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Isolation and identification of a metabolite of cidofovir from rat kidney

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Abstract

Cidofovir is an acyclic nucleotide analog with potent and broad-spectrum antiviral activity against adenoviruses and herpesviruses including cytomegalovirus (CMV). Cidofovir undergoes intracellular phosphorylation by host enzymes to cidofovir phosphate and cidofovir diphosphate (the active form). An unidentified metabolite has been observed previously in rat tissues and in urine of rabbits, rats and monkeys dosed with cidofovir. In the present study, this metabolite was isolated from rat kidney following an intravenous dose of 100 mg kg^{-1} cidofovir. The metabolite (metabolite I) was separated from cidofovir and impurities using extraction on anion-exchange resin followed by preparative normal and reversed-phase high-performance liquid chromatography (HPLC). The isolated metabolite I was subjected to proton, ^{13}C and phosphorus nuclear magnetic resonance (NMR) and matrix-assisted laser desorption/ionization mass spectroscopy, and confirmed to be cidofovir–phosphocholine. The uptake of cidofovir by rat kidney was saturated at an intravenous dose of 100 mg kg^{-1}, probably as a result of saturation of the renal tubular secretion pathway. However, the relative abundance of cidofovir phosphocholine was not affected by dose.

Keywords: Cidofovir; Cidofovir–phosphocholine; Drug metabolism; High-performance liquid chromatography; Kidney; Nuclear magnetic resonance

1. Introduction

Cidofovir (1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine; HPMPC) is an acyclic nucleotide analog with potent and broad-spectrum antiviral activity against adenoviruses and herpesviruses including cytomegalovirus (CMV) [1]. Cidofovir is currently being used as a systemic treatment for CMV retinitis in human immunodeficiency virus (HIV)-infected patients [2].

Cidofovir undergoes intracellular phosphorylation by host enzymes to form cidofovir phosphate, cidofovir diphosphate (the purported active form) [3–5] and two unidentified metabolites. These two metabolites of cidofovir have been observed previously in rat tissues and in the urine of rabbits, rats and monkeys dosed with [^{14}C]cidofovir [6–8]. One metabolite (metabolite I)

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eluted at a relative retention time consistent with the phosphocholine adduct of cidofovir. The other metabolite (metabolite II) coeluted with a cyclic analog of cidofovir, cyclic HPMPC (1-[((*S*)-2-hydroxy-2-oxo-1,4,2-dioxaphosphophosphorinan-5-yl)methyl]cytosine), on several different high-performance liquid chromatography (HPLC) systems.

Recently, metabolite I has been tentatively identified in vitro as an adduct of cidofovir with phosphocholine (cidofovir–phosphocholine) [9]. In that experiment, human embryonic lung cells were incubated in a medium containing [^{3}H]cidofovir and [^{14}C]choline. The mixed-label (^{3}H/^{14}C) metabolite formed after incubation was isolated, digested with phosphodiesterase and the resulting [^{14}C]choline phosphate and [^{3}H]cidofovir were identified by liquid chromatography.

The present study describes the preparative isolation of metabolite I from rat kidney, following administration of a high dose (100 mg kg^{-1}) of cidofovir to rats and confirmation of its identity using nuclear magnetic resonance (NMR) and mass spectroscopy (MS) analysis. This is the first report of the positive identification of a metabolite of cidofovir detected in vivo.

2. Experimental

2.1. Materials

Cidofovir was obtained from Gilead Sciences. [2-^{14}C]Cidofovir (3.0 mCi ml^{-1}, 17.3 mg ml^{-1}) was obtained from Moravek Biochemicals (Brea, CA). The cidofovir dosing solution was formulated as a sterile aqueous intravenous formulation containing 100 mg ml^{-1} cidofovir (58 μCi ml^{-1} [^{14}C]cidofovir). The 100 mg ml^{-1} solution was prepared aseptically using sterile saline. Dowex-1 ion-exchange resin was obtained from Sigma (St. Louis, MO). All other chemicals were obtained from Fluka (Ronkonkoma, NY).

2.2. Study design and sample collection

Eight male rats were used for the study. Each animal received a single intravenous bolus injection of [^{14}C]cidofovir (100 mg kg^{-1}, 1 ml kg^{-1}, 50 μCi kg^{-1}) via a tail vein. Animals were housed in metabolic cages for the duration of the study. All rats were sacrificed at 24 h post-dose. Kidneys were removed without exsanguination and stored at −70°C until sample preparation.

2.3. Extraction of the metabolite from rat kidneys

The primary metabolite of cidofovir (metabolite I) was isolated by extraction of rat kidney followed by purification on normal phase and reversed-phase preparative HPLC systems. Kidneys from all rats except one (used for determination of the concentration of total radioactivity) were completely thawed and combined with 25 ml of water. The tissue was homogenized with a Tekmar homogenizer (Janke and Kunkel, Germany) for 5 min and sonicated with a Vir Sonic 50 cell disruptor (Virtis, Gardiner, NY) at maximum output for 5 min. The homogenate was divided into two equal parts and transferred into two 50 ml centrifuge tubes. Methanol was added to each tube (1.5 part of methanol to 1 part of homogenate, v/v). The samples were re-homogenized and centrifuged at 4000 × *g* for 10 min. Following centrifugation, the supernatants were combined in a 50 ml centrifuge tube. Both of the remaining pellets were resuspended in 25 ml of methanol followed by sonication and centrifugation. After centrifugation, supernatants were combined with the original supernatants from the first extraction (total volume 75 ml).

The concentration of total radioactivity in kidney at 24 h was determined for one animal. Kidneys from one rat were thawed, combined with water (1/1, w/v) and processed as described above. Triplicate samples of the homogenate were weighed and then oxidized on a Model 307 Packard Sample Oxidizer and the resulting samples were counted on a TriCarb Model 2500 liquid scintillation counter (Packard, Meriden, CT).

2.4. Solid-phase extraction of metabolite

Dowex-1 strong anion-exchange resin (trimethylbenzylammonium chloride) was converted into the acetate form by two sequential

washes with 1 N acetic acid (5:1, v/w) and water (5:1, v/w). The treated anion-exchange resin (25 g) was loaded into an empty polypropylene cartridge and washed with 150 ml water. The rat kidney extract (75 ml) was applied and washed with 100 ml water. Following the wash, retained nucleotides were eluted with four 25 ml volumes of 1 M ammonium acetate. The collected eluant was evaporated under reduced pressure on a Savant evaporator (Savant Instruments, Farmingdale, NY) at room temperature and reconstituted with 3 ml methanol:water (1:1, v/v).

2.5. Preparative HPLC

The HPLC system comprised a Spectra Physics Model P4000 pump, a silica solvent saturation column (Chrompack, Raritan, NJ), a refrigerated AS3000 autoinjector with column oven and a Spectra-Physics UV detector followed by a Radiomatic A515A radioactive flow detector (Packard Instrument, Downers Grove, IL), equipped with a 0.5 ml flow cell and flow splitter. Scintillation cocktail (Redi-Solv, Packard Instrument, Downers Grove, IL) was delivered at 2 ml min^{-1}. Data from the A515A detector were analyzed using FLO-ONE for Windows data acquisition and processing software (Packard).

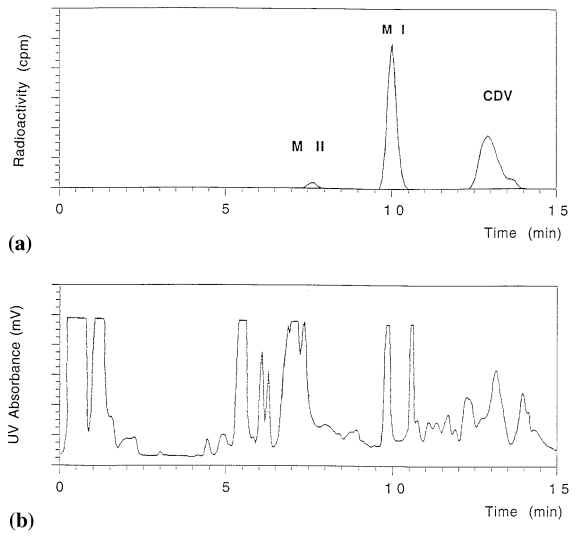

Fig. 1. Representative chromatograms of an extract from rat kidney obtained following intravenous administration of 100 mCi kg^{-1} ^{14}C cidofovir before clean-up; HPLC system I (A, radiochromatogram; B, UV at 272 nm; time delay from B to A is 0.3 min). MI, metabolite I; MII, Metabolite II; CDV, cidofovir.

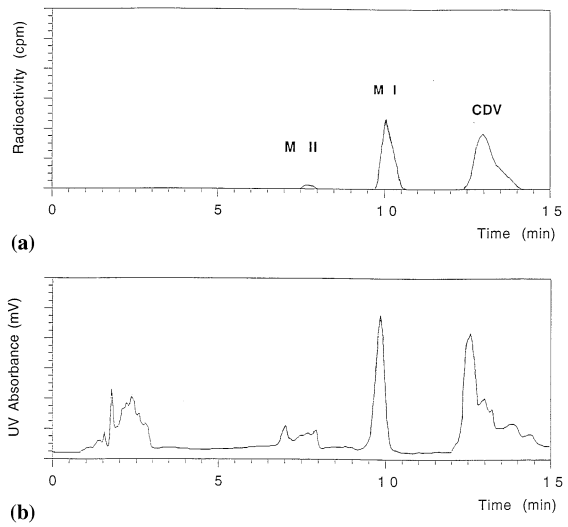

Fig. 2. Representative chromatograms of an extract from rat kidney following intravenous administration of 100 mCi kg^{-1} ^{14}C cidofovir after extraction on a Dowex-1 anion-exchange sorbent; HPLC system I (A, radiochromatogram; B, UV at 272 nm; time delay from B to A is 0.3 min). MI, metabolite I; MII, metabolite II; CDV, cidofovir.

HPLC system I: the analytical column used for initial preparative isolation of the metabolite was a Hypersil (APS-2) 250×4.6 mm, 5 μm (Phenomenex, Torrance, CA). Mobile phase A was acetonitrile:water (70:30, v/v); mobile phase B contained 200 mM ammonium formate and 0.2% (v/v) trifluoroacetic acid in water. The gradient program was 100% A to 100% B in 15 min. The flow rate was 2.2 ml min^{-1} and the column was maintained at 45°C. The injection volume was 100 μl. Total cycle time between injections was 25 min.

HPLC system II: for final desalting of the isolated metabolite, two Prodigy (ODS-2) columns (250×4.6 mm, 5 μm; and 150×4.6 mm, 5 μm, total length 400 mm) connected in series were used at room temperature. Deionized water was used as the mobile phase. Metabolite I was eluted under isocratic conditions at a flow rate of 0.8 ml min^{-1}. The UV detector was set at 214 nm

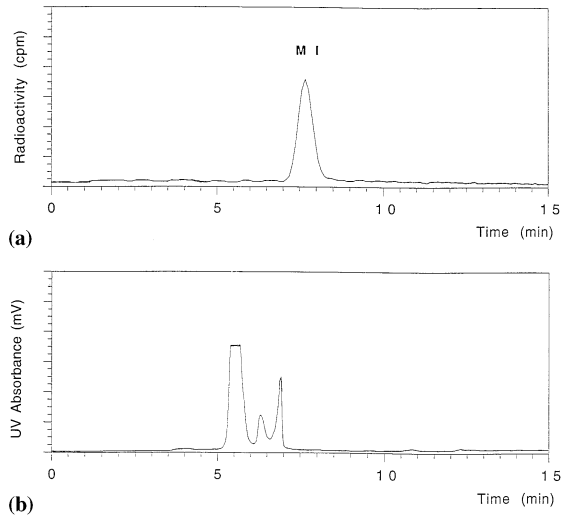

Fig. 3. Representative chromatograms of an extract from rat kidney following intravenous administration of 100 mCi kg^{-1} ^{14}C cidofovir after extraction on a Dowex-1 anion-exchange sorbent and isolation on a normal phase preparative chromatographic system; HPLC system II (A, radiochromatogram; B, UV at 214 nm; time delay from B to A is 0.5 min). MI, metabolite I.

wavelength. The injection volume was 100 μl and the retention time was 7.3 min for metabolite I and 6.7 min for the closest impurity. Total cycle time between injections was 10 min. Data was acquired and stored by a Peak Pro data acquisition system (Beckman).

2.6. Analysis

Approximately 200 μg of the dry isolated metabolite I was reconstituted in 600 μl D_2O and subjected to proton, ^{13}C and phosphorus NMR analyses on a Varian UnityPlus 500 MHz NMR spectrometer. The sample temperature was maintained at 25°C.

Approximately 200 μg of the dry isolated metabolite I was reconstituted in 200 μl H_2O. The solution (5 μl) was mixed with 5 μl of 0.5 M 3-hydroxypicolinic acid in acetonitrile:water (50:50, v/v) and subjected to MS analysis on a Charles Evans and Associates MALDI (matrix-assisted laser desorption/ionization) triple electrostatic analyzer time-of-flight mass spectrometer.

The concentration of total radioactivity in kidney at 24 h was determined for one animal. Kidneys from one rat were completely thawed, combined with water (1:1, w/v) and processed as described in Section 2.3. Triplicate samples of the homogenate were oxidized on a Model 307 Packard Sample Oxidizer and the resulting samples were counted on a TriCarb Model 2500 liquid scintillation counter (Packard, Meriden, CT).

Table 1
Structural assignment of the protons and carbons in metabolite I from proton and ^{13}C NMR spectra

Proton NMR resonance peak number	^{13}C shift, δ (ppm)	Carbon position assignment	Proton shift, δ (ppm)	Proton position assignment
1	50.21	1′	3.87	$H_{1'}$
2	50.21	1′	4.11	$H_{1'}$
3	80.44	2′	3.79	$H_{2'}$
4	60.77	3′	3.60	$H_{3'}$
5	60.77	3′	3.83	$H_{3'}$
6	66.35	6′	3.77	$H_{6'}$
7	66.35	6′	3.86	$H_{6'}$
8	60.04	5′	4.41	$H_{5'}$
9	66.22	4′	3.71	$H_{4'}$
10	54.26	7′	3.26	$H_{7'}$
11	95.79	5	6.05	H_5
12	148.37	6	7.71	H_6

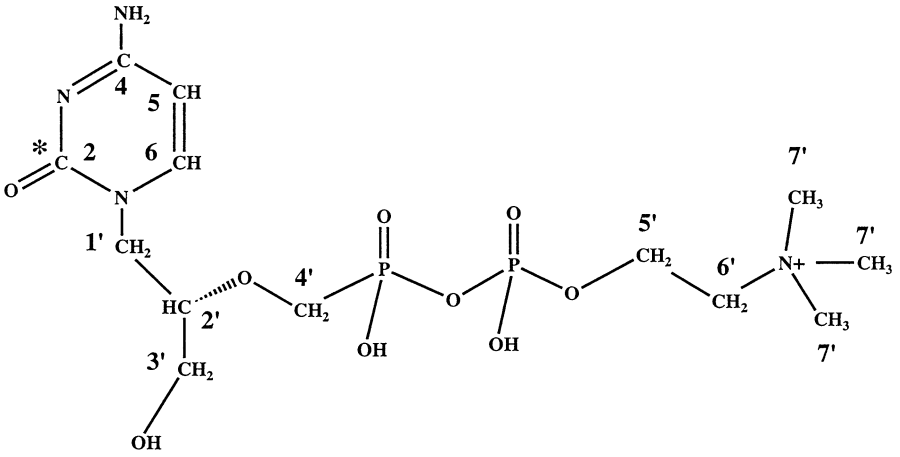

Fig. 4. Structure of metabolite I (cidofovir–phosphocholine) isolated from rat kidney following intravenous administration of cidofovir (the position of the labeled atom is denoted with an asterisk).

3. Results and discussion

3.1. Isolation and purification

Following intravenous administration of [^{14}C]cidofovir, the highest concentrations of radioactivity are found in the kidney [8]. Therefore, the kidney was selected as the most convenient source for isolation of metabolite I. Figs. 1–3 show representative radio- and UV chromatograms of samples of extracted kidney at various stages of the extraction obtained following intravenous administration of 100 mg kg^{-1} cidofovir (58 μCi ml^{-1} [^{14}C]cidofovir) to rats. From comparison of the UV profiles in Figs. 1 and 2, it is apparent that the solid-phase extraction step significantly improved preparative chromatography, since most of the endogenous compounds were removed. Recovery of metabolite I in the extraction step alone was about 50%. The overall recovery of the metabolite after the solid phase extraction and preparative chromatography was 8.8% of the total radioactivity present prior to purification.

3.2. NMR and MS analysis

The isolated solid was analyzed using proton, ^{13}C, phosphorus NMR and MALDI-MS. Table 1 summarizes data from the proton and ^{13}C NMR spectra of isolated metabolite I with corresponding structural assignment of carbon atoms and protons. The spectra displayed resonance signals corresponding to carbons and protons in the cidofovir molecule as well as characteristic signals for a $-H_2C-O-PO_2-$ group (at δ4.41 by proton NMR and at δ60.04 by ^{13}C NMR) and a $-N(CH_3)_3^+$ moiety of cidofovir–phosphocholine (at δ3.26 by proton NMR and at δ54.26 by ^{13}C NMR). Fig. 4 shows the corresponding structural assignment of the carbon atoms in metabolite I. Phosphorus NMR provided resonance signals for the cidofovir phosphorus at δ8.22 and the second phosphorus atom in phosphorylated cidofovir at δ12.12. For MALDI-MS analysis of the metabolite, a mixture of leucine enkephaline (MW = 556) and gramicidin S (MW = 1141) was used as the external mass calibration standard. The MS analysis of the metabolite provided a peak at m/z 445 corresponding to cidofovir–phosphocholine ($C_{12}H_{24}N_4O_5P_2$, MW = 445) and an additional peak at m/z 467 which was attributed to a monosodium salt of the metabolite. Both NMR and MS analyses supported the assigned structure for the choline adduct of monophosphorylated cidofovir shown in Fig. 4.

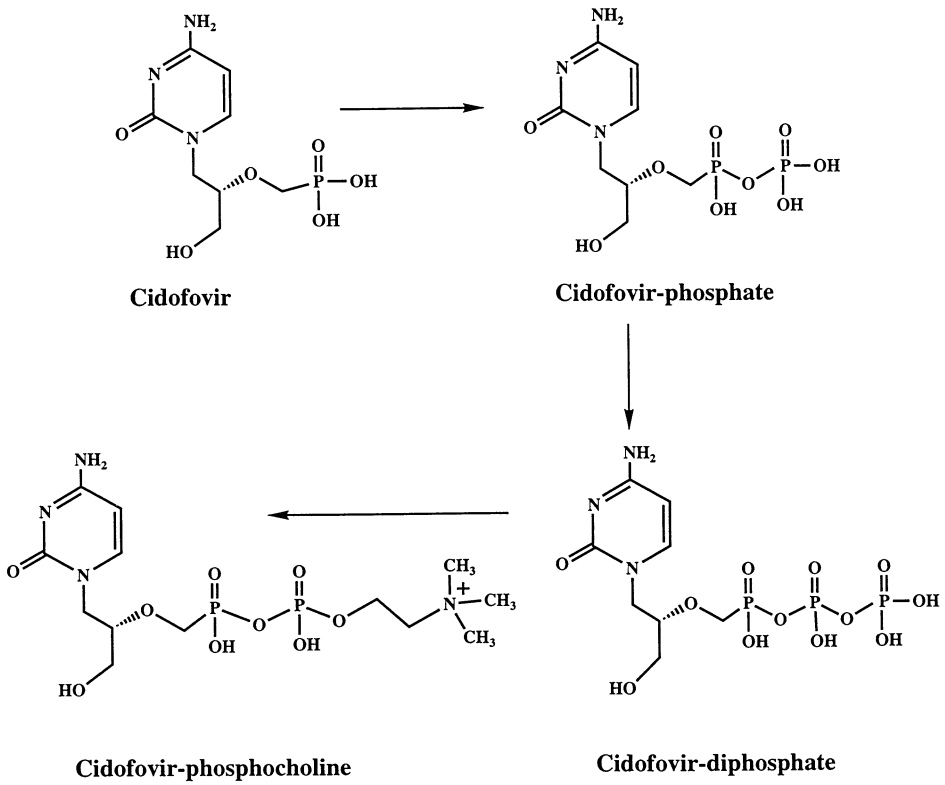

Fig. 5. Proposed intracellular metabolic scheme for cidofovir.

3.3. Metabolism

The identification of metabolite I as cidofovir–phosphocholine supports the proposed in vivo metabolic route for cidofovir shown in Fig. 5. Cidofovir is metabolized within cells to form cidofovir phosphate, cidofovir diphosphate (the active form) and cidofovir–phosphocholine. The long intracellular half-lives of these phosphorylated metabolites are responsible for the extended duration of action of the drug [10]. Metabolite II, also detected in many tissues and in urine, appears to be the cyclic analog of cidofovir, cyclic HPMPC [11]. The observed presence of cyclic HPMPC likely results from internal cyclization of the phosphorylated species ex vivo. Cyclic HPMPC is easily formed in aqueous solutions of authentic, chemically synthesized cidofovir diphosphate and cidofovir–phosphocholine as a result of their chemical hydrolysis at low pH. Preliminary evidence also suggests that metabolite II may be formed as a result of the extraction process (G.M. Lynch, K.C. Cundy, unpublished data).

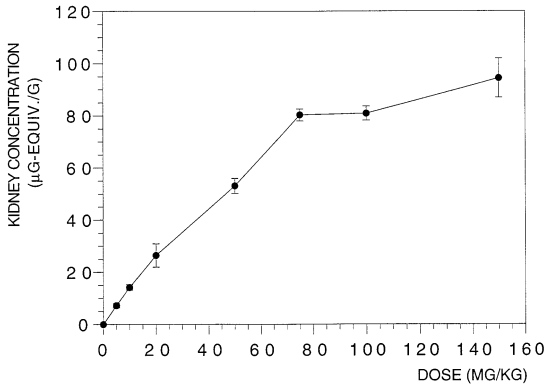

Fig. 6. Relationship between dose and concentration of radioactivity (mean ± S.D.) in rat kidney following intravenous administration of [^{14}C]cidofovir to rats.

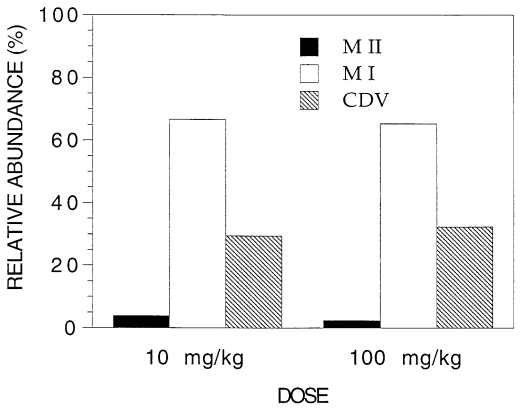

Fig. 7. Relative abundance of radiolabeled metabolites in rat kidney after intravenous administration of cidofovir. Metabolite II was attributed to cyclic HPMPC, based on coelution with cyclic HPMPC on three different HPLC systems. MI, metabolite I (cidofovir–phosphocholine); MII, metabolite II; CDV, cidofovir.

3.4. Effect of dose on concentration of cidofovir and metabolites in kidney

The concentration of total radioactivity in the kidney at 24 h after a 100 mg kg^{-1} [^{14}C]cidofovir dose was 80.9 μg-equiv. g^{-1} (0.67% of a total dose). Fig. 6 shows the effect of the administered intravenous cidofovir dose on the concentration of radioactivity in kidney of rats at 24 h post-dose. The concentration of radioactivity in kidney appears to reach a maximum value, suggesting saturation of the uptake process. Cidofovir is transported into kidney cells by a probenecid-sensitive anion transport system located on the basolateral surface of proximal tubule cells [9]. It is likely that high concentrations of cidofovir overload the capacity of this anion transport system, leading to a lower than expected kidney concentration. Analysis of the kidney concentration data using a Michaelis–Menten saturation model provided a maximum kidney concentration of 134 μg g^{-1} and a Michaelis constant of 61 mg kg^{-1} (Fig. 6).

Fig. 7 shows the relative abundance of metabolites of cidofovir in the extracts prepared from rat kidneys obtained 24 h after intravenous administration of cidofovir at doses of 10 and 100 mg kg^{-1}. The graphs show comparable metabolite profiles at the two doses, suggesting that phosphorylating enzymes were not saturated at intracellular concentrations of radioactivity as high as 80.9 μg-equiv. g^{-1}. Since the uptake of cidofovir was already saturated at the 100 mg kg^{-1} dose, it may not be possible to produce high enough levels of intracellular cidofovir to saturate the enzymes responsible for its phosphorylation.

4. Conclusions

A metabolite of cidofovir was isolated from rat kidneys obtained at 24 h after intravenous administration of [^{14}C]cidofovir (100 mg kg^{-1}, 58 μCi kg^{-1}) to rats. The metabolite was identified as cidofovir–phosphocholine using NMR and mass spectrometry. This finding is consistent with the tentative assignment of cidofovir–phosphocholine as the metabolite observed in cultured cells. This is a first report of the definitive isolation and identification of a metabolite of cidofovir obtained in vivo.

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