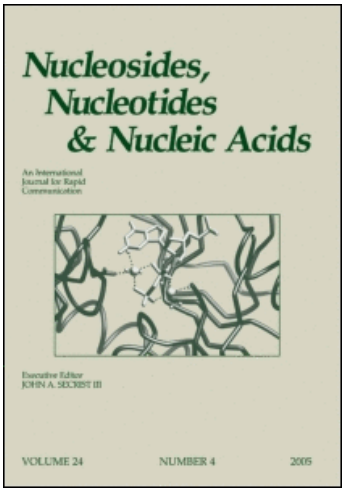

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LC/MS DETERMINATION OF THE INTRACELLULAR CONCENTRATION OF TWO NOVEL ARYL PHOSPHORAMIDATE PRODRUGS OF PMPA AND THEIR METABOLITES IN DOG PBMC

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ABSTRACT

LC/MS assays were developed to determine the plasma and intracellular concentrations of two aryl phosphoramidate prodrugs of the nucleotide analog 9-[2-*R*-(phosphonomethoxy)propyl]adenine. LC/MS was used to demonstrate the presence of high concentrations of PMPA in peripheral blood mononucleocytes following oral administration of prodrugs in dogs. High concentrations of PMPA and active metabolite were detected in MT-2 cells incubated with prodrug using an ion-pairing LC/MS assay.

INTRODUCTION

The nucleotide analog 9-[2-*R*-(phosphonomethoxy)propyl]adenine (PMPA) has shown potent and selective activity against HIV (1). PMPA has low bioavailability due to its ionic phosphonate moeity (2). To overcome this limitation, a series of novel aryl phosphoramidate derivatives of PMPA have been synthesized, with two compounds, GS-7097 and GS-7171 demonstrating potent *in vitro* activity against HIV. GS-7340, one of two GS-7171 diastereomers, was selected for further development.

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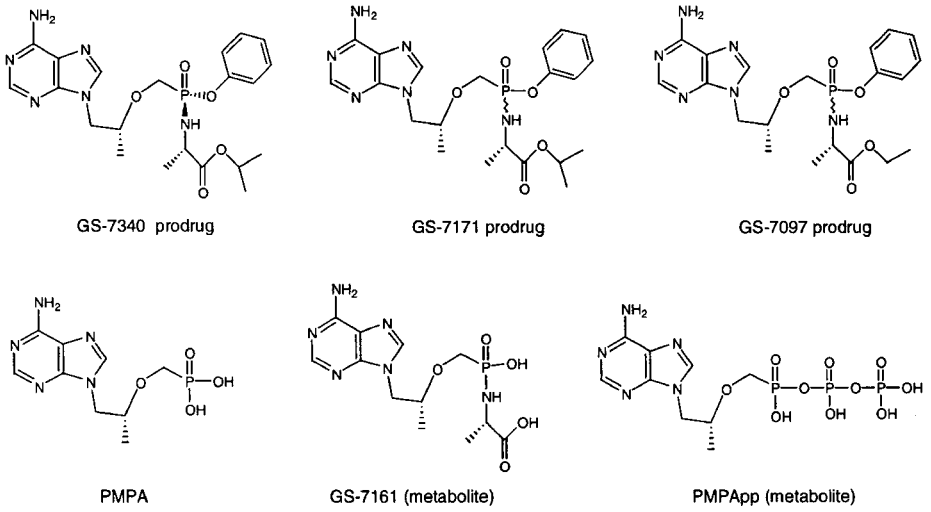

Figure 1. Structures of prodrugs, PMPA and metabolites.

A method was needed to determine concentrations of prodrugs and PMPA in plasma and peripheral blood mononuclear cells (PBMC) following oral administration in dogs. A previous method based on fluorescence detection was unsuitable for some metabolites. Liquid chromatography/mass spectrometry (LC/MS) offers improved sensitivity and may allow simultaneous determination of PMPA, prodrug and metabolites (Fig. 1) including PMPA diphosphate (PMPApp) and GS-7161.

LC/MS requires volatile mobile phase for compatibility with the MS source. The volatile ion-pairing reagent N',N'-dimethylhexylamine (DMHA) has been shown to be MS compatible and to improve the chromatographic results for phosphorylated nucleotide analogs (3,4). Herein, we describe results obtained using LC/MS methods developed with and without DMHA ion-pairing reagent.

METHOD

An Agilent HP-1090 or a Thermoquest P4000/A3000 LC was used. A Finnigan LCQ^{TM} ion-trap MS was operated in positive electrospray ionization (ESI) mode. The MS was set to monitor in segments the $[M+H]^+$ precursor ion of each analyte and to collect the full MS/MS product ion scans. Reconstructed ion chromatograms (RIC) were generated by summing the ion current intensities of the major product ions versus chromatographic retention time. Calibration curves were created by plotting the standard analyte area versus analyte concentration. Sample extracts were injected directly onto the LC/MS.

Two LC/MS methods were developed. The first used an ammonium acetate mobile phase with an acetonitrile gradient (0–60%, 15 min.) on an Inertsil ODS

column (4.6 × 150 mm). The second used DMHA acetate (20 mM, pH 7) as an ion-pair with a methanol gradient (15–80%, 12 min.) on a Luna C8(2) column (2.1 × 50 mm).

Dog plasma and PBMC extracts were prepared as described (1) and analyzed using LC/MS method #1. Intracellular levels were calculated by converting LC/MS concentrations using a conversion factor based on cell volume and sample dilution.

MT-2 cell cultures were incubated (37°C, 3 hrs.) in the presence of GS-7340 (10 μM). Cells were separated by centrifugation at 1000 rpm for 5 minutes, lysed with a 2:1 methanol/water solution and analyzed using LC/MS method #2.

RESULTS AND DISCUSSIONS

An LC/MS method was developed to allow analysis of plasma and PBMC extract samples obtained from dogs dosed orally with GS-7097 and GS-7171. The results demonstrated good bioavailability and high levels of intact prodrug in plasma. LC/MS analysis of PBMC extracts showed high levels of PMPA, but undetectable levels of prodrug, indicating that the prodrugs were rapidly converted inside cells into PMPA (Table 1 and Fig. 2). Low levels of a metabolite, GS-7161, were also detected. The results were consistent with results previously obtained using LC/fluorescence. Compared to the LC/fluorescence method, the first LC/MS method showed improved sensitivity for the prodrug with a simpler sample preparation to minimize metabolite degradation. Neither of the methods allowed detection of PMPApp due to its poor chromatographic retention and degradation during sample preparation/derivatization. Determination of PMPApp is desirable as it is the active species involved in reverse transcriptase inhibition.

An improved LC/MS method using DMHA ion-pairing reagent gave excellent chromatographic results for PMPA and GS-7340 (Fig. 3), and also allowed detection of PMPApp. The new LC/MS method had good sensitivity for all three

Table 1. Intracellular Concentrations of GS-7171 Prodrug and Metabolites by Non Ion-Pairing LC/MS in Dog PBMC Extracts, Following Oral Administration of GS-7171 at 16 mg/kg

	Concentration by LC/MS (μM)		
Time (hours)	PMPA	GS-7171	GS-7161
0	n/d	n/d	n/d
2	0.16	n/d	trace
8	0.14	n/d	n/d
24	0.052	n/d	nd

Note: n/d = none detected.

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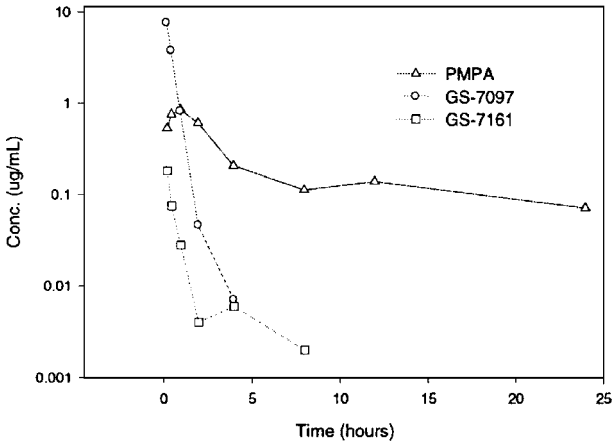

Figure 2. Plasma concentrations of GS-7097 prodrug and metabolites by non ion-pairing LC/MS in dog PBMC extracts, following oral administration of GS-7097 at 16 mg/kg.

analytes: PMPA, PMPApp and GS-7340 detection limits in water were 100, 20 and 2 nM, respectively. The application of the method was demonstrated in MT-2 cells incubated in the presence of 10 μM GS-7340 for 3 hours. MT-2 cell extracts analyzed using LC/MS method #2 showed high intracellular levels of both PMPA (120 μM) and PMPApp (340 μM), demonstrating that prodrug is efficiently converted into PMPA and PMPApp inside cells.

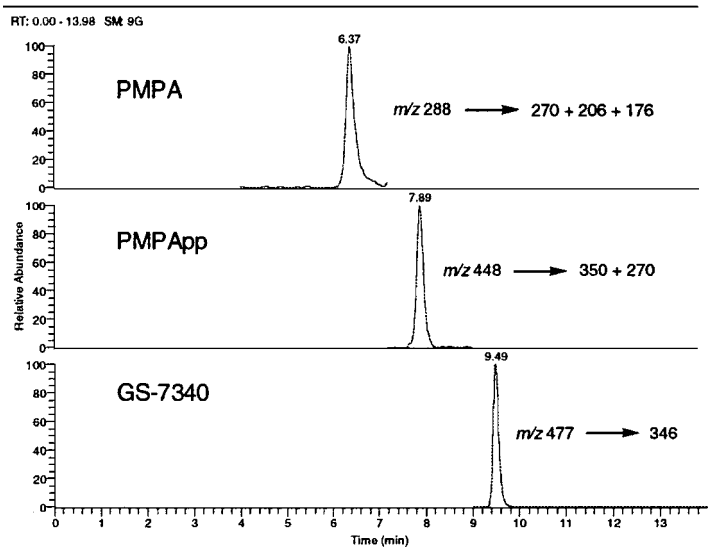

Figure 3. +ESI LC/MS/MS reconstructed ion chromatograms from a standard injection containing 1 μM PMPA, PMPApp and GS-7340 using ion-pairing chromatography.

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CONCLUSIONS

Two LC/MS assays were developed to determine the plasma and intracellular concentrations of two aryl phosphoramidate prodrugs and their metabolites. The first LC/MS method demonstrated the presence of high concentrations of PMPA in PBMC following oral administration of prodrug in dogs. An improved LC/MS method using an ion-pairing reagent allowed simultaneous detection of prodrug and the metabolites PMPA and PMPApp. High concentrations of PMPA and PMPApp were detected in MT-2 cells after incubation with prodrug using the new LC/MS assay. The results indicate the potential of these prodrugs as orally active anti-HIV agents.

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