Journal of Chromatography, 577 (1992) 299–304
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6337

Thermospray liquid chromatographic–mass spectrometric analysis of anti-AIDS nucleosides: quantification of 2′,3′-dideoxycytidine in plasma samples

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(First received November 27th, 1991; revised manuscript received February 20th, 1992)

ABSTRACT

Thermospray liquid chromatography–mass spectrometry was investigated as a method for quantification of 2′,3′-dideoxycytidine (DDC) from human plasma. A stable isotope analog of DDC ([$^{15}N_2$,$^{2}H_2$]DDC) was used as an internal standard. Selected ion monitoring of the protonated molecular ions for DDC and the internal standard was used to record mass chromatograms. The areas of the peaks in the mass chromatograms were used for quantification. The detection limit of DDC in this assay was 50 pg on-column. The calibration curve was linear over the desired range, 0.25–20 ng/ml. The major advantages of this assay over others are: no derivatization, high sensitivity, high specificity and short assay time.

INTRODUCTION

The human immunodeficiency virus (HIV) has been recognized as the etiological agent of the acquired immunodeficiency syndrome (AIDS) [1,2]. A number of 2′,3′-dideoxynucleosides and related compounds inhibit the *in vitro* infectivity and the cytopathic effect of the HIV retrovirus [3–5]. One of the nucleoside analogues, zidovudine (AZT), has already been approved for clinical use [6]. 2′,3′-Dideoxycytidine (DDC, Fig. 1a), is currently undergoing clinical evaluation for the treatment of AIDS [7–9]. At a therapeutic dose of 0.01 mg/kg every 4 h, DDC reduces the serum p24 antigen levels with minimal toxicity.

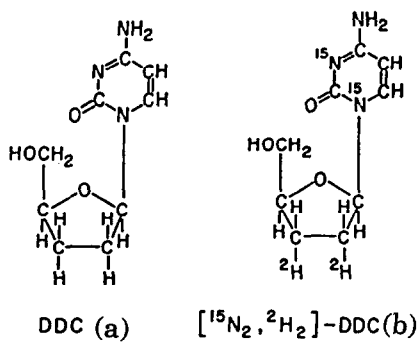

Fig. 1. Structures of DDC (a) and the internal standard (b).

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0378-4347/92/$05.00

Plasma concentrations of DDC at such low doses are in the concentration range 0–10 ng/ml [10]. Conventional analytical method such as high-performance liquid chromatography (HPLC) are not suitable due to inadequate sensitivity and specificity. Mass spectrometry (MS) is a potential method for detection, since it may provide adequate sensitivity and specificity as the ions corresponding to the compound of interest can be monitored. This eliminates the interferences from other compounds present in the biological fluids. Gas chromatography–mass spectrometry (GC–MS) has been used previously for quantification of DDC. However, the published GC–MS assay for DDC requires multiple derivatizations [11]; it is extremely tedious and losses during derivatizations limit the sensitivity of the assay.

LC–MS is finding wide applications for quantitative analysis of drugs [12,13]. The HPLC system can be interfaced with a mass spectrometer in a variety of ways such as direct liquid introduction (DLI), atmospheric pressure ionization (API), moving belt and thermospray interfaces [12,13]. Thermospray is a convenient method since it allows the entire HPLC effluent to be introduced directly into the ion source of the mass spectrometer at the flow-rates (1–2 ml/min) commonly used in conventional chromatography [14]. In addition, there is no need for an external ionization source as the analyte is ionized in the thermospray interface. LC–MS has been applied previously to the analysis of nucleosides [15–18]. Nucleoside analogues produced protonated molecular ions as the major peak in the spectra together with some structurally informative fragment ions when subjected to thermospray ionization (unpublished results). This makes them suitable for quantitative studies. Thermospray LC–MS has been investigated for quantitation of DDC in plasma samples as no derivatizations are needed for LC–MS analysis. A stable isotope analogue of DDC ([$^{15}N_2$,$^2H_2$]DDC, Fig. 1b) was used as an internal standard.

EXPERIMENTAL

Materials

DDC was purchased from Sigma (St. Louis, MO, USA), and the internal standard ([$^{15}N_2$,$^2H_2$]DDC; >98% isotopic purity) was provided by Dr. W. H. Garland of Hoffman La-Roche (Nutley, NJ, USA). Solid-phase extraction (SPE) cartridges (Supelclean LC-18, 3-ml tubes) were purchased from Supelco (Bellefonte, PA, USA). All solvents were of HPLC grade and reagents were the best grades commercially available.

Sample preparation

Standard samples. Standards for the calibration curve were made by spiking 1 ml of water with 0.25, 0.50, 1.0, 2.5, 5.0, 10.0, 15.0 and 20.0 ng DDC. A fixed amount of internal standard (5.0 ng) was added to all samples. The samples were loaded onto LC-18 SPE cartridges, which were prewashed with 2 ml of methanol followed by 2 ml of water. The SPE cartridges were washed with 2 ml of water and DDC was eluted by washing with 2 ml of methanol–water (20:80, v/v). The eluent was evaporated to dryness and redissolved in 50 μl of methanol–water (20:80, v/v). The recovery of DDC from plasma after SPE on LC-18 cartridges as determined by radioactive counting was greater than 90%. A 25-μl sample was injected on the thermospray LC–MS instrument used in the selected-ion monitoring (SIM) mode.

Plasma samples. DDC was isolated from the plasma by SPE on LC-18 cartridges. To 1 ml of the plasma sample 5 ng of the internal standard were added followed by vortex-mixing. The plasma sample was subjected to SPE on LC-18 cartridges as described above. The eluent was evaporated to dryness and the residue was redissolved in 50 μl of methanol–water (10:90, v/v). A 25-μl sample was analyzed by thermospray LC–MS.

Thermospray LC–MS

The purified DDC sample was injected onto the HPLC system, which was interfaced with the VG Trio-3 triple quadrupole mass spectrometer

via a thermospray interface, based on the Vestec design using a heated capillary [13]. The HPLC system used a Hitachi L-6200 intelligent HPLC pump, a Valco C6W injector and an Altex Ultrasphere ODS analytical column (5 μm, 250 mm × 4.6 mm I.D.). The mobile phase was methanol–0.05 *M* ammonium acetate (10:90, v/v) at a flow-rate of 1.0 ml/min. Under these conditions, the retention time for DDC was 8.9 min. Thermospray conditions were optimized to achieve maximum sensitivity by proper selection of the capillary temperature (280°C), the source temperature (200°C) and the voltage on the repeller electrode (200 V).

Selected-ion monitoring

SIM mode was used for the determination of DDC in order to obtain the desired sensitivity. The protonated molecular ions at *m/z* 212 for DDC and *m/z* 216 for the internal standard were used for recording the selected-ion mass chromatograms. The area of the peaks in the selected-ion mass chromatograms was used for quantitation of the samples.

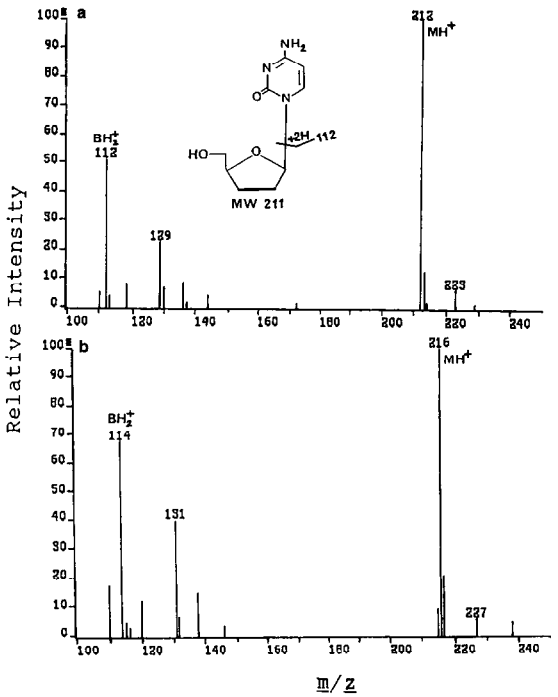

Fig. 2. Thermospray mass spectra for (a) DDC and (b) internal standard.

RESULTS AND DISCUSSION

The thermospray mass spectra of DDC and the internal standard (Fig. 2a and b, respectively) showed dominant protonated molecular ions (MH^+) together with some structurally informative fragment ions. The thermospray mass spectrum of DDC showed an ion at *m/z* 112 corresponding to the protonated base (BH_2^+), together with the MH^+ ion at *m/z* 212. The thermospray mass spectrum of the internal standard ($[^{15}N_2,^2H_2]$DDC) showed ions at *m/z* 216 and 114 corresponding to the MH^+ and BH_2^+, respectively. The BH_2^+ ion for the internal standard was 2 daltons higher than the corresponding ion for DDC due to the presence of two ^{15}N stable isotope labels in the base portion of the molecule (see Fig. 1b). The overall molecular mass of the internal standard was 4 daltons higher than that for DDC due to the presence of two ^{15}N and two ^2H stable isotope labels in the molecule. There was no ion at *m/z* 216 in the mass spectrum of DDC and at *m/z* 212 in the mass spectrum of the internal standard. Hence, there was no interference of one in the measurement of the other.

In order to attain the low detection limits needed for the assay, the SIM mode was used to record mass chromatograms. The selected-ion mass chromatograms were recorded at *m/z* 212 for DDC and at *m/z* 216 for the internal standard. The selected-ion mass chromatogram were recorded at *m/z* 212 for DDC for a buffer blank (Fig. 3a) and a 100-pg sample of DDC on-column (Fig. 3b). No peak appeared at 8.9 min in the chromatogram from the buffer blank indicating that there was no contribution of DDC from the injector or the column. The signal-to-nois ratio for the 100-pg sample was better than 6:1 for the peak at 8.9 min corresponding to DDC. The detection limit for DDC by this method was 50 pg. The selected-ion mass chromatograms were recorded at *m/z* 212 and 216 for the standard samples and plasma unknowns. The selected-ion chromatograms at *m/z* 212 and 216 for a 10-ng

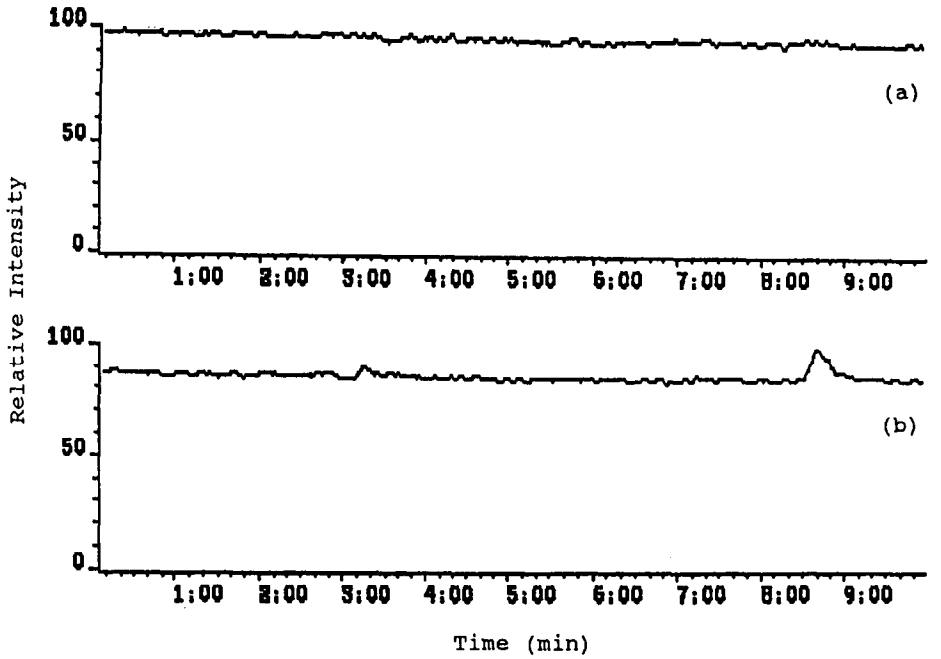

Fig. 3. Selected-ion chromatogram traces for *m/z* 212 for injections of (a) buffer blank and (b) 100 pg DDC on-column.

DDC standard sample are shown in Fig. 4. The peak areas were calculated by manually selecting the beginning and end points and using the software to calculate the areas.

The calibration curve was obtained by plotting the concentration of DDC as abscissa against the ratio of the area of the DDC peak to that of the internal standard (I.S.) peak as ordinate. The cal-

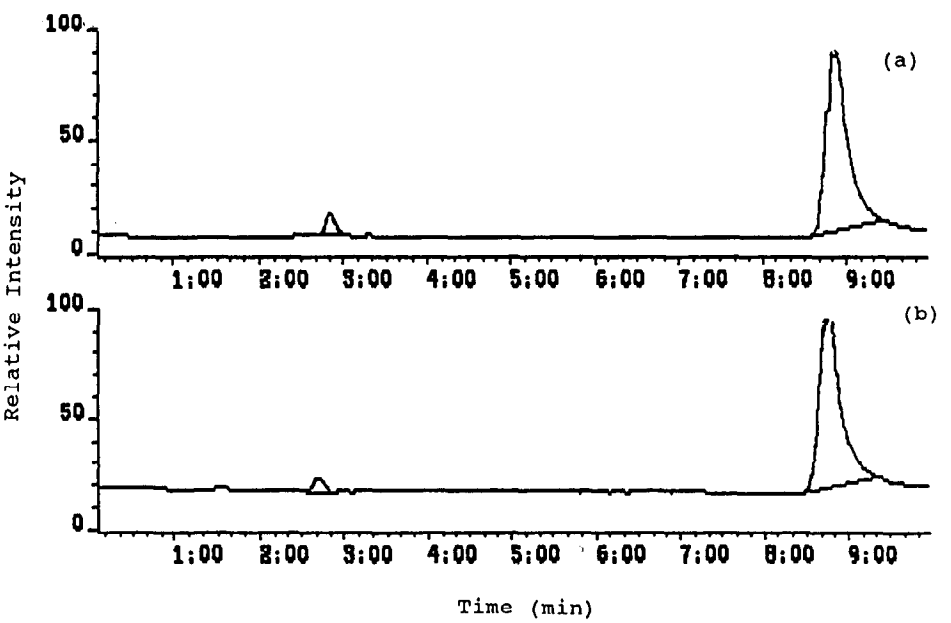

Fig. 4. Selected-ion chromatograms for the 10-ng DDC standard sample: (a) DDC at *m/z* 212 and (b) internal standard at *m/z* 216.

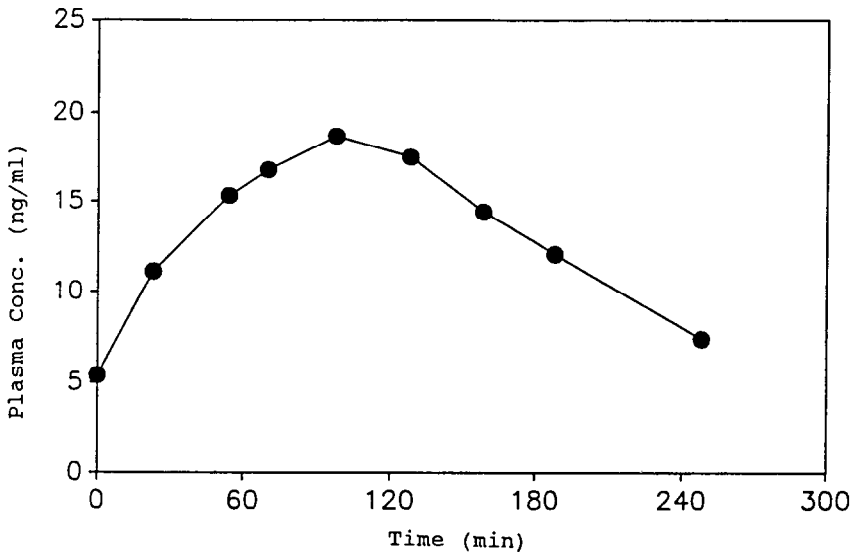

Fig. 5. Concentration profile of DDC in the human plasma as a function of time after a single oral dose of 0.03 mg/kg DDC.

ibration curve was linear over the desired range (0.25–20 ng/ml). The regression equation for the calibration curve was $A_{(DDC)}/A_{(I.S.)} = 0.135 \times$ (concentration) − 0.015 and the correlation coefficient was 0.9998. Concentration of unknowns were calculated from the regression equation. The accuracy and precision of the assay was determined by replicate measurements ($n = 5$) on three quality control standards. The values obtained for 3-, 6- and 12-ng quality control samples made up in plasma were 2.9 ± 0.1, 5.8 ± 0.2 and 11.9 ± 0.3, respectively. A number of plasma samples obtained at different times from a patient dosed orally with a single dose of 0.03 mg/kg DDC were analyzed. The plasma levels of DDC as a function of time after the single oral dose are shown in Fig. 5. It is evident that there was some residual drug present in the plasma of this subject at time zero. This most likely arises from the previous intravenous dose, which was given two days earlier. It can be argued that, due to short half-life of the drug, the concentration of the drug should be almost zero after two days. DDC is known to show delayed toxicity days after the drug administration has been stopped [8], hence, there may be a slower elimination phase in the disposition of DDC. This many account for the drug concentration at time zero. The peak plasma concentration occurred at about 2 h, which is consistent with that reported previously [9].

In summary, the thermospray mass spectrum of DDC gives a dominant protonated molecular ion at m/z 212, which makes it suitable for quantification by SIM. The detection limit for DDC in this assay was 50 pg injected on-column. The calibration curve was linear over the desired range, *i.e.* 0.25–20 ng/ml. The major advantages of this method over others are: no derivatization, high sensitivity, high specificity and short analysis time.

ACKNOWLEDGEMENTS

This work was supported by the NIH Grant RR000236, Clinical Research Center and AIDS Clinical Trials Group of the Johns Hopkins University. We are thankful to Drs. R. J. Cotter and P. S. Lietman for guidance and helpful discussions.

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