Journal of Chromatography, 420 (1987) 1–12
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3729

APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THERMOSPRAY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY TO THE ANALYSIS AND IDENTIFICATION OF 2′,3′-DIDEOXYADENOSINE AND ITS METABOLITE IN BIOLOGICAL MEDIA

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(First received January 20th, 1987; revised manuscript received March 31st, 1987)

SUMMARY

Reversed-phase high-performance liquid chromatographic (HPLC) procedures are described for determining the stability of 2′,3′-dideoxyadenosine (DDA) in biological fluids at therapeutic dosages. The validated methodology uses both direct injection and solid-phase extraction techniques. Deamination of DDA to 2′,3′-dideoxyinosine (DDI) in plasma by adenosine deaminase was monitored by HPLC, and the identification of DDI verified by thermospray HPLC–mass spectrometry. This methodology should prove useful in future studies concerning the stability and metabolism of dideoxynucleosides.

INTRODUCTION

2′,3′-Dideoxyadenosine (DDA) has been identified, in in vitro studies, as a potential anti-viral agent against human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV), the causative agent of acquired immunodeficiency syndrome (AIDS) and associated maladies [1]. The methodology described herein has application to activity evaluations in vitro and to metabolism and clinical pharmacokinetic studies of 2′,3′-dideoxynucleosides. Preclinical toxicological studies with DDA have recently begun.

Numerous publications have described reversed-phase high-performance liquid chromatographic (HPLC) assays for nucleosides in blood fluids [2–17]. Procedures generally require deproteinization of the medium as well as preconcentration of the sample to detect low levels of analyte. Preconcentration methods must be compatible with HPLC analysis while providing sufficient res-

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olution of the nucleoside(s) from interferents. Solid-phase extraction (SPE) columns have been successfully used to separate a variety of drugs from biological fluids and nucleosides in water [18,19].

To date, no analytical method has been described in the literature for assay of DDA by HPLC. This report presents (i) the development of a rapid and sensitive HPLC assay for monitoring the stability of DDA in RPMI-1640 growth medium, human and mouse plasma and (ii) the identification of 2′,3′-dideoxyinosine (DDI) formed from the enzymatic deamination of DDA in biological media by adenosine deaminase (ADA).

EXPERIMENTAL

Apparatus

HPLC. The HPLC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) M6000A pump, M440 fixed-wavelength UV detector (254 nm) and U6K injector. The assays were carried out using a Zorbax C_8 reversed-phase column (6-μm diameter particles; 250×4.6 mm I.D.) (DuPont, Wilmington, DE, U.S.A.) with a 5-μm RP-8 guard column (30×4.6 mm I.D.) (Brownlee, Santa Clara, CA, U.S.A.), at a flow-rate of 2.0 ml/min. Quantification was based on integrated peak areas. Integration was performed using a 4400 chromatography data system (Nelson Analytical, Cupertino, CA, U.S.A.). The HPLC mobile phase for DDA assay was acetonitrile–water (10:90, v/v) containing 0.05 *M* ammonium dihydrogen phosphate. The organic content of the mobile phase was reduced to 5% acetonitrile for identification of DDI.

HPLC–mass spectrometry (HPLC–MS). Details of the HPLC and MS instrumentation and operating procedures have been previously reported [20,21] and are briefly described below. The HPLC system consisted of a Waters Assoc. M6000A pump, U6K injector and an M440 UV detector (254 nm). A Vestec (Houston, TX, U.S.A.) thermospray (TSP)–HPLC–MS interface on a Finnigan MAT 4500 mass spectrometer (San Jose, CA, U.S.A.) was used to acquire the HPLC–MS data. The HPLC column was the same as described above. The flow-rate was reduced to 1.2 ml/min, and 0.1 *M* ammonium acetate (pH 4.7) was added to the acetonitrile–water (5:95, v/v) mobile phase for thermospray ionization. The thermospray interface temperature was optimized for maximum sensitivity for DDA and DDI. In the positive-ion mode, the optimum vaporizer and source temperatures were 204 ± 10°C and 250°C, respectively. The mass spectrometer was scanned from m/z 100 to 550 in 2-s intervals throughout the analysis.

Reagents and materials

DDA, RPMI-1640 growth medium (containing 10% fetal bovine serum and glutathione), and pentostatin were furnished by the National Cancer Institute (Bethesda, MD, U.S.A.). Human plasma was purchased from the American Red Cross (Durham, NC, U.S.A.). Mouse plasma, containing sodium citrate, was obtained from Pelfreeze Biologicals (Rogers, AR, U.S.A.). DDI was purchased from Calbiochem (San Diego, CA, U.S.A.).

All solvents (HPLC grade), ammonium acetate, ammonium dihydrogen phos-

phate and Prep-Sep C_{18} cartridges were purchased from Fisher Scientific (Raleigh, NC, U.S.A.). Acetic acid was purchased from American Scientific Products (Charlotte, NC, U.S.A.). The mobile phases were prepared with reagent-grade water obtained from a Milli-Q system (Millipore, Bedford, MA, U.S.A.) and filtered through a 5-μm Mitex LS filter (Millipore) prior to use. A Baker-10 SPE system (J.T. Baker, Phillipsburg, NJ, U.S.A.) was used for sample preparation. Reagent-grade water was used to prepare solutions.

HPLC assay procedure for stability of DDA in RPMI-1640 growth medium

This assay was designed to determine drug stability at a concentration of 5 μM drug ($\sim$1.2 μg/ml) in RPMI-1640 growth medium. Previous in vitro studies had indicated that DDA showed some ability to inhibit the infectivity and replication of HTLV-III/LAV and to inhibit its cytopathic effect against target T cells [1] at this concentration.

A 10-ml aliquot of an aqueous solution of DDA (1.2 mg/ml) was added to a 10-ml volumetric flask and diluted to volume with RPMI-1640 growth medium which had been filtered through a 5-μm PTFE filter. The contents of the flask were thoroughly mixed (inverted 5$\times$) and the sample was analyzed directly by HPLC without further treatment. A 25-μl aliquot was removed and analyzed at time periods corresponding to 0 (initial preparation), 0.25, 0.5, 0.75, 1.0, 2.0, 3.0 and 4.0 h of storage at 37°C in the light.

HPLC assay procedure for stability of DDA in mouse and human plasma

This assay was designed to determine drug stability at a concentration of 1 μM drug ($\sim$235 ng/ml) in both mouse and human plasma. The final drug concentration was chosen on the basis of (i) the limit of detection for the HPLC method and (ii) a possibility of low-drug bioavailability after oral administration due to instability under acidic conditions. A 240-μl aliquot of an aqueous solution of DDA (10 μg/ml) was introduced into a 20-ml PTFE-lined screw-cap vial containing 10 ml of plasma, and the vial was vortexed for 10 s. The fortified plasma sample was analyzed by HPLC after SPE treatment (discussed below) at time points corresponding to 0 (initial preparation), 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 h of storage at 37°C in the light.

SPE procedure for mouse and human plasma

The SPE columns (Prep-Sep C_{18}, 500 mg octadecyl) were mounted on a Baker vacuum manifold, which can process up to ten samples simultaneously. Each SPE column was conditioned prior to use by aspiration of 1.5 ml of methanol through the column followed by two 1.5-ml aliquots of water, without allowing the column to go dry. At each time point, a 1-ml aliquot of plasma was aspirated through the cartridge which was then washed with two 1.5-ml aliquots of 0.05 *M* ammonium dihydrogen phosphate. Air was aspirated through the cartridge for $\sim$15 s and the analyte (DDA) eluted with three 500-μl aliquots of 45% methanol in 0.05 *M* ammonium dihydrogen phosphate. The sample was vortexed $\sim$5 s and a 250-μl aliquot analyzed by HPLC.

Determination of drug stability

The stability of DDA was determined from the percentage drug remaining in the biological fluid at each time point. The precentage DDA was calculated from the HPLC assay result as: $DDA_x = (R_x/R_0) \cdot 100\%$ where $DDA_x(\%)$ = concentration of drug at t_x as a percentage of the analyte level at t_0 ($t_0 = 100\%$); R_0 = response at t_0; R_x = response at t_x.

Assay procedure for the stability of DDA and DDI at pH 1

This assay (performed in triplicate) was designed to determine the stability of DDA at a concentration of $\sim 40\ \mu M$ in an acidic medium. A 10-ml aliquot of DDA, at 10 μg/ml in water, was acidified to pH 1 with 500 μl of 1 *M* hydrochloric acid. The sample was incubated at 37 °C with 1-ml aliquots removed periodically. Each 1-ml aliquot was immediately neutralized to pH 4.5–5 with 88 μl of 0.5 *M* sodium hydroxide and analyzed directly by HPLC. A control standard was prepared as above except that equivalent aliquots of water were substituted for addition of acid and base.

The stability of DDI at pH 1 was examined in the same manner except that HPLC analysis was performed using the mobile phase specified for identification of DDI.

Recovery

Recovery of DDA from human and mouse plasma was determined from a comparison of a spiked plasma sample to an aqueous standard of the drug, using the SPE procedure discussed above. The spiked drug sample was processed immediately after preparation (which included 10 s of vortexing). The recovery was calculated as: $\text{recovery} = (R_p/R_s) \cdot 100\%$ where recovery (%) = percentage DDA recovered from the plasma; R_p = DDA response in spiked plasma sample; R_s = DDA response in standard solution.

Precision of the assays

The HPLC assay procedures for DDA in RPMI-1640 growth medium and human plasma were examined for precision. Filtered RPMI-1640 growth medium was spiked with DDA at a concentration of 5 μM, and 25-ml aliquots were injected nine separate times onto the HPLC column without further treatment. The observed relative standard deviation (R.S.D.) in RPMI-1640 growth medium was $< 1.5\%$ for the retention time and $< 4.0\%$ for the DDA response. Filtered human plasma was spiked with DDA at a concentration of 1 μM, and an aliquot of this solution processed using the SPE procedure discussed above. The eluates from two SPE cartridges were combined and 250-μl aliquots injected nine separate times onto the HPLC column. The observed R.S.D. in plasma was $< 0.4\%$ for retention time and $< 5.0\%$ for response.

Linearity and accuracy of the assays

The linearity and accuracy of each assay were determined by single analysis of each of seven solutions ranging from 10 to 200% of the stated assay concentration. For RPMI-1640 growth medium, 99.2% of the total variance was explained

TABLE I

STABILITY OF DDA IN BIOLOGICAL MEDIA

Values represent mean ± S.D. of three separate experiments at 37°C.

t_x (h)	Recovery (%)		
	RPMI-1640*	Human plasma**	Mouse plasma**
0	100	100	100
0.25	96.8 ± 0.1	56.5 ± 13.7	32.0 ± 4.0
0.50	86.7 ± 0.7	41.4 ± 14.2	32.0 ± 2.4
0.75	80.9 ± 1.0	36.5 ± 8.5	2.9 ± 4.1
1.0	73.4 ± 4.1	29.2 ± 8.6	0
1.5		17.2 ± 5.7	
2.0	53.2 ± 0.8	13.7 ± 7.5	
3.0	40.0 ± 5.2		
4.0	24.5 ± 3.6		

*Performed at 1.2 μg/ml (~5 μM).
**Performed at 235 ng/ml (~1 μM).

by the regression line over the concentration range 0.122–2.44 μg/ml. The overall accuracy of the assay, assessed from the deviation between measured versus calculated (linear regression) concentration levels, and expressed in terms of the mean deviation (absolute difference) as a percentage of the assay concentration, was ±5.3%. For human plasma, 99.5% of the total variance was explained by the regression line over the concentration range 46.4–464 ng/ml, and the accuracy was ±3.1%.

RESULTS AND DISCUSSION

The stability data for DDA in RPMI-1640 growth medium at 5 μM and for human and mouse plasma at 1 μM at 37°C are presented in Table I. Representative chromatograms are illustrated in Figs. 1–3.

A rapid disappearance of DDA was observed in all three biological matrices examined. This observation led to an attempt to identify the metabolite(s) formed. It had been previously reported that DDA could undergo rapid deamination to DDI by ADA [22,23]. Thus, an authentic sample of DDI chromatographed in mouse plasma was compared with DDA under the same conditions. The DDI was found to be poorly retained and coeluted with a large endogeneous interference from plasma near the column void volume. The retention of DDI was increased beyond the interferences by reduction of the acetonitrile content of the mobile phase to 5% for subsequent work. The overall buffer concentration in the mobile phase remained at 0.05 *M* ammonium dihydrogen phosphate.

The degradation of DDA to DDI was monitored in RPMI-1640 growth medium at 100 μM and in mouse plasma at 25 μM (direct injection, without SPE treatment). In agreement with the previous stability studies (discussed above), observed increases in DDI concentration were most rapid in mouse plasma with

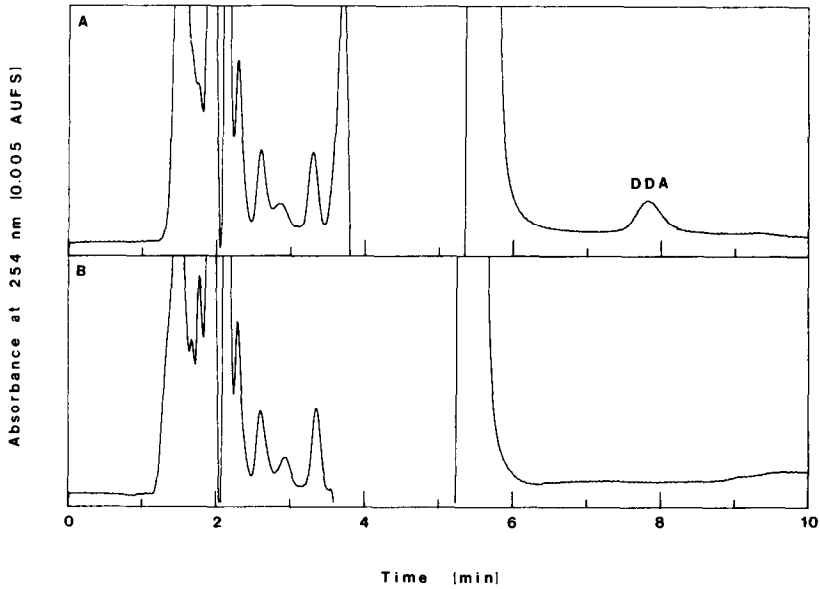

Fig. 1. Chromatograms of DDA in RPMI-1640 growth medium. (A) 25 μl of growth medium spiked with 1.22 μg/ml DDA at t_0 and analyzed at $t_{3.0h}$ (40% recovery of DDA); (B) 25 μl of filtered RPMI-1640 growth medium (sample blank). Conditions as in the text.

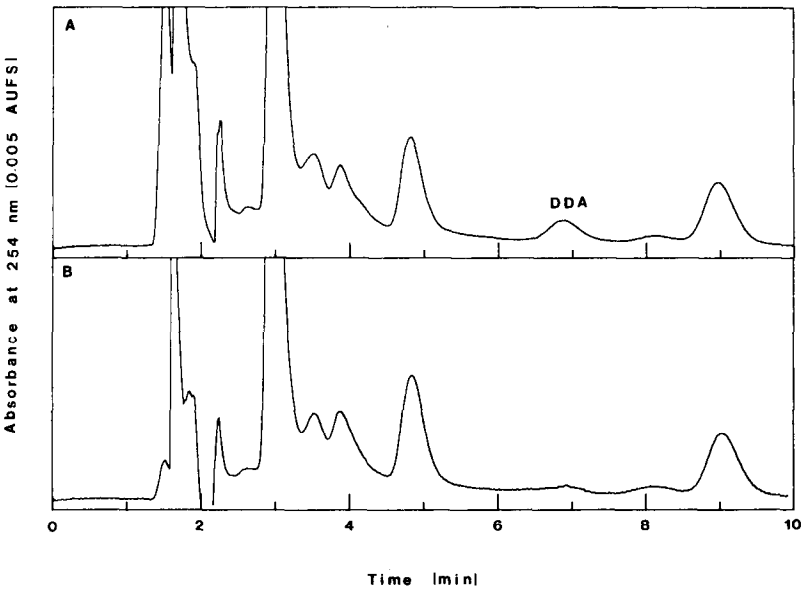

Fig. 2. Chromatograms of human plasma extracts obtained by solid-phase extraction. (A) Human plasma (250 μl) spiked at t_0 with 235 ng/ml DDA and analyzed at $t_{1.0h}$ (29% recovery of DDA); (B) 250 μl of human plasma (sample blank). Conditions as in Fig. 1.

a concomitant loss of DDA for both cases. These results are plotted in Fig. 4 for the RPMI-1640 growth medium and mouse plasma. Representative HPLC chromatograms are presented in Figs. 5 and 6.

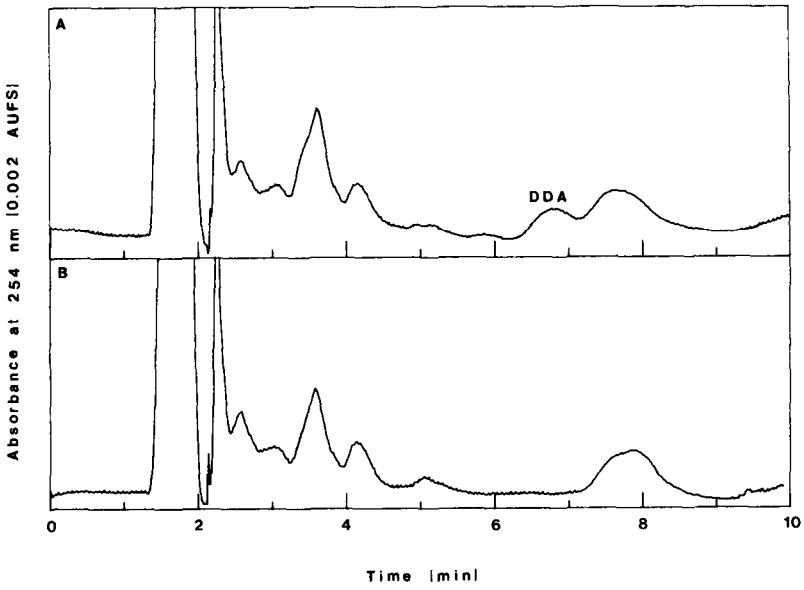

Fig. 3. Chromatograms of mouse plasma extracts obtained from solid-phase extraction. (A) Human plasma (250 μl) spiked at t_0 with 235 ng/ml DDA and analyzed at $t_{0.25h}$ (32% recovery of DDA); (B) 250 μl of mouse plasma (sample blank). Conditions as in Fig. 1.

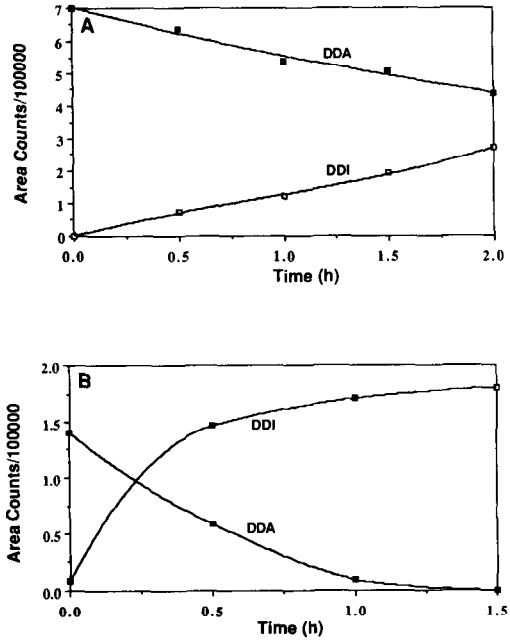

Fig. 4. Plots of peak area as a function of time for the enzymatic degradation of DDA to DDI. (A) Spiking of RPMI-1640 growth medium with DDA at 100 μM; (B) spiking of mouse plasma with DDA at 25 μM.

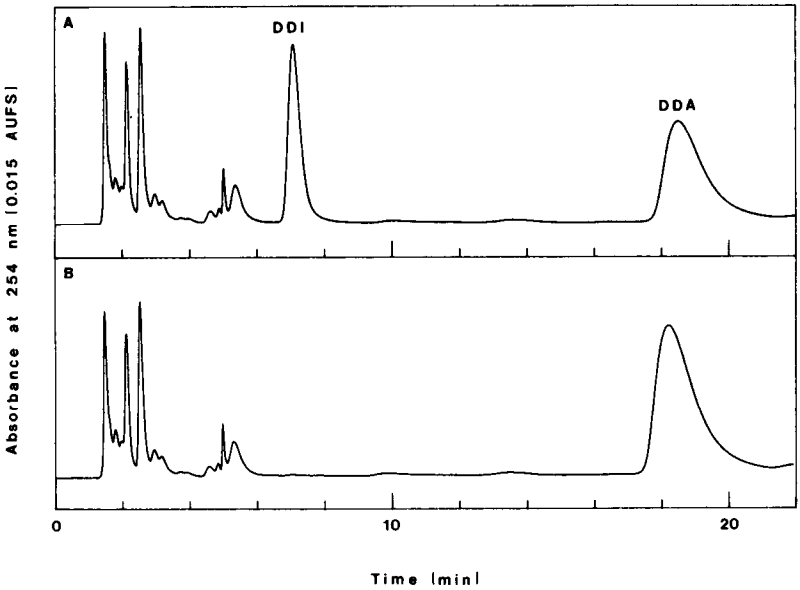

Fig. 5. Chromatograms of enzymatic degradation of DDA in RPMI-1640 growth medium. (A) DDA-spiked growth medium at $t_{2.0h}$; (B) DDA-spiked growth medium at t_0. Conditions as in Fig. 1 except for the mobile phase which was acetonitrile–water (5:95, v/v) containing phosphate buffer.

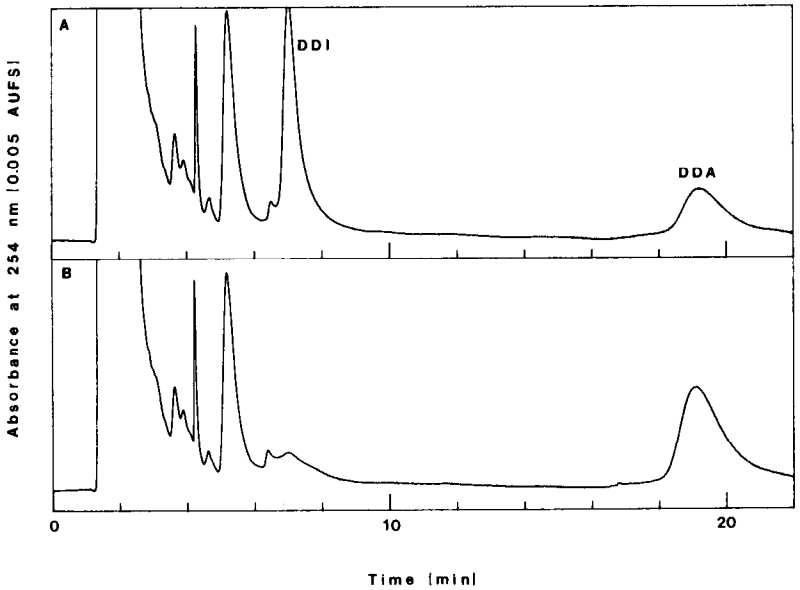

Fig. 6. Chromatograms of enzymatic degradation of DDA to DDI in mouse plasma. (A) Mouse plasma spiked with DDA at t_0 and analyzed at $t_{0.5h}$; (B) DDA-spiked mouse plasma at t_0. Conditions as in Fig. 5.

The identity of the DDI peak was confirmed using TSP-HPLC–MS in the positive-ion detection mode. The TSP-HPLC–MS profiles, shown in Fig. 7, proved to be very specific for each nucleoside. The mass spectrum for DDA exhibited an

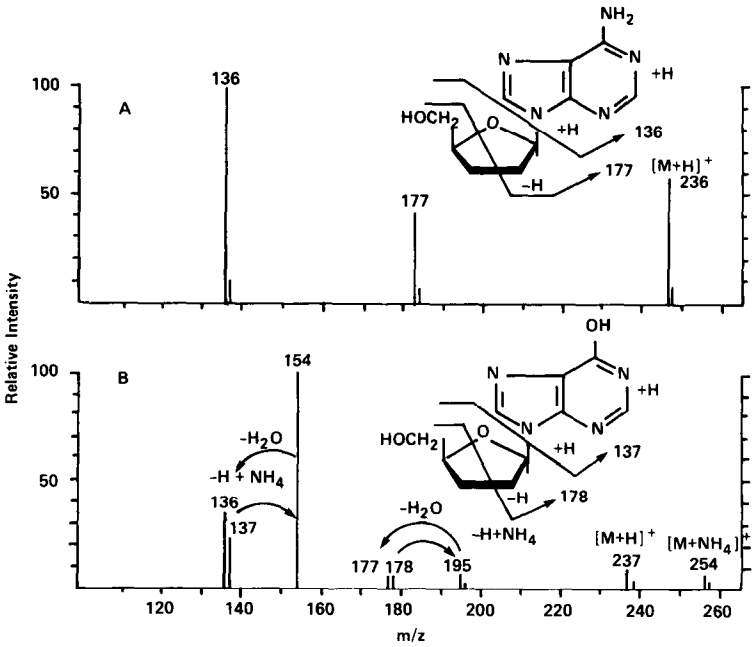

Fig. 7. Thermospray positive-ion spectrum for 500 ng of (A) DDA and (B) DDI.

$[M+H]^+$ ion at *m/z* 236 with two major fragment ions at *m/z* 136 and 177. These fragment ions were due to the loss of the sugar ($C_5H_9O_2$) from the protonated molecular ion along with a proton transfer from the sugar and loss of C_3H_7O from the $[M+H]^+$ ion. The fragment ions due to the loss of the sugar and cleavage of the sugar are similar to those observed for fast atom bombardment (FAB) MS of nucleosides [24,25]. However, the formation of these fragment ions in thermospray by pyrolytic processes cannot be ruled out.

DDI exhibited ions due to addition of ammonium and protonation of both the fragment ions and molecular ion. The major ions observed included $[M+H]^+$ at *m/z* 237, $[M+NH_4]^+$ at *m/z* 254, $[M+H-C_5H_9O_2+H]^+$ at *m/z* 137 and $[M+NH_4-C_5H_9O+H]^+$ at *m/z* 154. Since TSP-HPLC–MS in the presence of ammonium acetate can resemble ammonia chemical ionization, compounds with proton affinities near or lower than the conjugate base of ammonia will show NH_4 adduct ions [26]. The hydroxyl functionality on the DDI lowers the proton affinity of the molecule enough to favor ammonium addition. The DDA, which contains a primary amine, has a sufficiently high proton affinity to form only protonated ions. This difference in proton affinities between DDI and DDA adds further specificity to the analysis of these nucleosides.

The TSP-HPLC–MS analysis verified degradation of DDA to DDI with time as shown by the selected-ion current chromatogram for the $[M+H]^+$ ion of DDA (Fig. 8) in mouse plasma. No interferences were detected for the analysis in plasma and the observed signals matched the selected ion and retention time for the authentic standard. The ion chromatograms for DDI at *m/z* 154 showed a gradual increase in intensity with sample storage time (Fig. 9). The ion at *m/z*

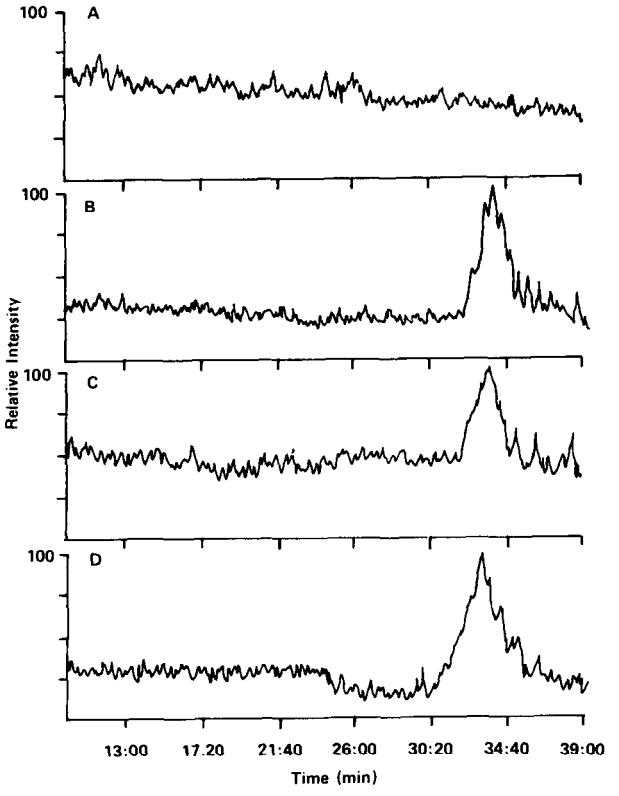

Fig. 8. TSP-HPLC-MS selected-ion profiles of the $[M+H]^+$ ion for DDA (m/z 236) in mouse plasma. (A) Blank; (B) 0.2 h; (C) 0.9 h; (D) 100 ng reference standard. Conditions as described in text.

154 ($[M+NH_4-C_5H_9O_2+H]^+$) proved to be the most specific for DDI since DDA (which has a higher proton affinity than DDI) does not exhibit an $[M+NH_4-C_5H_9O_2+H]^+$ ion (m/z 153) which could produce interference from ^{13}C isotopic contribution.

The degradation of DDA in mouse plasma was sufficiently rapid as to require direct injection of samples in the HPLC analysis. Application of any type of sample preparation, such as the SPE technique used for RPMI-1640 growth medium, produced loss of DDA during the preparation step. The magnitude of these losses is demonstrated by the average ($n=3$) recoveries of DDA obtained at the 1 μM level (235 ng/ml) for spiked human and mouse plasma samples analyzed without storage (immediately after fortification) using the SPE procedure. Although the recovery of DDA from human plasma was $101 \pm 3\%$, mouse plasma yielded a recovery of only $67 \pm 6\%$. The lower recovery obtained for mouse plasma may be attributed to immediate degradation of the DDA in this case. The high recovery of DDA from RPMI-1640 growth medium and human plasma rules out loss of drug from degradation under the applied extraction conditions and/or from irreversible adsorption to the bonded-phase SPE material.

Pentostatin (2′-deoxycoformycin) has been reported to be a potent and selec-

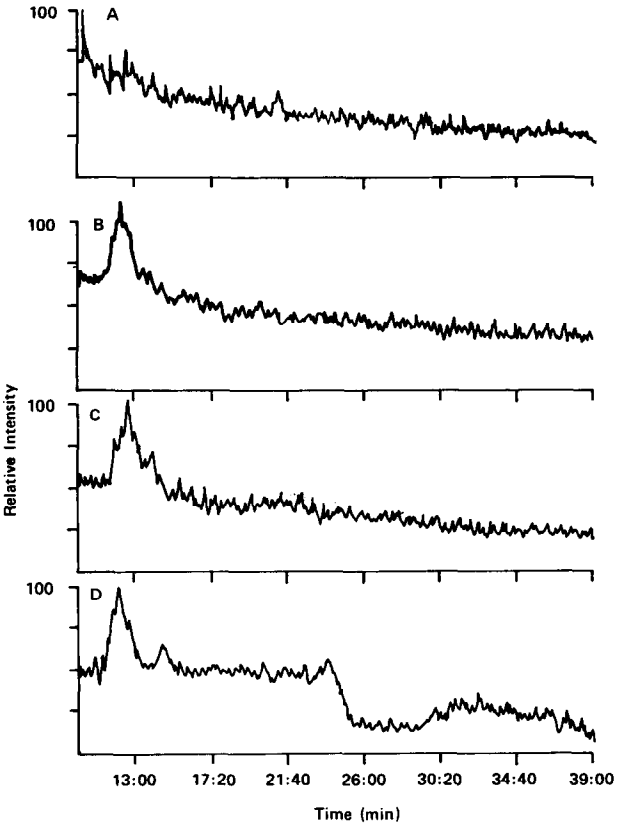

Fig. 9. TSP-HPLC-MS selected-ion profiles of the $[M+NH_4-C_5H_8O_2]^+$ ion for DDI (m/z 154) formed in mouse plasma. (A) Blank; (B) 0.2 h; (C) 0.9 h; (D) 100 ng reference standard. Conditions as in Fig. 8.

tive inhibitor of ADA [27,28], and, therefore, might be potentially useful as a means of preventing further loss of DDA from mouse plasma samples following their collection in pre-clinical studies. To study this possibility, a 5-ml aliquot of filtered mouse plasma was incubated for 30 min at 37°C with 1.5 mg (1mM) of pentostatin. Following this incubation, 30 μg of DDA were added and the resulting solution ($\sim$25 μM DDA in plasma) was vortexed and analyzed directly by HPLC. Results of this study showed no loss of DDA in the mouse plasma over a 4-h period; pentostatin completely inhibited the enzymatic deamination of DDA.

Both DDA and DDI were found to undergo rapid degradation when exposed to low pH conditions (pH 1) simulating oral administration. Very low recoveries ($<5\%$) were obtained from an initial concentration of $\sim$40 μM for either compound after only 10 min in the acidic medium. Thus, oral administration of these compounds will require special formulation methods in order to achieve a reasonable level of bioavailability.

In conclusion, this methodology has been shown to be sufficiently sensitive and accurate for determining ng/ml levels of DDA and DDI in RPMI-1640 growth medium, human and mouse plasma. Information on the stability of these com-

pounds in biological and acidic media should also prove useful in future pharmacokinetic and formulation studies.

ACKNOWLEDGEMENTS

The technical assistance of Mr. Terry Pack is greatly appreciated. This work was supported by NCI, Pharmaceutical Resources Branch, Bethesda, MD, U.S.A., under Contract No. NO1-CM-37619.

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