High-performance liquid chromatographic determination of cytosine-containing compounds by precolumn fluorescence derivatization with phenacyl bromide: application to antiviral nucleosides and nucleotides

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Abstract

A novel precolumn derivatization method for the HPLC determination of cytosine-containing compounds by HPLC is described. Highly fluorescent 2-phenyl-3,N^4-ethenocytosine derivatives are produced by a reaction of non-fluorescent cytosine-containing compounds with phenacyl bromide in weakly acidic acetonitrile solution at elevated temperature. The applicability of the method to various biogenic and antiviral compounds is demonstrated. Quantitative determination of cidofovir, a potent antiviral drug currently undergoing evaluation in the clinic for treatment of cytomegalovirus retinitis is also reported. The limit of detection for cidofovir in cynomolgus monkey plasma was 5 ng/ml (ca. 100 fmol on column) with the between-day precision of 16.6, 6.4 and 2.4% for five replicate samples at 20, 160 and 320 ng/ml, respectively. The within-day precision was 15.9, 5.9 and 2.1%, respectively. The method described has broad applicability and may offer significant advantages over existing HPLC methods in antiviral drug development as well as in nucleic acid research.

1. Introduction

Cytosine-based compounds serve many important functions in cell replication and metabolism in mammals. A number of cytosine-based therapeutics are analogs of endogenous species and possess broad-spectrum anti-DNA virus and anti-retrovirus activity. Among these antiviral drugs are dideoxycytidine (ddC) [1], (−)-*cis*-5-(4-amino-1,2-dihydro-

2 - oxo - 1 - pyrimidinyl) - 1,3 - oxothiolane - 2 - methanol (3-TC) [2] and a number of nucleotide analogs including cidofovir (HPMPC; (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine). Cidofovir, is an acyclic cytidine monophosphate analog that has shown potent anti-cytomegalovirus and anti-herpesvirus activity in vitro and in vivo [3]. It is currently undergoing evaluation in clinical trials as a systemic agent for treatment of cytomegalovirus (CMV) retinitis in AIDS patients.

The search for suitable techniques for selective and sensitive determination of the cytosine moiety in biological matrices is of great interest to biomedical

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and physiological research. Most analytical techniques for determination of the cytosine-based compounds in biological media use reversed-phase or ion-exchange HPLC with UV detection [4,5]. However, the polar character of the cytosine group combined with the complexity of biological matrices, including large numbers of endogenous UV-absorbing nucleotides and nucleosides, makes selective detection at low concentration levels exceedingly difficult. Even with extensive sample clean-up, the sensitivity and selectivity of UV detection may not be sufficient for the detection of the biogenic cytosine containing compounds or for monitoring of cytosine therapeutics in physiological fluids. As a result, more sensitive methods [e.g. radioimmunoassays (RIA)] are often required to monitor clinical drug levels [6]. However, the development of RIAs requires a considerable investment of time and effort, and the resulting methods are only applicable to a single compound.

It has been reported that cytosine- and adenine-containing compounds respectively form fluorescent $3,N^4$-ethenocytosine and $2,N^6$-ethenoadenine derivatives with haloacetaldehydes [7,8]. The $2,N^6$-ethenoadenine derivatives are highly fluorescent, and the reactions with chloroacetaldehyde and bromoacetaldehyde have become widely used for HPLC analyses of adenine-containing compounds [9,10]. In contrast to $2,N^6$-ethenoadenine derivatives, the $3,N^4$-ethenocytosine derivatives are only weakly fluorescent [11], and the derivatization of cytosine-containing compounds with haloacetaldehydes offers no advantage over direct UV detection. It has been reported that 2-phenyl-substituted $3,N^4$-ethenocytosine derivatives possess much stronger fluorescence than the unsubstituted derivatives and a synthetic route via a reaction with ω-bromoacetophenone (phenacyl bromide) has been suggested [12]. Based on this reaction, we have developed a novel HPLC fluorescence derivatization assay for the detection of cytosine-containing compounds, which is both sensitive and highly selective.

In this report, we describe the application of precolumn derivatization with phenacyl bromide to the HPLC detection of various cytosine-containing compounds, including cidofovir.

2. Experimental

2.1. Materials

Cidofovir was synthesized by Gilead Sciences (Foster City, CA, USA). 3-TC was kindly donated by Glaxo (Research Triangle Park, NC, USA). Other nucleotides and nucleosides and ddC were purchased from Sigma (St. Louis, MO, USA). Radiolabeled [2-^{14}C]cidofovir was obtained from Moravek Biochemicals (Brea, CA, USA). Phenacyl bromide was obtained from Fluka (Ronkonkoma, NY, USA). The ion-pairing reagent, dodecyltrietylammonium phosphate (Q12), was purchased from Bodman (Aston, PA, USA). All other chemicals and solvents (of analytical grade) were purchased from Baxter (Muskegon, MI, USA). Water was purified using a Milli-Q UF purification system (Millipore, Bedford, MA, USA).

2.2. Chromatography

The HPLC system consisted of a Spectra Physics Model P4000 pump, a silica solvent saturation column (Chrompack, Raritan, NJ, USA), a refrigerated AS3000 autoinjector with column oven, and an Hitachi F-7480 fluorescence detector. The Peak Pro data acquisition system (Beckman, Palo Alto, CA, USA) was used for data acquisition and processing. For radiochromatography, a Radiomatic A515A radioactive flow detector (Packard Instrument, Downers Grove, IL, USA), equipped with a 0.5-ml flow cell and detector splitter set at 50%, was added to the system after the fluorescence detector. Scintillation cocktail Redi-Solv (Packard) was delivered at a flow-rate of 2 ml/min. Data from the A515A were analyzed using FLO-ONE for Windows data acquisition and processing software (Packard). The analytical column used in all HPLC separations was a Prodigy (ODS-2) 100×4.6 mm, 5 μm (Phenomenex, Torrance, CA, USA).

2.3. Chromatographic conditions

System I: For demonstration of the general applicability of the method for derivatization and

detection of various cytosine-containing compounds including 3-TC and ddC, an isocratic reversed-phase HPLC method was used. The mobile phase contained 0.1% (v/v) trifluoroacetic acid in acetonitrile–water (16:84, v/v). The flow-rate was 2.2 ml/min and the column was maintained at 45°C. The fluorescence detector was set at the excitation wavelength of 305 nm and the emission wavelength of 370 nm. The injection volume was 20 µl.

System II: The most efficient separation of the derivatives of cidofovir and cytidine-5′-monophosphate (5′-CMP) from endogenous compounds in plasma was achieved using an isocratic ion-pair reversed-phase HPLC method. The mobile phase consisted of Q12 (6 mmol/l) and phosphoric acid (12 mmol/l) in acetonitrile–water (30:70, v/v). The final pH of the mobile phase ranged from 3.0 to 3.1. 5′-CMP was used as an internal standard. Cidofovir and 5′-CMP were eluted under isocratic conditions at a flow-rate of 3 ml/min with the column temperature set at 45°C. The fluorescence detector was set at 305 nm excitation wavelength and 370 nm emission wavelength. The injection volume was 20 µl and the retention time was 3.1 min for cidofovir and 5.3 min for 5′-CMP.

2.4. Solutions

Stock solutions (0.25 mg/ml each) of cytosine, cytidine,5′-CMP, 3-TC, and ddC were prepared in 10 mmol/l phosphate buffer, pH 7. Stock solutions of guanidine, uracyl, thymine, xanthine and hypoxanthine were prepared in a similar fashion. Individual working standard solutions of each compound (100 ng/ml) were prepared in phosphate buffer. In addition, a mixture 5′-CMP, cytosine, cytidine, 3-TC and ddC was prepared in phosphate buffer to obtain 100 ng/ml concentrations for each individual compound. Phenacyl bromide solution was prepared in acetonitrile and was stable for at least 2 weeks at room temperature. Cidofovir stock solution was prepared by dissolving an appropriate amount of the compound in 10 mmol/l phosphate buffer, pH 7, to obtain a 0.25 mg/ml solution. For working standards, the appropriate volumes of this stock solution were diluted with phosphate buffer to obtain six

standards of cidofovir with concentrations ranging from 40 to 1280 ng/ml.

2.5. Derivatization procedure for aqueous solutions of cytosine-containing compounds

Samples of working solutions were processed by placing aliquots (100 µl) of aqueous working standards into 1.5-ml polypropylene centrifuge tubes. To each of these tubes, 300 µl of acetic acid–acetonitrile–water solution (1:80:19, v/v) was added and the resulting mixture was briefly vortex-mixed, followed by centrifugation for 5 min (Biofuge, 15 000 g). The supernatants were transferred into screw-capped polypropylene centrifuge tubes and 100 µl of a phenacyl bromide solution (1.25 mmol/l in acetonitrile) was added to each tube. The tubes were sealed and incubated for 45 min at 80°C (unless stated otherwise). After incubation, the samples were evaporated to dryness under reduced pressure at room temperature (SpeedVac sample concentrator, Savant, Farmingale, NY, USA). Following evaporation, the residues were reconstituted in 60 µl of water. The reconstituted samples were briefly vortex-mixed, centrifuged for 5 min (Microfuge, 15 000 g) and then transferred into autosampler vials for HPLC analysis.

2.6. Derivatization procedure for cidofovir in plasma

Plasma standards were prepared by spiking 20 µl of each working standard into 80 µl of blank plasma to obtain a six-point standard curve with final concentrations ranging from 10 to 320 ng/ml. Quality control samples, used to validate the method, were prepared by spiking the corresponding working standards of cidofovir into blank cynomolgus monkey plasma to obtain concentrations of 320, 160 and 20 ng/ml. The working internal standard/protein precipitation solution was prepared by adding a sufficient volume of the 0.25 mg/ml stock 5′-CMP solution to 100 ml of acetonitrile–water–acetic acid solution (80:19:1, v/v) to obtain a 500 ng/ml solution. Plasma samples (100 µl) were processed as described for working standard solutions.

2.7. Recovery and derivatization efficiency

To determine the overall recovery and derivatization efficiency of the method, the procedure using radiochromatography of ^{14}C-labeled cidofovir was used. Three plasma standards and three aqueous standards were prepared at concentrations of unlabeled cidofovir of 20, 160, and 320 ng/ml, respectively. A sufficient volume of ^{14}C-labeled cidofovir was added to each standard to obtain a specific activity of 1 μCi/ml. The radiolabeled plasma standards were derivatized as described in Section 2.5 and Section 2.6 and injected onto the HPLC system with a Radiomatic scintillation flow detector. The aqueous standards were injected onto the system underivatized. To determine recovery of the method, the total peak area of the derivatized plasma samples was compared to the total peak area of the corresponding underivatized aqueous standards. To assess the derivatization efficiency, the peak area of the desired derivative was compared to the total peak area in each derivatized plasma standard (including underivatized cidofovir).

3. Results

3.1. Method optimization

To find the optimum conditions for the derivatization reaction, several parameters of the reaction were investigated, including the concentration of phenacyl bromide, pH, temperature and organic solvent. The

effects of these variables on the reaction were estimated by the relative rate of formation of the derivative as measured by direct injection of the reaction mixture (5 μl) onto the HPLC system. The fluorescence responses of the corresponding derivative peaks were monitored at their respective maximum excitation and emission wavelengths.

To examine the effect of pH of the reaction mixture on the reaction yield, a phosphate–acetate buffer (0.02 mol/l of each salt in the reaction mixture) was used. The response remained constant within the pH range of 3.5–5, but decreased outside that range (at pH 2 the fluorescence responses were about 50% of the maximum value and at pH 7, they were about 25% of the maximum). The reaction yield was the highest at pH values of between 3 and 5, and the required pH range could be obtained by addition of 10 mmol/l acetic acid.

Fig. 2 demonstrates the effect of the reagent concentration on the fluorescence response of the cidofovir derivative in water and plasma. A plateau in the response curve was generally reached at about 100 mmol/l phenacyl bromide for the aqueous standards and 350 mmol/l phenacyl bromide for plasma samples. A significantly greater excess of the reagent was required to reach a plateau in the fluorescence response of plasma samples compared to that found in aqueous standards. This is probably due to the presence of high concentrations of reactive endogenous compounds competing with cidofovir for the reagent. Since phenacyl bromide itself is not fluorescent, the high concentration of the unreacted reagent does not interfere with chromatography. A
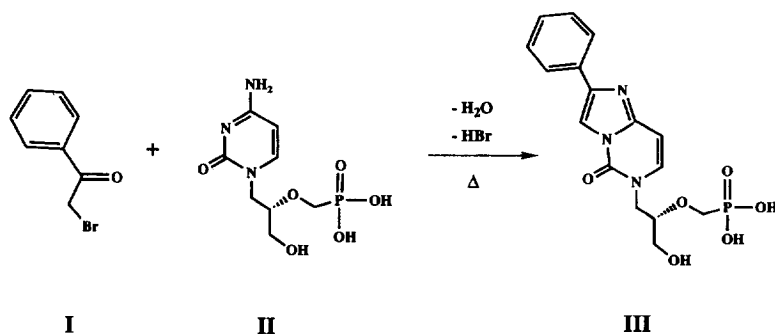

I   II   III

Fig. 1. Schematic reaction of phenacyl bromide with cidofovir (conditions for the reaction are given in Section 2. Compound identification: I=phenacyl bromide, II=cidofovir, III=fluorescent 2-phenyl-3,N[4]-ethenocytosine derivative of cidofovir.
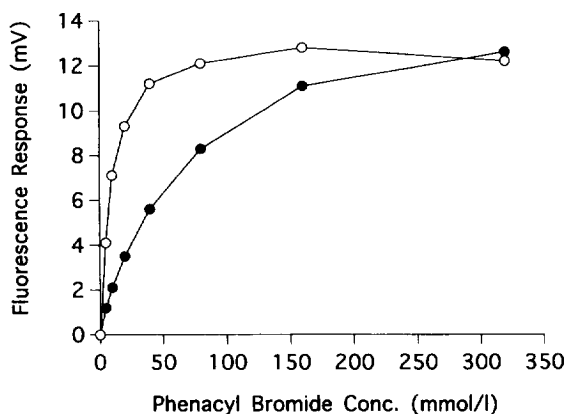
Fig. 2. Effect of phenacyl bromide concentration on the yield of the fluorescent derivatives following derivatization of aqueous and plasma cidofovir standards. (○=Aqueous standard, ●=plasma standard). Reaction and chromatographic conditions, except phenacyl bromide concentration, are as described in Section 2.

Fig. 3. Time course of the reaction of phenacyl bromide with cidofovir. (△=60°C, ○=80°C, ●=100°C).Reaction and chromatographic conditions, except the reaction time and temperature, are as described in Section 2.

concentration of 250 mmol/l phenacyl bromide provided a sufficient excess of the reagent with no visible interference in the chromatography.

Addition of an organic solvent was necessary to dissolve phenacyl bromide in the reaction mixture. Of the solvents tested, acetonitrile produced a better yield than methanol, methyl cellosolve or dimethylsulfoxide (DMSO).

The highest overall yields for cidofovir and the other cytosine-containing compounds evaluated were achieved in 0.1 mol/l acetic acid in water–acetonitrile (60:40, v/v) at 80°C for 45 min. A reaction temperature of 80°C and a reaction time of 45 min generally provided the optimal combination of the highest yields in the shortest period of time (Fig. 3). Higher temperatures allowed the derivatization to proceed more rapidly (approximately 30 min), but the final derivatives' peaks were smaller than those obtained at 80°C for 45 min.

We found that adenine-containing compounds also form fluorescent derivatives with phenacyl bromide under the described derivatization conditions. However, the excitation and emission wavelength maxima for these derivatives (236 and 412 nm, respectively, at pH 3) were different from those of the cytosine-containing compounds. Other nucleic acid bases, guanidine, uracyl, thymine, xanthine and hypoxanthine, did not yield fluorescent derivatives
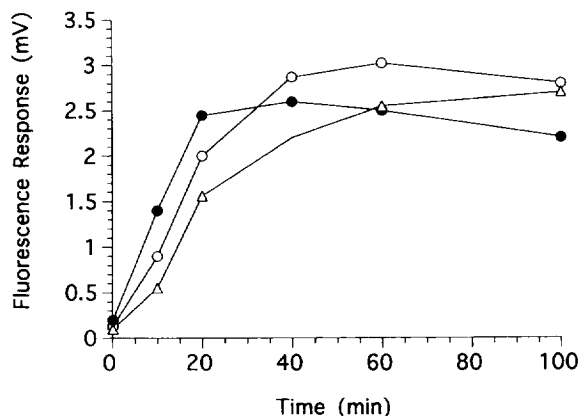
when treated with phenacyl bromide under the described conditions.

The derivatives of all cytosine-containing compounds displayed almost identical fluorescence spectra. Fig. 4 shows the corrected excitation (A) and emission spectra (B) for the 3,N⁴-ethenoderivative of cidofovir. The spectra were taken "on-the-fly" after injecting derivatized 160 ng/ml aqueous standards.

3.2. Separation of cytosine derivatives

The addition of a phenyl group to a cytosine-containing compound greatly enhances its partition properties on a reversed-phase column, diminishing the need for ion-pair modifiers, allowing higher concentration of organic modifiers, and, as a result, leading to increased column stability and greater chromatographic reproducibility. Various combinations of buffer solutions and organic modifiers were examined for the optimal separation on the Prodigy (ODS-2) column. The use of a trifluoroacetic acid–acetonitrile mobile phase appeared to produce the sharpest peaks with no tailing or fronting, leading to the selection of 0.1% (v/v) trifluoroacetic acid, in acetonitrile–water (16:84, v/v) as the final mobile phase. Fig. 5 shows a representative chromatogram of a derivatized mixture of six cytosine-containing compounds.
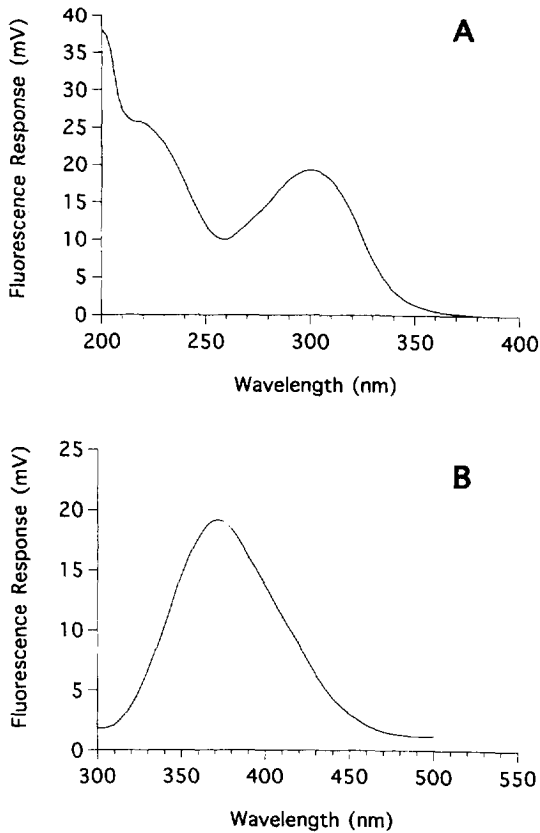
Fig. 4. Excitation–emission spectra of the 2-phenyl-3,N[4] ethenocytosine derivative of cidofovir (A=excitation spectrum, B=emission spectrum). Conditions are described in Section 2.
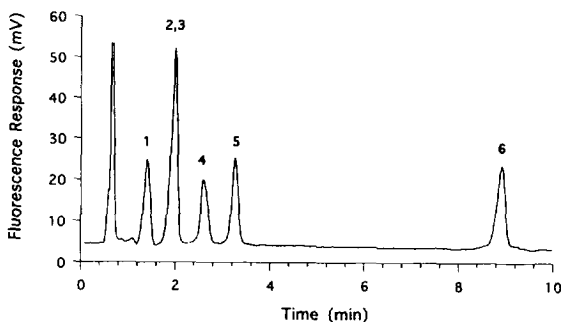

Fig. 5. Representative chromatograms of derivatized cytosine-containing compounds (200 ng/ml each) at excitation/emission wavelengths of 310/370 nm. Chromatographic and derivatization conditions are as described in Section 2. Peak identification: (1), 5'-CMP; (2), cytosine; (3), ddC; (4), cytidine; (5), deoxycytidine; (6), 3-TC.

3.3. Separation of the cidofovir derivative in plasma

Because cidofovir is protonated throughout the useful HPLC pH range (2–7), ion-pair chromatography could be used to facilitate its separation from endogenous plasma material. A mobile phase containing 6 mmol/l of Q12 and 12 mmol/l of phosphoric acid in acetonitrile–water (30:70, v/v) was selected. This mobile phase provided good separation of cidofovir and 5'-CMP (internal standard) from the endogenous plasma peaks. Representative chromatograms of cidofovir in cynomolgus monkey plasma are shown in Fig. 6. An example of a derivatized drug-free plasma sample spiked with 5'-CMP is displayed in Fig. 6A. A representative chromatogram of a derivatized plasma sample spiked with 40 ng/ml of cidofovir and 200 ng/ml of 5'-CMP is displayed in Fig. 6B. A typical chromatogram of a plasma sample obtained from a cynomolgus monkey 8 h after intravenous administration of 10 mg/kg cidofovir (spiked with 5'-CMP) is shown in Fig. 6C. The total chromatography time was less than 8 min, the peaks were symmetrical and well resolved, and the baseline was clean and free from interfering peaks.

3.4. Assay validation

The method was validated by performing replicate analyses (n=5) of pooled cynomolgus monkey plasma spiked with cidofovir (20, 160, and 320 ng/ml) on three separate days. Concentrations were determined using the respective standard curves prepared on the day of analysis. The precision and the overall accuracy of the method was estimated (Table 1). The within-day precision, defined as the mean of the daily coefficients of variation at each concentration (n=5), ranged from 2.1 to 15.9%. The between-day precision, expressed as the coefficient of variation of the pooled three-day data at each concentration (n=15), was in the range of 2.4 to 16.6%. The accuracy of the method, expressed as the ratio of actual to predicted concentration (C-ratio), was in the range of 99.8% to 104.0%. The derivatives of cidofovir and 5'-CMP were stable in the reconstitution solvent for over 48 h at room temperature and for over 1 month at −20°C.
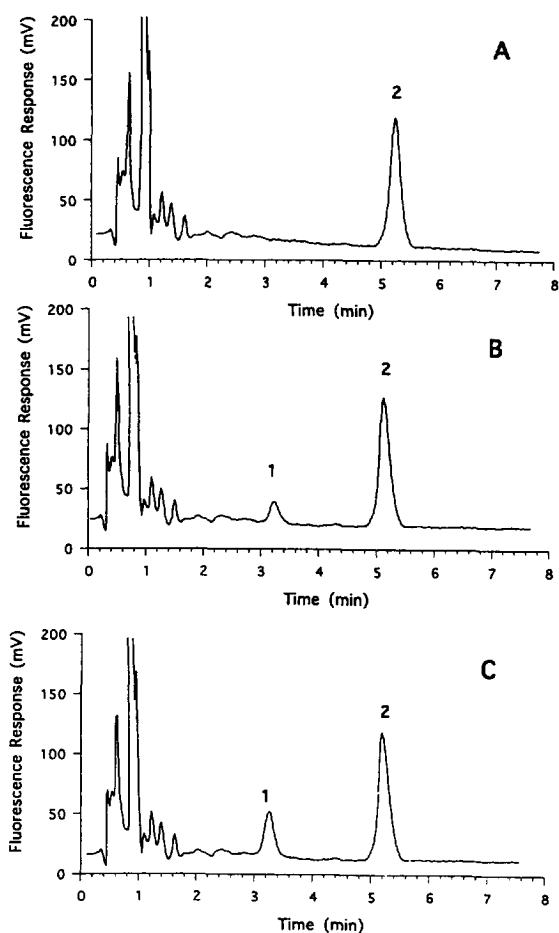
Fig. 6. Representative chromatograms of (A) derivatized blank plasma; (B) derivatized plasma sample spiked with 40 ng/ml of cidofovir (20 μl injection); (C) derivatized plasma sample taken from a cynomolgus monkey 12 h following oral administration of 3 mg/kg cidofovir. Samples in chromatograms A, B and C were spiked with 200 ng/ml of 5'-CMP (internal standard). Chromatographic and derivatization conditions are as described in Section 2. Peaks: 1=cidofovir; 2=5'-CMP (internal standard).

The specificity of the method was assessed by comparing chromatograms of derivatized blank plasma from cynomolgus monkey, rat, dog and human. All chromatograms were free of interfering peaks and endogenous materials were well separated from the cidofovir peak. To ensure that other cytosine-based antiviral drugs used in AIDS patients would not interfere with analysis of cidofovir in clinical plasma samples, blank plasma was spiked with 1 μg/ml each of 3-TC and ddC, and derivatized with

phenacyl bromide. Both drugs produced fluorescent derivatives from the reaction. However, the derivatives were well separated from the cidofovir peak. Neither of these drugs possesses an acidic group that can interact with the ion-pairing reagent Q12 and therefore their derivatives are not retained as strongly as derivatives of cidofovir or 5'-CMP.

3.5. Linearity, limit of detection and limit of quantitation

Peak-area ratios of drug to internal standard were plotted versus drug concentrations, and the slope and intercept of the standard curves were estimated by least-squares linear regression. Standard curves from 10 to 320 ng/ml cidofovir were linear and gave correlation coefficients that were better than 0.997. The method detection limit, determined at the signal-to-noise ratio of 3, was 5 ng/ml for cidofovir. The detection limit on column was 0.1 ng (ca. 300 fmol).

3.6. Recovery and derivatization efficiency

Fig. 7A and Fig. 7B compare the radiochromatograms for representative radiochromatograms of underivatized ^{14}C-labeled cidofovir in aqueous solution with a derivatized plasma sample containing [^{14}C]cidofovir. Fig. 7B shows that about 45% of cidofovir was converted to the fluorescent derivative, with no other radioactive reaction products. Comparison of the peak area of the derivative (peak 2 in Fig. 7B) to the peak area of the underivatized cidofovir standard (peak 1 in Fig. 7A) gave a total method recovery of 80 to 95% over the concentration range. Overall, 41% to 55% of available cidofovir was recovered as the fluorescent derivative.

4. Discussion

Precolumn derivatization is widely used in HPLC assays to enhance the sensitivity and selectivity of detection as well as to improve chromatographic properties of the compounds of interest. Simple fusion of an additional ring onto the pyrimidine system of cytosine compounds using haloacetaldehyde produces only weakly fluorescent imidazo[1,2-c]pyrimidine   (3,N^4-ethenocytosine)

Table 1
Accuracy and precision data for the determination of cidofovir in cynomolgus monkey plasma using precolumn derivatization with phenacyl bromide.

Concentration (ng/ml)	Day	Assay (mean ± S.D., $n=5$) (mg/ml)	Within-day (mean ± S.D., $n=5$) (%)	Between-day precision[a] ($n=15$) (%)	Accuracy[a] (mean ± S.D., $n=5$) (%)
20	1	20.8 ± 3.3	15.9 ± 0.4	16.6	104.0 ± 6.3
	2	19.5 ± 3.0			
	3	22.0 ± 3.5			
160	1	166.6 ± 9.8	5.9 ± 3.2	6.4	105.3 ± 1.5
	2	171.1 ± 5.4			
	3	167.6 ± 16.1			
320	1	315.6 ± 5.3	2.1 ± 0.5	2.4	99.8 ± 0.9
	2	322.0 ± 6.2			
	3	320.8 ± 8.2			

[a] The within-day, between-day precision and accuracy are defined in Section 3.4.

[13]. Introduction of a phenyl moiety into the C^2 position of the imidazopyrimidine heterocycle, through reaction with phenacyl bromide (Fig. 1), vastly increases fluorescence of these compounds. Through mesomeric or inductive interactions, the additional phenyl group removes the n–π^* transition between the carbonyl group and the ring that is

Fig. 7. Representative radiochromatogram of an aqueous underivatized standard spiked with 320 ng/ml cidofovir/1 μCi/ml [^{14}C]cidofovir (A) and a derivatized plasma sample of the same concentration (B). Chromatographic conditions are as described in Section 2. Peaks: 1=cidofovir; 2=fluorescent 2-phenyl-3,N^4-ethenocytosine derivative of cidofovir.

responsible for the weak fluorescence from the unsubstituted ethenocytosine compounds [13]. The resulting 2-phenyl-substituted derivatives of cytosine-containing compounds produced from the reaction [11] are well suited for reversed-phase HPLC analysis and the reaction is readily adaptable for precolumn derivatization. The 2-phenyl-3,N^4-ethenocytosine derivatives are not only highly fluorescent, but also significantly more lipophilic than cytosine-containing compounds which generally are poorly retained on reversed-phase sorbents.

Cidofovir, a cytosine monophosphate analog, undergoes phosphorylation within cells by cellular enzymes to produce the purported active metabolite cidofovir diphosphate [3]. The long intracellular half-life of the phosphorylated metabolite is responsible for the prolonged antiviral effect of the drug. As a result, the drug is expected to be therapeutically active when its plasma levels are well below 100 ng/ml. It is, therefore, necessary to have a highly sensitive technique for detection of cidofovir in order to carry out reliable pharmacokinetic studies. One of the most difficult problems in detection of cidofovir in biological fluids is the presence of a large number of endogenous bases and their nucleotides and nucleosides. These endogenous compounds possess very similar chromatographic and detection characteristics to those of cidofovir. The combination of derivatization of the drug and ion-pair reversed-phase HPLC separation greatly improves selectivity of detection. An ion-pair modifier, dodecyltrietylam-
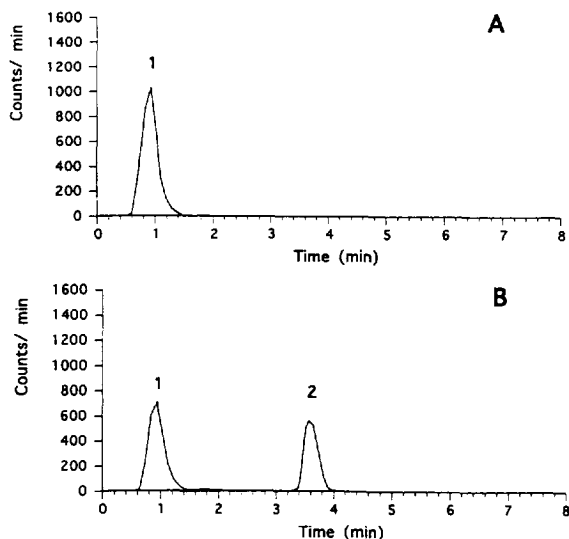
monium phosphate, helps to produce the very clean baseline, free of interfering peaks. 5'-CMP was selected as the internal standard. It has similar physico-chemical properties to those of cidofovir, allowing simultaneous derivatization by phenacyl bromide to a fluorescent analog, and the derivatized internal standard is well resolved from the cidofovir peak during HPLC separation.

The technical simplicity, speed and robustness of the method offer a reliable procedure. The assay appears to be suitable for the determination of cidofovir in plasma from multiple species, allowing accurate estimation of the pharmacokinetic parameters of the drug. The selectivity of the method eliminates the need for extensive clean-up of biological samples and, since phenacyl bromide is not fluorescent, the derivatization does not require any additional steps for removal of the reagent.

The fluorescent method described here for plasma is applicable to other biological systems as well (e.g., urine, cell extracts, etc.). In addition, fluorescence derivatization with phenacyl bromide should be useful for the fluorescence labeling of the cytosine moiety in oligonucleotides, high molecular mass nucleic acids (RNA and DNA) and in pharmacokinetic analysis of other cytosine-based antiviral drugs.

Phenacyl bromide also reacts with adenine-containing compounds, forming fluorescent derivatives. Apparently, as in the case of the cytosine moiety, the reaction produces 2-phenyl-substituted ethenoadenine derivatives (2-phenyl-2,N^6-ethenoadenine). The fluorescent properties of the derivatives are similar to those of the well described unsubstituted 2,N^6-ethenoadenine compounds [3–5].

Because of the universal character of the phenacyl bromide reaction for cytosine- and adenine-containing compounds, including oligonucleotides and nucleic acids, the method should find wide application in biomedical research.

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