlength. The desired concentration of the dichloromethane extract from a biological sample was obtained by using a Vapour Mix apparatus (Tokyo Rikakikai Co., Ltd.).

Materials——4-Hydroxyifosfamide [2-(2-chloroethyl)amino-3-(2-chloroethyl)-4-hydroxy-tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide], carboxyifosfamide [2-carboxyethyl-*N*,*N*′-bis(2-chloroethyl)phosphorodiamidate] and ifosfamide mustard [*N*,*N*′-bis(2-chloroethyl)phosphorodiamidic acid] were kept in a refrigerator until just before use. Ifosfamide [2-(2-chloroethyl)amino-3-(2-chloroethyl)-tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide] and mesna (sodium 2-mercaptoethanesulfonate) were used as received from Asta-Werke AG, West Germany. Mesna is a

therapeutic drug used to treat the urotoxic side-effect of ifosfamide and other oxazaphosphorines. 3-Aminophenol was recrystallized from distilled water and dissolved in 2 N HCl under shielding from light. Hydroxylamine hydrochloride was added to the reagent solution to prevent the oxidation of 3-aminophenol. 7-Hydroxyquinoline was synthesized from glycerol and 3-aminophenol by the method of Bradford *et al.*[4] and purified by repeated sublimations at 160—180 °C in an oil bath under reduced pressure. mp 247 °C (differential thermal analysis) (mp 238 °C[4]). Citric acid–phosphate buffer (pH 3.5) was adjusted by mixing of 0.1 M citric acid and 0.2 M disodium phosphate. Dichloromethane of nonfluorescent reagent grade (Dotite Luminazol, Dojindo Laboratories, Japan) was used without further purification. Other chemicals were of reagent grade. Commercially available human serum (Flow Laboratories, U.S.A.) was used.

Standard Solution——A stock solution was prepared by dissolving about 3 mg of 7-hydroxyquinoline in 100 ml of 1 N HCl. Further dilutions with 1 N HCl were made to obtain the desired concentrations. The calibration curve was linear from 0.2 to 10 nmol/ml of 7-hydroxyquinoline in standard solutions.

Procedure——Sample Preparation: Serum and urine samples were mixed well with four volumes of citric acid–phosphate buffer (pH 3.5) precooled to 0—4 °C in ice-cold water. A 3 ml aliquot of the buffered sample solution of serum was taken, and 15 ml of precooled dichloromethane and 1.5 g of NaCl were added. The mixture was shaken for 10 min and centrifuged for 5 min at 4 °C. After the removal of the aqueous phase by aspiration, 8 ml of the dichloromethane extract was transferred to a 12-ml centrifuge tube and evaporated to dryness under reduced pressure at room temperature.

In the case of urine, 6 ml of precooled dichloromethane and 0.5 g of NaCl were used for 1 ml of the buffered sample solution. A 4-ml portion of the dichloromethane extract was taken for evaporation.

Assay by Fluorometry: The residue from the dichloromethane extract was reconstituted with 1 ml of distilled water, followed by addition of 1 ml of the mixed reagent solution of 3-aminophenol (3.3 mg/ml) and hydroxylamine hydrochloride (4 mg/ml) in 2 N HCl. The fluorescence reaction was performed by heating the mixture for 20 min in a boiling water bath. After being cooled to room temperature, the fluorescence intensity of the reaction mixture was read at 512 nm with excitation at 352 nm. At the same time, blank solutions from the control serum and urine and standard solutions of 7-hydroxyquinoline for the calibration curve were measured. The contents of 4-hydroxyifosfamide in the serum and urine were found from the calibration curve.

Results and Discussion

Fluorescence Reaction of 4-Hydroxyifosfamide with 3-Aminophenol

Acrolein reacts with 3-aminophenol in acidic solution to yield 7-hydroxyquinoline,[5] and 4-hydroxyifosfamide undergoes a similar reaction. The fluorescence excitation and emission spectra of the reaction mixture of 4-hydroxyifosfamide with 3-aminophenol showed maxima at 352 and 512 nm, respectively, as shown in Fig. 1, and coincided with those of the standard solution of 7-hydroxyquinoline. Therefore, the fluorescence product derived from 4-hydroxyifosfamide and 3-aminophenol was confirmed to be 7-hydroxyquinoline.

The conditions for the reaction of 4-hydroxyifosfamide with 3-aminophenol were examined. The reaction was run under acidic conditions with 0.25 to 3 N hydrochloric acid, and the maximum fluorescence intensity was obtained with 1 N hydrochloric acid. The intensity was affected by the concentration of 3-aminophenol and reached a maximum at 1.5×10^{-2} M (3.3 mg/ml in the reagent solution) or above. A reaction time of 20 min in a

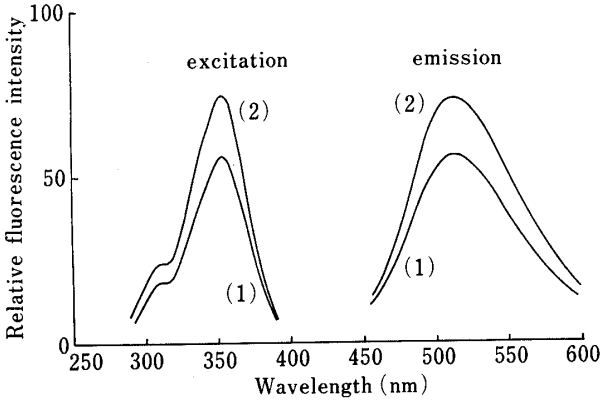

Fig. 1. Fluorescence Excitation and Emission Spectra of the Reaction Mixture and 7-Hydroxyquinoline

(1) Uncorrected excitation and emission spectra of the reaction mixture: 4-hydroxyifosfamide (3.9 nmol/ml) in aqueous solution was treated as described in the text.

(2) Uncorrected excitation and emission spectra of 7-hydroxyquinoline (1.5 nmol/ml) in 1 N HCl.

boiling water bath was sufficient to provide maximum and constant fluorescence intensity, which was stable for 2 h.

To evaluate the reaction yield of the fluorescent product under the conditions described above, the fluorescence intensities of reaction mixtures initially containing various amounts of 4-hydroxyifosfamide were compared with those of 7-hydroxyquinoline in standard solutions. The yield of the fluorescent product was calculated to be 55.6%, which is essentially the same as the 55% yield of 7-hydroxyquinoline obtained from acrolein and 3-aminophenol under comparable conditions,[5] and therefore indicated that acrolein was produced quantitatively from 4-hydroxyifosfamide.

From these results, we assumed that 4-hydroxyifosfamide was converted to 7-hydroxyquinoline by condensation of acrolein, liberated from its tautomer aldoifosfamide, with 3-aminophenol, as shown in Chart 1.

Stability of 4-Hydroxyifosfamide

The effect of pH on the stability of 4-hydroxyifosfamide in aqueous solution was examined in the pH range of 2.6 to 7.5, adjusted with citric acid–phosphate buffers. The fluorescence intensities of the samples in solutions at various pH values were measured for 0.5 to 5 h at room temperature. As shown in Fig. 2, 4-hydroxyifosfamide was relatively stable in the weakly acid region of pH 3—4.5. The optimum pH for the stability was at about 3.5. This observation was applied to stabilize 4-hydroxyifosfamide in serum and urine samples.

Figure 3 shows that 4-hydroxyifosfamide in human serum was degraded rapidly at room temperature, whereas it was reasonably stable at 0—4 °C in ice-cold water, though the degradation loss was still not negligible. In the serum treated with citric acid-phosphate buffer

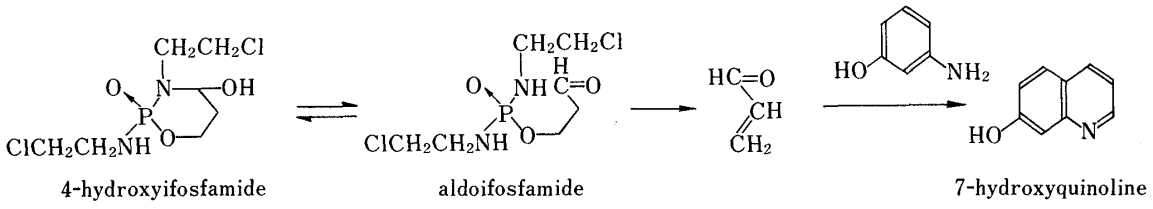

Chart 1

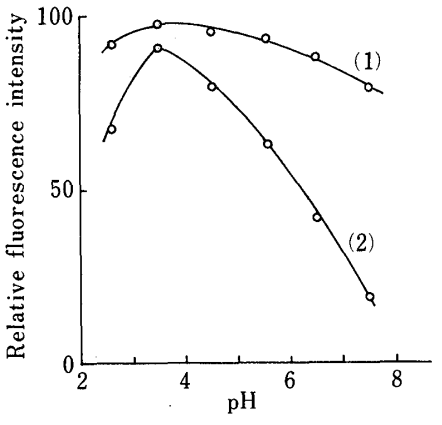

Fig. 2. Effects of pH on the Stability of 4-Hydroxyifosfamide in Aqueous Solution

Sample solutions of various pH values (adjusted with citric acid–phosphate buffers) were treated as described in the text. 4-Hydroxyifosfamide: 5.4 nmol/ml.
(1) 0.5 h after pH adjustment.
(2) 5 h after pH adjustment.

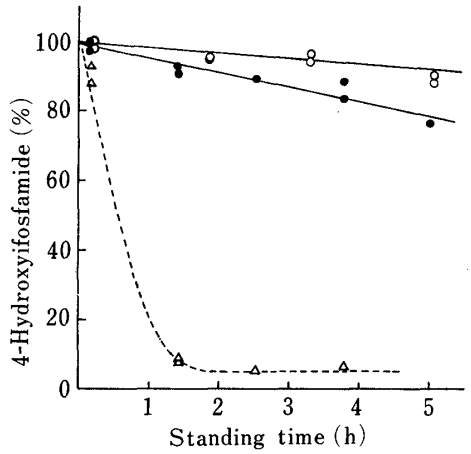

Fig. 3. Stability of 4-Hydroxyifosfamide in Human Serum

Serum samples containing 4-hydroxyifosfamide (5.3 nmol/ml) were treated as described in the text.
△, serum, 23—25 °C; ●, serum, 0—4 °C; ○, serum diluted to five times its volume with citric acid–phosphate buffer (pH 3.5), 0—4 °C.

(pH 3.5) at 0—4 °C, 4-hydroxyifosfamide was further stabilized and its degradation decreased significantly.

The stability of 4-hydroxyifosfamide in human urine was also examined at 0—4 °C using citric acid–phosphate buffers of pH 3.5 and 5.5. It was slightly more stable when buffered at pH 3.5 and its stability was about the same as that in the buffered serum. Thus, citric acid–phosphate buffer (pH 3.5) could stabilize 4-hydroxyifosfamide in serum and urine to a sufficient extent that the loss due to degradation could be neglected for the purpose of the present assay.

Recovery of 4-Hydroxyifosfamide in Human Serum and Urine

The recoveries of 4-hydroxyifosfamide from human serum and urine were examined in the concentration ranges of 1—10 nmol/ml (0.3—3 μg/ml) and 5—50 nmol/ml (1.5—15 μg/ml), respectively. The average recoveries of 4-hydroxyifosfamide from the serum and urine were calculated to be 79% and 73%, respectively. These values were used as recovery factors in calculating the 4-hydroxyifosfamide contents of blood and urine samples. The lower detection limits were 0.5 and 2 nmol/ml in serum and urine, respectively.

The effects of ifosfamide and related compounds on the recoveries of 4-hydroxyifosfamide were examined using mixed serum and urine samples in which ifosfamide, carboxyifosfamide, ifosfamide mustard, and mesna were present in up to 10-fold excess. As shown in Table I, the recovery of 4-hydroxyifosfamide from the serum was calculated to be 76.7%. In the case of urine samples, the recovery was in the range of 69.6 to 77.4% and the average value was calculated to be 74.2%. These values indicated that 4-hydroxyifosfamide was recovered from the mixed serum and urine samples without interference from ifosfamide and related compounds, that is, ifosfamide, carboxyifosfamide, ifosfamide mustard and mesna did not affect the assay.

The effect of acrolein on the assay of 4-hydroxyifosfamide was examined in the presence of equimolar amounts of acrolein and 4-hydroxyifosfamide. No difference in the fluorescence intensities was observed between the solutions with and without acrolein, because acrolein extracted with dichloromethane was removed together with the solvent during evaporation to concentrate the mixture. This result suggested that acrolein as a metabolite of ifosfamide

TABLE I. Recovery of 4-Hydroxyifosfamide from Human Serum and Urine in the Presence of Ifosfamide, Carboxyifosfamide, Ifosfamide Mustard and Mesna

Sample	No.	Concentration range (nmol/ml)	Recovery of 4-hydroxyifosfamide (%)	(c.v., %)	(*n*)
Serum	1	1.0—10.9	76.7	5.7	24
Urine	2	4.9—48.9	72.7	3.2	12
	3	5.1—50.8	69.6	5.2	12
	4	4.9—49.1	75.6	2.9	11
	5	5.2—51.8	77.4	3.6	11
	6	5.1—50.6	75.1	2.6	12
			Mean 74.2	5.9	58

The values are based on the slope of the regression equation between the added and found values of 4-hydroxyifosfamide in the following mixed samples. Sample 1 contained ifosfamide 9.6 nmol/ml, carboxyifosfamide 9.4 nmol/ml, ifosfamide mustard 9.8 nmol/ml and mesna 10.1 nmol/ml. Sample 2 contained ifosfamide at 4.6 to 46.3 nmol/ml. Sample 3 contained carboxyifosfamide at 4.9 to 48.9 nmol/ml. Sample 4 contained ifosfamide mustard at 5.0 to 49.7 nmol/ml. Sample 5 contained mesna at 5.8 to 57.9 nmol/ml. Sample 6 contained ifosfamide 51.5 nmol/ml, carboxyifosfamide 48.1 nmol/ml, ifosfamide mustard 49.0 nmol/ml and mesna 48.7 nmol/ml. c.v. = coefficient of variation.

Table II. Determination of 4-Hydroxyifosfamide in Rat Blood Plasma and Urine after Intravenous Administration of Ifosfamide (Dose: 200 mg/kg)

	Plasma concentration (μg/ml) Time after administration			Urinary excretion 0—120 min	
	15 min	30 min	120 min	(mg)	(% of dose)
	20.1	16.0	2.8	0.82	1.24
	24.8	17.7	3.3	0.66	1.02
	21.0	17.1	3.3	1.07	1.67
Mean±S.D.	22.0±2.5	16.9±0.9	3.1±0.3	0.85±0.21	1.31±0.33

should not interfere with meaurements of actual blood and urine samples.

Determination of 4-Hydroxyifosfamide in Rat Blood Plasma and Urine

The present method was used to determine the 4-hydroxyifosfamide levels in rat blood plasma and urine. Ifosfamide was intravenously administered at 200 mg/kg to 12 male rats weighing 305 to 330 g. Blood plasma samples were collected from the animals at 15, 30 and 120 min after drug administration. Urine was removed from the ligated urinary bladder at 120 min after drug administration. 4-Hydroxyifosfamide was detected from rat blood plasma and urine samples by fluorescence spectroscopy according to the standard method, and the results are summarized in Table II.

The blood plasma concentrations of 4-hydroxyifosfamide decreased with time after administration and its disappearance was considered to follow quasi first-order kinetics. Excretions of 4-hydroxyifosfamide in rat urine amounted to 0.66 to 1.07 mg in 120 min and the mean urinary recovery was calculated to be about 1.3% of the dose.

The present method is simple and suitable for the assay of 4-hydroxyifosfamide in blood and urine samples, and should be applicable to pharmacokinetic studies of ifosfamide.

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