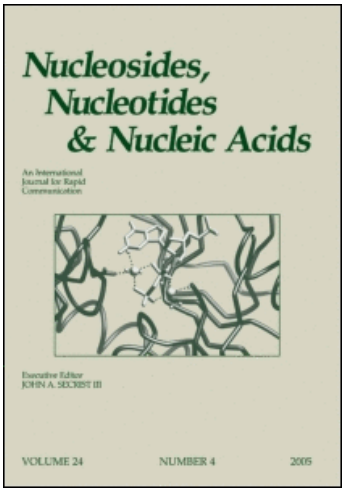

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ANALYSIS OF DEOXYCYTIDINE ACCUMULATION IN GEMCITABINE TREATED PATIENTS

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□ *Deoxycytidine (CdR) analogs are increasingly popular as chemotherapeutic agents and their effectiveness can be linked to the direct competition with active forms of endogenous CdR. A tandem mass spectrometric assay was developed to determine the plasma concentrations of CdR. Plasma extracts were prepared by protein precipitation and an ethyl acetate/water back extraction, and then separated chromatographically. Detection parameters were optimized for multi-reaction monitoring (MRM) tandem mass spectrometry and assay efficiency was improved using 15N3 CdR as an isotopic internal standard. Preliminary results from a gemcitabine trial are shown which indicate that CdR concentrations increase systemically during infusion, from about 5 nM to 78 nM after hepatic artery infusion and to 102 nM after systemic infusion for 24 hours. The developed assay demonstrated good sensitivity and selectivity for CdR.*

Keywords Deoxycytidine; Gemcitabine; Pharmacokinetics; Tandem mass spectrometry

INTRODUCTION

Deoxycytidine (CdR) is an essential component necessary for the formation of DNA strands; CdR is the base composed of cytosine and deoxyribose. Cellular CdR is in equilibrium with the systemic circulation and is activated to its triphosphate form prior to DNA incorporation.[1] Various mechanisms exist for the cellular control of CdR levels, maintaining a consistent level.

CdR analogs have been shown to demonstrate antitumor activity in a variety of tumor types.[2] The analog gemcitabine consists of the CdR

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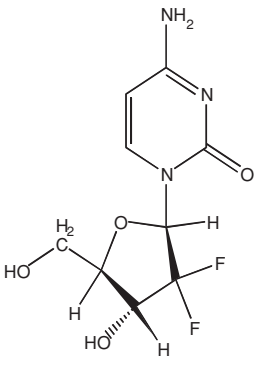
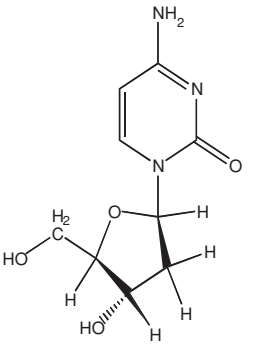


$C_9H_{11}F_2N_3O_4$
Exact Mass: 263.07
Mol. Wt.: 263.20
C, 41.07; H, 4.21; F, 14.44; N, 15.97; O, 24.32



$C_9H_{13}N_3O_4$
Exact Mass: 227.09
Mol. Wt.: 227.22
C, 47.57;H,5.77;N,18.49;O,28.17

FIGURE 1 Molecular representation of (a) gemcitabine (difluorodeoxycytidine) and (b) deoxycytidine. For gemcitabine the addition of 2 fluorine atoms in the sugar moiety represents the single difference in comparison to CdR.

molecule with the addition of two fluoride atoms on the 2′ position of the deoxyribose ring structure (Figure 1).[3] Generally, the mode of action of CdR analogs involves the incorporation of the active triphosphate metabolite into DNA strands in direct competition with the endogenous deoxycytidine triphosphate, dCTP.[3,4] In the case of gemcitabine during and just after intravenous administration the cellular levels of gemcitabine triphosphate (dFdCTP) increase[5] in comparison to endogenous dCTP creating more favorable conditions for the incorporation into DNA.[6] One of the intermediate metabolites (gemcitabine diphosphate, dFdCDP) is a known inhibitor of ribonucleotide reductase (RR),[7–9] which can possibly prevent the formation of dCDP from cytidine diphosphate, (CDP). dFdCTP is a potent inhibitor of dCMP-D, which catalyzes the deamination of dCMP to dUMP as well as that of dFdCMP to dFdUMP (Figure 2). The inhibition of dFdCMP deamination represents a self potentiation mechanism for Gemcitabine.[10,11]

To investigate the role of CdR, UdR (deoxyuridine), CDP, and dCTP in the chemotherapeutic action of gemcitabine, we developed an analytical method to monitor CdR plasma levels with tandem liquid chromatography mass spectrometry. Using the same principle, we also monitored UdR plasma concentrations with different chromatographic conditions. Utilizing this method, the change in concentration of CdR and UdR during and postinfusion of gemcitabine could be examined under a variety of different schedules. Initial results are reported for a 24-hour intravenous infusion.

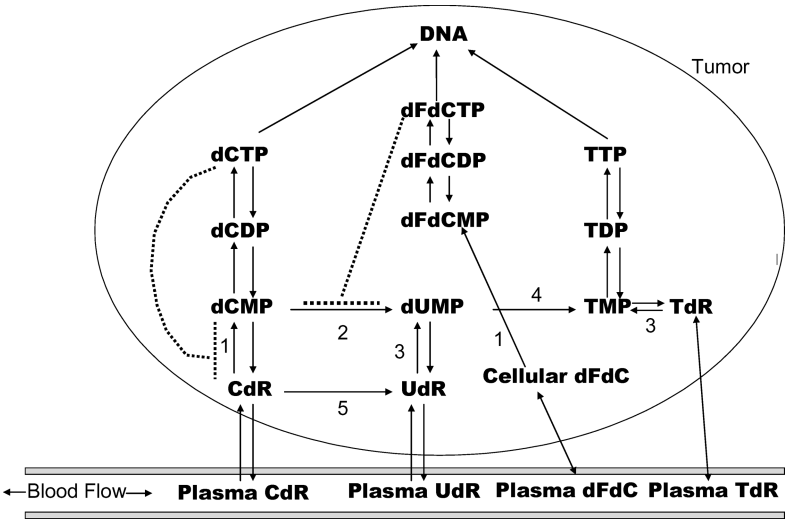


FIGURE 2 A schematic representation of the metabolic pathway for gemcitabine (dFdC), deoxycytidine (CdR), deoxyuridine (UdR), and thymidine. The rate limiting enzymes are indicated by 1 (deoxycytidine kinase (dCK)), 2 (dCMP deaminase), 3 (thymidine kinase), 4 (thymidylate synthase), and 5 (cytidine deaminase). dCTP has an inhibitory role on dCK and dFdCDP has an inhibitory effect on ribonucleotide reductase, whereas dFdCTP has an inhibitory effect on dCMP-deaminase.

Reagents

Analytical grade solvents such as Acetonitrile, Formic acid, Ethyl acetate and iso-Propanol were supplied by Merck & Co. (VWR, the Netherlands). HPLC grade water was supplied via a MilliQ water purification system (Millipore, the Netherlands) purified at 18 μohm into glass using a QPAK2 cartridge and a UF cartridge directly into glass containers. 2′ CdR and UdR were obtained commercially from Sigma-Aldrich (the Netherlands); $^{15}N_3$ CdR was supplied by Cambridge Isotope Laboratories (UK) and Tetrahydrouridine was obtained from Calbiochem (distributed by VWR International B.V., the Netherlands).

Equipment

Freeze drying was performed with a Christ bench top freeze drier (Salm and Kipp, the Netherlands) at −85°C. Chromatography was conducted using a Perkin Elmer series 200 HPLC system coupled via atmospheric pressure chemical ionization source to an Applied Biosciences SCIEX API 3000 mass spectrometer. Interpretation of response was performed with version 1.1 Analyst software (Applied Bioscience, the Netherlands).

ANALYTICAL PROCEDURE

Chromatographic separation was performed on 2-Spherisorb phenyl Econosphere glass columns fitted in series with a zero dead volume holder supplied by Chrompack (distributed by Varian, the Netherlands). Isocratic elution was performed with 50 mM formic acid: acetonitrile (9:1; v/v) at 1ml/min with a 1:10 split at the ionization source. Injection volumes used were between 4 and 70 μl depending on sample. Component detection was performed with APCI (atmospheric pressure chemical ionization) as the ion source. Mass spectrometric parameters were optimized for each component individually.

All standard and sample preparations were performed on ice. Whole blood samples were spiked with tetrahydrouridine (25 μl of 10 mM) to prevent deamination of CdR and dFdC. Plasma was prepared by centrifugation at 4°C and stored at −20°C until required. Aliquots of control plasma spiked with stock solutions of dFdC, CdR, and UdR were prepared in a concentration range of 0–220 nM, $^{15}N_3$ CdR was used as an isotopic internal standard. Sample and standard plasmas were protein precipitated with cold isopropyl alcohol which was subsequently evaporated to dryness by freeze

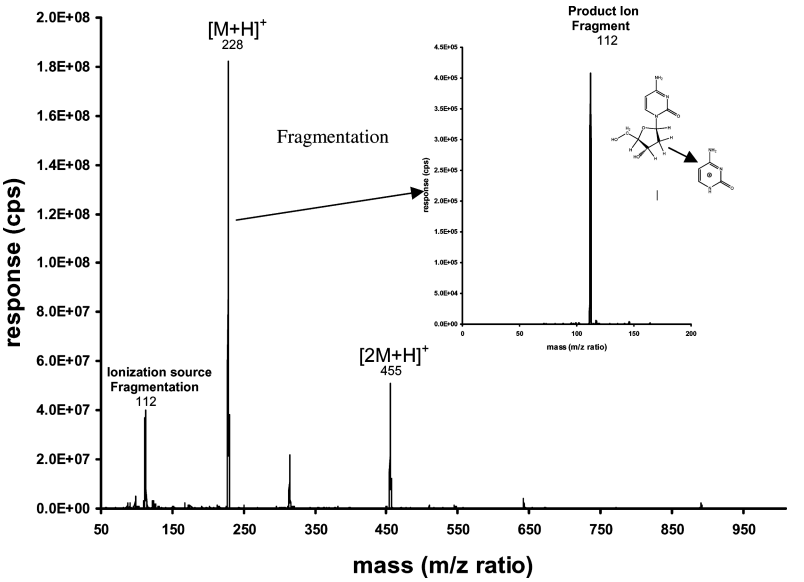


FIGURE 3 The 50–350 m/z segment from a full spectrum scan (50–2000 m/z) of CdR in 90% 50 mM formic acid: 10% acetonitrile. The molecular ions ($[M+H]^+$) for CdR can be observed at 228 m/z. Beyond 350 m/z no peaks were observed up to and including 2000 m/z. Within the range 50 to 120 m/z several breakdown products can be observed. Other mass responses observed are related to the solvent. Also shown is the 50 to 270 m/z product ion spectrum (fragmentation insert) of the $[M+H]^+$ ion, only a single response was observed at 112.2 m/z. This corresponds to the loss of the ribose sugar unit from the CdR molecule and represents a highly selective and sensitive means of separating deoxycytidine from other similar analogs.

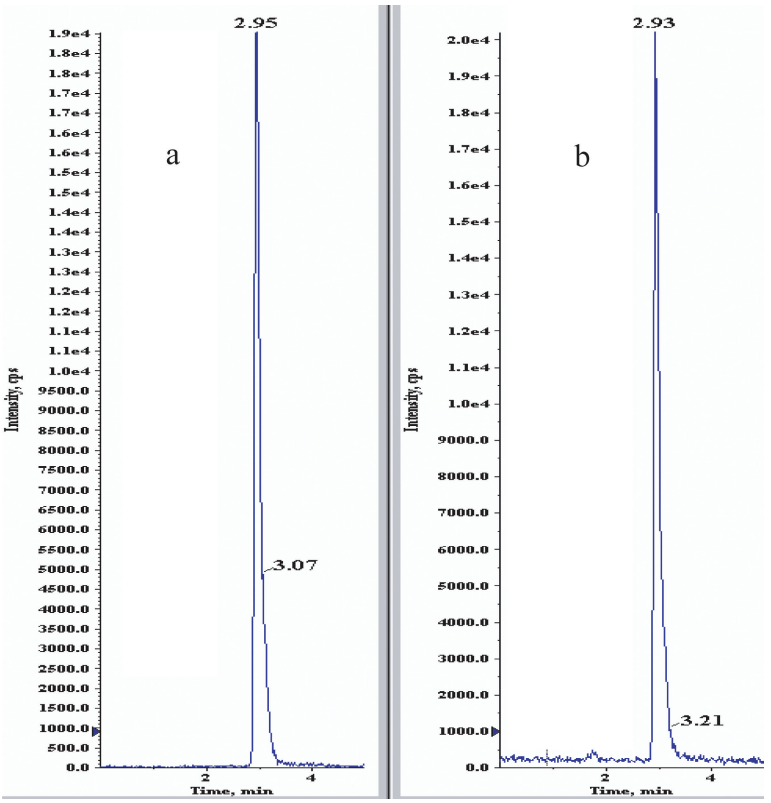


FIGURE 4 Illustration of the chromatographic response of (a) deoxycytidine and (b) $^{15}N_3$ deoxycytidine using 90% 50mM formic acid: 10% acetonitrile as a mobile phase. The chromatogram is clear from all other interference and peak shape is symmetrical.

drying. The dried samples were reconstituted in ethyl acetate and back extracted with 100 μl of purified water.

RESULTS AND DISCUSSION

The APCI conditions were optimized as follows: nebulising gas flow, 11 l/min; curtain gas flow, 9 l/min; collision activated dissociation gas flow (CAD), 4 l/min; nebulisier current, 3.0; probe temperature, 425°C. Quantitation was developed in positive MRM (multireaction monitoring) mode by the monitoring of the determined transition pairs of m/z m/z 228 (molecular ion)/112 (major fragment ion) for CdR (Figure 3), m/z 229 (molecular ion)/113 (major fragment ion) for UdR and m/z 231 (molecular ion)/115 (major fragment ion) for $^{15}N_3$ CdR. Chromatographic conditions were validated for selectivity, accuracy, precision, and robustness, expected retention times were 2.95 and 2.93 minutes for CdR and $^{15}N_3$ CdR, respectively (Figure 4).

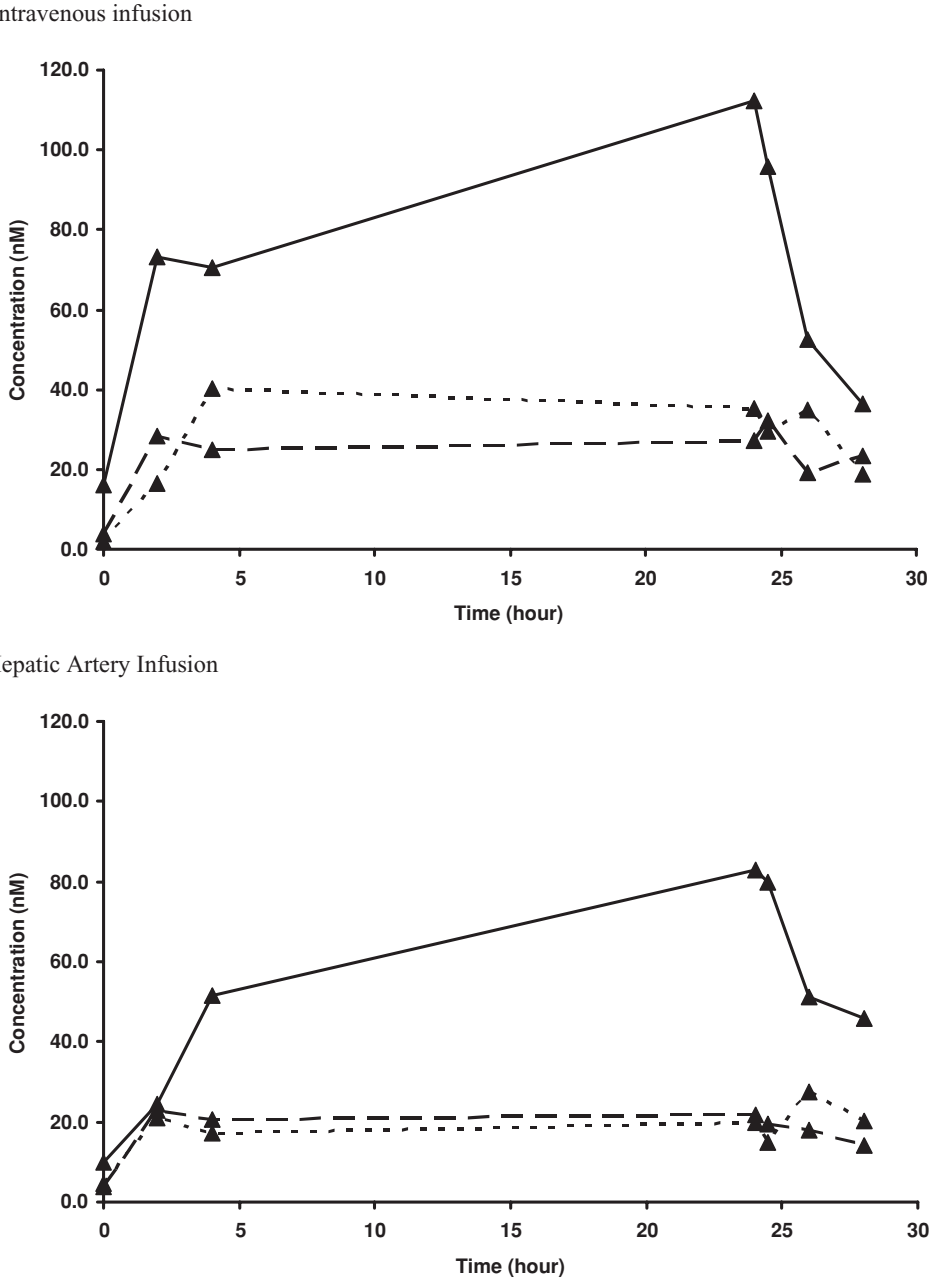


FIGURE 5 Concentration (nM) versus time profile for deoxycytidine following intravenous and hepatic artery infusions of gemcitabine. Three different dosing levels (75, 135, and 180 mg/m^2) are shown. Deoxycytidine plasma concentrations increase over the course of the infusion reaching a plateau concentration dependent on dose level. Post infusion levels can be seen to decrease back toward baseline endogenous concentrations. Similar profiles are observed for both intravenous and hepatic artery infusion techniques.

TABLE 1 Pharmacokinetic Parameters for Deoxycytidine after Treatment with a 24-hour Gemcitabine Infusion Either Via the Hepatic Artery or Intravenously. All Parameters were Determined Using WinNonLin Software

Treatment	Hepatic Artery Infusion			Intravenous Infusion		
Dose (mg/m^2)	75	135	180	75	135	180
C_{max} (nM)	22.5	29.8	78.2	32.3	48.2	101.8
T_{max} (Hrs)	24.7	17.1	24.7	10.2	17.9	23.6
AUC_{inf} nM hrs	455	388	1400	594	881	1829

Selectivity testing was performed for all components and the degree of crosstalk observed was estimated. Gemcitabine did not demonstrate any response for the transition pairs used for CdR and $^{15}N_3$ CdR. The same also was observed for CdR and $^{15}N_3$ CdR when compared against each of the other components. It also was observed that high concentrations of each component did not have a suppression effect on low concentrations of each other component, respectively. The chromatography was established to be reliable and accurate within ±15% in the concentration range of 1–100 ng/ml (4.4–440 nM). The above assay was used to determine the CdR plasma concentration in subjects from a clinical trial using gemcitabine.

Following intravenous infusion with gemcitabine at 75, 135, and 180 mg/m^2 over a 24-hour period, the plasma concentration of CdR increased in line with increasing concentration of gemcitabine (Figure 5, Table 1). After the completion of the infusion the gemcitabine levels rapidly decayed to baseline, however, CdR decayed less rapidly and had not returned to baseline at the end of the sampling period. This rise was observed in all patients tested (n = 13) and at all dosing levels. Correspondingly, the UdR plasma levels did not demonstrate a rise in concentration for either treatment and for any of the 3 dose levels tested and remained at levels between 40 and 60 nM (data not shown).

CONCLUSIONS

The developed assay shows good selectivity and sensitivity toward CdR, capable of detecting plasma baseline levels from a 200 μl sample size. To the best of our knowledge no other similarly sensitive method for plasma CdR has been published. HPLC methods are all limited to deoxynucleoside concentrations >0.5 μM. The initial results shown using clinical samples of a gemcitabine Phase I trial demonstrated a clear increase in plasma concentration during and shortly after intravenous infusion. During the 24-hour infusion, the CdR levels came to a maximum after 4 hours in line with maximum concentrations of dFdCTP (data not shown). This increase, therefore, potentially can be explained by an inhibition of dCMP-D by

dFdCTP and an accumulation of dCTP due to competition with dFdCTP. To stabilise cellular concentrations dCMP will be dephosporylated to CdR. Increasing intracellular CdR concentrations will be fluxed from the cell to the plasma to maintain safe physiological levels.

When dFdCTP decreased post-dFdC infusion, a decrease in the inhibition of dCMP-D would be reflected in a decrease in CdR plasma concentration, as is observed in the study investigated. Hence, CdR plasma accumulation could be considered as a simple marker for the accumulation of dFdCTP in tissue.

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