

Determination of 2',3'-Dideoxyinosine and 2',3'-Dideoxyadenosine in Rat Plasma by High-Performance Liquid Chromatography with Precolumn Fluorescence Derivatization

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A high-performance liquid chromatographic method with precolumn fluorescence derivatization using 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran is described for the quantification of 2',3'-dideoxyinosine, a therapeutic drug for acquired immunodeficiency syndrome, and 2',3'-dideoxyadenosine, an anti-human-immunodeficiency-viral agent, in rat plasma. The dideoxyribonucleosides and 3'-deoxythymidine (internal standard) in rat plasma (0.1 ml) are cleaned up by a solid-phase extraction technique using an octadecyl silica (ODS) cartridge, Toyopak ODS M, and the dideoxyribonucleosides in the eluate are reacted with the reagent to produce the corresponding fluorescent esters. The esters are separated by chromatography on a reversed phase column, TSKgel ODS-80T_M. The detection limits (signal-to-noise ratio = 3) for the dideoxyribonucleosides are 1.3—5.4 pmol on column. Plasma concentrations of 2',3'-dideoxyinosine after intra-jugular-veinous administration to rat can be monitored by this method.

Keywords 2',3'-dideoxyinosine; 2',3'-dideoxyadenosine; 3'-deoxythymidine; precolumn fluorescence derivatization; high-performance liquid chromatography; 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran; rat plasma; acquired immunodeficiency syndrome

2',3'-Dideoxyinosine (ddI) and 2',3'-dideoxyadenosine (ddA) exhibit potent activity against human immunodeficiency virus, and ddI has been used as a therapeutic drug for acquired immunodeficiency syndrome.¹⁻⁶ ddI has various side effects (*e.g.* bone marrow toxicity,⁷ nausea,⁸ diarrhea,⁸ pancreatitis,⁸ peripheral neuropathy,⁸ rash,⁸ hepatic toxicity,⁸ hyperuricemia,⁸ hypertriglyceridemia,⁸ hyperamylasemia⁸ and hyrokalemia⁸), and the concentration monitoring of ddI in plasma or serum is required throughout therapy. High-performance liquid chromatographic (HPLC) methods with ultraviolet detection have been reported so far for the quantification of ddI.^{6,9} The methods require a large amount of plasma (1 ml).

We reported that 2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran (OMB-COCl) reacted with ribo- and 2'-deoxyribonucleosides at the 5'-hydroxyl group (a hydroxyl group of the sugar moieties) to produce the corresponding fluorescent esters.¹⁰ Recently, we have found that 2',3'-dideoxynucleosides such as ddI, ddA and 3'-deoxythymidine (dT) also react with OMB-COCl to yield the corresponding fluorescent products. This study aims to establish a precolumn fluorescence derivatization of the HPLC method for the determination of ddI and ddA in rat plasma based on the above-mentioned reaction. dT was used as an internal standard. Plasma concentration monitoring of ddI after intra-jugular-veinous administration to rat was also studied.

Experimental

Chemicals OMB-COCl was obtained from Dojindo Laboratories (Kumamoto, Japan), and ddI, ddA and dT were purchased from Yuki Gosei Kogyo (Tokyo, Japan). Toyopak ODS M cartridges for sample cleanup were obtained from Tosoh (Tokyo, Japan), which were washed successively with methanol (6 ml), water (12 ml) and 0.1 M phosphate buffer (pH 4.5; 2 ml) before use.

HPLC The HPLC system and its operating conditions were the same as those described previously.¹⁰ The mobile phase was acetonitrile-0.1 M phosphate buffer (pH 7.0) (7:13, v/v) which was pumped at a flow rate of 1.0 ml/min (column pressure, approximately 150 kg/cm²). Peak heights were used for the quantification of 2',3'-dideoxyribonucleosides.

Administration of ddI to Rat and Preparation of Plasma Samples Male Sprague-Dawley rats (235—275 g, 8 weeks old) were maintained in a

temperature-(25°C), humidity-(50%) and light-controlled environment. Sixteen rats were divided into 2 groups (8 in each group). Rats received the ddI solution (50 mg/ml in saline, 2.0 ml/kg) (5 in each group) or saline (2.0 ml/kg) (3 in each group) as control by single intra-jugular-veinous administration. Heparinized (1000 units/ml) blood samples (1 ml) were taken from the jugular veins in the first group at 5, 10, 15 and 30 min after administration and from the second group at 45, 60, 90 and 120 min. The blood (1 ml) was centrifuged (10 min, 1500 g) and plasma (0.4 ml) was obtained. The plasma samples were stored at -20°C until assay. Drug-free plasma spiked with ddI and ddA was also prepared.

Cleanup of Plasma Sample and Fluorescence Derivatization To a plasma sample (0.1 ml) were added 85 nmol/ml dT (internal standard) solution (10 µl) and 0.1 M phosphate buffer (pH 4.5; 0.89 ml). The mixture was passed through a Toyopak ODS M cartridge at a flow rate of 0.12—0.15 ml/min. The cartridge was washed with 0.1 M phosphate buffer (pH 4.5) (2 ml) and water (2 ml). The adsorbed ddI, ddA and dT were eluted with methanol (1 ml). The eluate placed in a screw-capped vial (3.5 ml) was evaporated to dryness under a stream of nitrogen at 37°C. To the residue dissolved in dried pyridine (0.1 ml) was added 3 mM OMB-COCl in dried benzene (0.9 ml). The vial was tightly closed and heated at 100°C for 50 min in the dark. After cooling, the reaction mixture was concentrated to dryness under a stream of nitrogen at 60°C. The residue was dissolved in the mobile phase for HPLC (1 ml). The resulting solution (20 µl) was subjected to HPLC.

Results and Discussion

The optimal derivatization reaction conditions (reaction temperature and time, pyridine concentration and OMB-COCl concentration) were almost identical to those described previously.¹⁰ The fluorescent derivatives from ddI, ddA and dT were stable for at least 72 h at room temperature in the mobile phase.

A typical chromatogram obtained with rat plasma to which was added ddI and ddA (concentrations, 8.5 nmol/ml each), according to the procedure, is given in Fig. 1. The retention times of the OMB derivatives of ddI, ddA and dT were 14.6, 16.6 and 21.5 min, respectively.

ddI, ddA and dT (concentrations, 8.5 nmol/ml each) spiked to human plasma and then treated according to the procedure gave almost identical chromatograms.

The OMB derivatives of ddI, ddA and dT were separated with isocratic elution on a reversed phase column, TSKgel ODS-80T_M, using a mixture of acetonitrile and phosphate buffer as a mobile phase. At an acetonitrile concentration

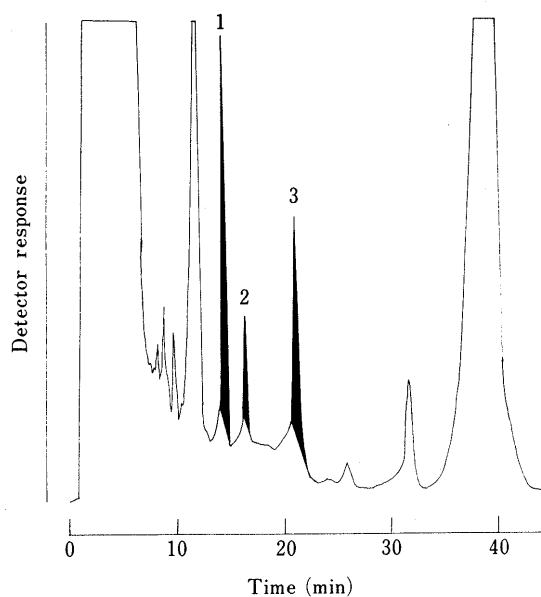


Fig. 1. Chromatogram Obtained with Rat Plasma Added with ddI, ddA and dT

The peaks for ddI, ddA and dT are shaded. Peaks: 1=ddI, 2=ddA, 3=dT, others=the reagent blank and the plasma components. Amount (pmol on column): 17 pmol each.

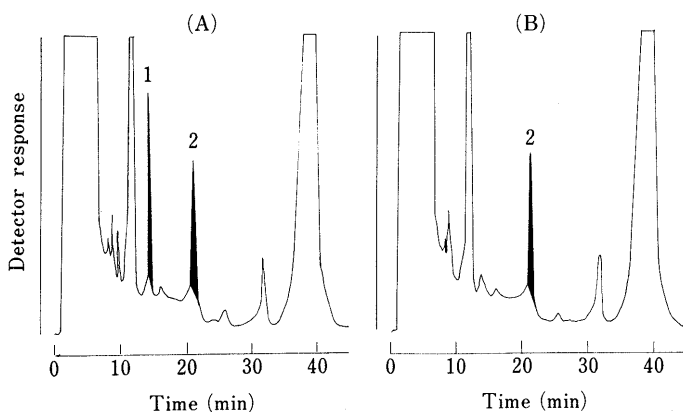


Fig. 2. Chromatograms Obtained with Rat Plasma 15 min after Administration of (A) ddI and (B) Saline

The peaks for ddI and dT are shaded. Doses: A=420 μ mol (100 mg)/kg, B=2 ml (saline)/kg. Peaks and amount (pmol on column) in parentheses: 1=ddI (6.3), 2=dT (17), others=the reagent blank and the plasma components.

of 40% (v/v) or greater, the peak for ddI was overlapped with a preceding blank peak, whereas at concentrations less than 30% (v/v), the peaks for ddI, ddA and dT were not observed even after 1 h. The phosphate buffer (0.1 M) of pH 6 or less resulted in a delay in the elution of ddI, ddA and dT with a broadening of the peaks. Concentrations of the phosphate buffer (pH 7.0) ranging from 0.05 to 0.15 M had no effect on the separation or the peak heights for ddI, ddA and dT. The most satisfactory separation was attained when acetonitrile-0.1 M phosphate buffer (pH 7.0), (7:13, v/v) was used.

ddI, ddA and dT are more hydrophobic than ribonucleosides and 2'-deoxyribonucleosides and can be retained on an ODS cartridge and eluted with methanol; the cleanup of the plasma sample was performed by a solid phase extraction technique using Toyopak ODS M cartridge. The recoveries of ddI, ddA and dT (85 nmol/ml each) added

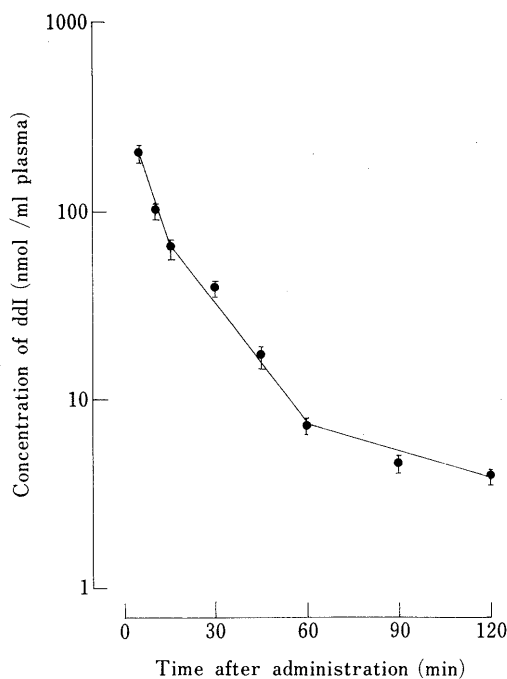


Fig. 3. Time-Concentration Curve for Plasma ddI Following the Intra-Jugular-Venous Administration of ddI Solution to Rat

Dose: 420 μ mol/kg. Each point and vertical line represents the mean ($n=5$) and standard deviation, respectively.

to drug-free rat plasma (1.0 ml) were $85 \pm 10\%$ (ddI), $73 \pm 4\%$ (ddA) and $80 \pm 5\%$ (dT) (mean \pm relative standard deviation; $n=5$ each), respectively.

Figure 2 depicts chromatograms obtained with rat plasma 15 min after single intra-jugular-venous administration of ddI (100 mg/kg) and saline (2.0 ml/kg) as a control. The peak for ddI was identified on the basis of the retention time in comparison with the standard solution and by co-chromatography of the standard and the rat plasma.

Figure 3 shows a typical time-concentration curve obtained with rat plasma after intravenous administration of ddI (100 mg/kg; 420 μ mol/kg). The concentration in plasma after administration showed a triphasic decrease; the biological half-lives in α , β and γ phases were 2.6, 19.4 and 41.9 min, respectively. The pattern of the curve was almost identical with those reported by others^{8,11} (dose, 100 mg/kg).

The calibration graph of ddI, constructed by plotting the ratios of the peak heights of the spiked ddI to that of internal standard *versus* the amounts (0-300 nmol) of ddI added to plasma (0.1 ml), was linear. The calibration graph of ddA was also linear in the range of 0-300 nmol. The correlation coefficients for the lines were greater than 0.99. The precision was established by repeated determinations ($n=5$) using the rat plasma obtained 30 min after the administration of ddI. The relative standard deviation (%) was 10.2 (mean, 30.8 nmol/ml). The detection limits (pmol/20- μ l injection volume at a signal to-noise ratio of 3) for the dideoxyribonucleosides in plasma were 1.3 (ddI), 5.4 (ddA) and 4.3 (dT).

In conclusion, this study has provided the first fluorometric HPLC method for the determination of ddI and ddA in rat plasma. This method is simple and offers the

necessary sensitivity of permit the monitoring of ddI in a small amount (0.1 ml) of plasma. The method should be useful in biomedical investigations of ddI and ddA.

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