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Short Communication

Determination of 9-[(2-phosphonylmethoxy)ethyl]adenine in rat urine by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method for the determination of 9-[(2-phosphonylmethoxy)ethyl]adenine (PMEA) in urine is described. The procedure includes treatment of the urine sample with chloroacetaldehyde to form the fluorescent 1,N^6-ethenoadenosine derivative, which was analyzed by reversed-phase HPLC with fluorometric detection. Validation of the method showed good sensitivity, precision and reproducibility. The method is useful for the study of urinary excretion of PMEA in the rat.

INTRODUCTION

9-[(2-Phosphonylmethoxy)ethyl]adenine (PMEA, Fig. 1A) is an acyclic phosphonate nucleotide analogue which exhibits broad-spectrum antiviral activity. *In vitro*, PMEA has shown activity against herpes simplex viruses [1] and is a potent inhibitor of retroviruses, which include the Moloney murine sarcoma virus [2] and the human immunodeficiency virus [3]. In animal models, PMEA has an antiretrovirus potency against murine AIDS similar to AZT [4] and, like acyclovir, was effective against herpes simplex virus type 1 infections.

The monosodium salt of PMEA has an aqueous solubility of >100 mg/ml at pH 6.5. The apparent pK_a values for the ionization of the first phosphonic acid group, the adenine moiety and the second phosphonic acid group are 1.7, 4.0 and 6.8, respectively. As a consequence of these chemical properties, the development

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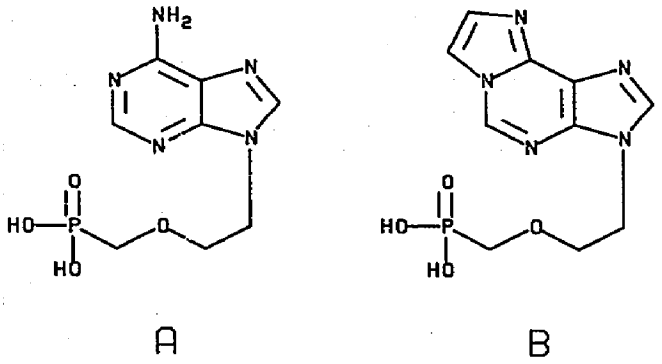

Fig. 1. Structures of PMEA (A) and the 1,N^6-etheno derivative of PMEA (B).

of an assay for PMEA in urine proved to be a challenging task. Our attempts to develop a sufficiently sensitive (1 μg/ml) and specific assay which employed direct injection, liquid–liquid or solid-phase extraction techniques coupled with conventional high-performance liquid chromatographic (HPLC) techniques such as reversed-phase, ion-pairing and ion-exchange chromatography with UV detection were unsuccessful.

Adenosine and related nucleotides AMP, ADP and ATP react with chloroacetaldehyde to form highly fluorescent 1,N^6-ethenoadenosine derivatives [5]. These 1,N^6-etheno derivatives of the adenine nucleotides have been separated by reversed-phase HPLC [6,7]. Therefore, we proceeded to develop a sensitive assay for PMEA in rat urine based on the fluorescent detection of the 1,N^6-etheno derivative of PMEA (Fig. 1B) [8]. The validity of this approach has been demonstrated by the determination of adenosine and c-AMP in urine by using solid-phase extraction combined with HPLC with fluorimetric detection [9].

EXPERIMENTAL

Chemicals and reagents

The monosodium salt of PMEA was obtained from Bristol-Meyers Squibb (Wallingford, CT, USA). Optima HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade water was purchased from J. T. Baker (Phillipsburg, NJ, USA). Tetrabutylammonium phosphate (PIC A) was obtained from Waters Assoc. (Milford, MA, USA). Chloroacetaldehyde, 50 wt.% solution in water, was purchased from Aldrich (Milwaukee, WI, USA). Sodium acetate and glacial acetic acid, AR grade, were obtained from Mallinckrodt (Paris, KY, USA). The derivatization reagent was a solution containing 17% (v/v) of 50% chloroacetaldehyde in 0.1 mol/l sodium acetate buffer (pH 4.5). The resulting solution was stored in an amber glass reagent bottle at 4°C and prepared fresh weekly.

Control rat urine was collected from native Sprague–Dawley rats (Harlan Labs., Indianapolis, IN, UAS) and frozen at −20°C until used.

Equipment

The HPLC system consisted of two Model 510 pumps, a Model 680 gradient controller and a Model 712 WISP autoinjector, all from Waters Assoc. The detector was a Model 650-10S fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) with the detector excitation and emission wavelengths set at 240 and 420 nm, respectively, and the bandwidth of each monochromator set at 10 nm.

The analog signal from the detector was digitized by a P-E Nelson Analytical Model 2700 laboratory data system (P-E Nelson System, Cupertino, CA, USA). The quantification of the detector response was based on the measurement of the peak height of the PMEA derivative.

A constant-temperature water bath, Model 12700 from Thermolyne (Dubuque, IA, USA), was used for derivatization of the urine samples.

Chromatography

A reversed-phase HPLC technique with gradient elution was used. The sample (10 μl) was injected onto a 250 mm × 4.6 mm I.D. PRP Polymer RP 10 μm column preceded by a New Guard PRP cartridge guard column, both from Brownlee Labs. (Santa Clara, CA, USA). Mobile phase A consisted of methanol–acetonitrile (2:1, v/v), and mobile phase B was 0.1% (v/v) triethylamine in 0.005 mol/l tetrabutylammonium phosphate (pH 9.5). The flow-rate was 1 ml/min, and the chromatography was performed at ambient temperature. After an initial hold of 2 min, the gradient was developed from 20% A and 80% B to 45% A and 55% B over 10 min using curve 8 on the gradient controller and held at the final conditions for 2 min. The initial solvent composition was reestablished over the next 3 min (curve 6), after which the column was reequilibrated for 5 min before the next sample injection.

Standard solutions

A primary standard solution of PMEA was prepared at a concentration of 10 mg/ml in HPLC-grade water, and dilutions of this standard were made to give secondary standard solutions of 1 mg/ml, 100 μg/ml and 10 μg/ml. These solutions were stored at 4°C and prepared monthly.

Procedure

A standard curve consisting of duplicates at seven concentration levels over the range 2–200 μg/ml was prepared daily with each set of samples. Standard curves were prepared by pipetting between 25 and 100 μl of the appropriate secondary standard solution into a 13 × 100 mm PTFE-lined screw-cap tube and adding sufficient control rat urine to obtain a final volume of 0.5 ml. Both samples and standards were prepared by adding 0.5 ml of the derivatization reagent to 0.5 ml of urine. The contents were vortex-mixed, the tube was capped, and the

samples were then heated for a minimum of 4 h at 50°C in a water bath. After derivatization, the samples were allowed to cool to room temperature and then filtered through a 0.45 μm syringe tip filter (Gelman Acrodisc, No. LCPVDF) before injection onto the HPLC column.

Validation

Intra-day precision and accuracy for this assay were determined by analyzing multiple identically spiked urine samples at two concentration levels against a duplicate standard curve. Inter-day precision and accuracy were determined by analyzing a separate set of identically spiked urine samples at two concentration levels on four separate days. Duplicate quality control (QC) samples at these same two concentration levels were analyzed daily with animal study samples.

The linearity of the assay was determined by analyzing a duplicate standard curve ranging from 2 to 200 μg/ml and evaluating the data using a lack-of-fit statistic [10].

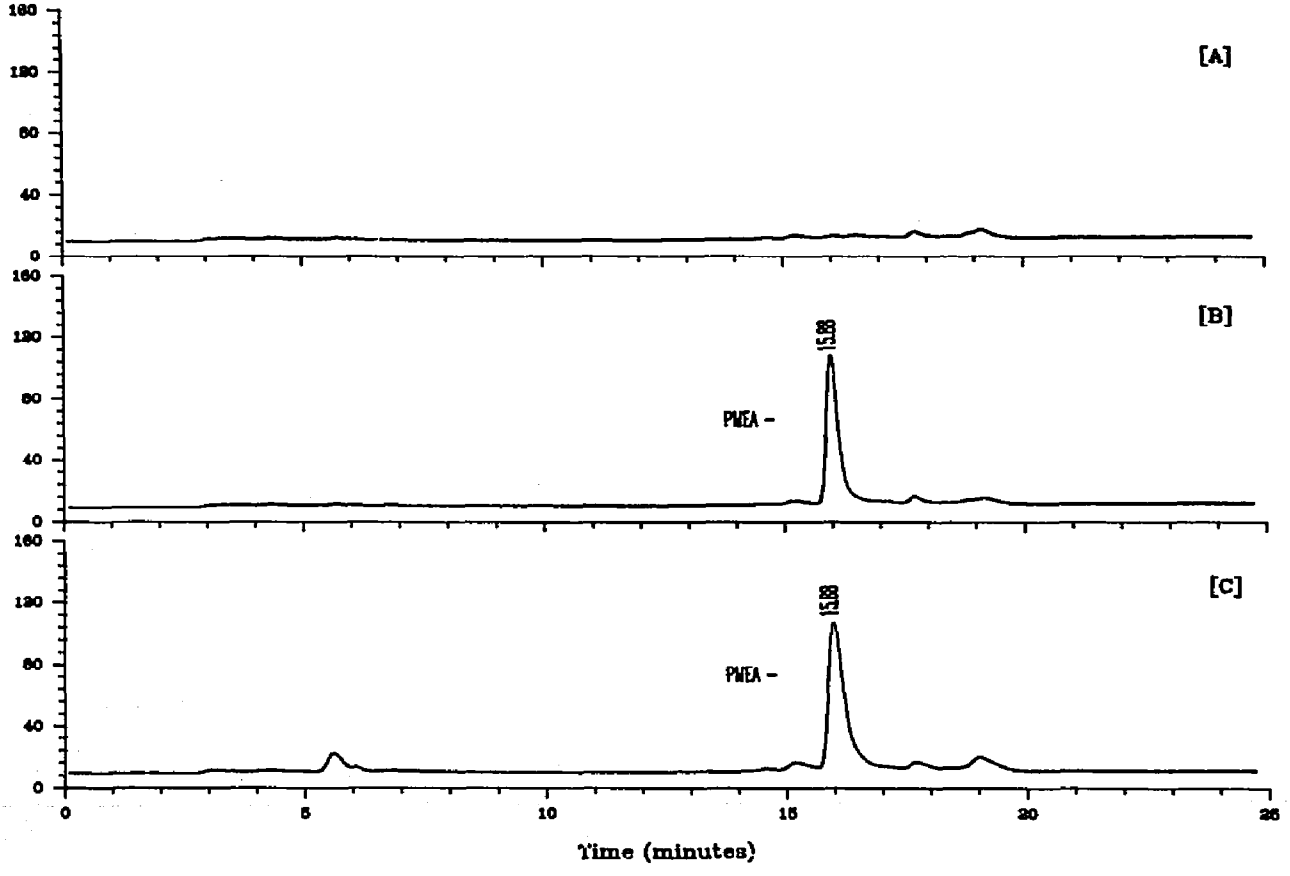

Fig. 2. Representative chromatograms of derivatized urine samples. (A) Control urine; (B) control urine spiked with PMEA (50 μg/ml); (C) a 0–24 h urine sample from a rat given an oral dose of PMEA at 30 mg/kg. The PMEA derivative has a retention time of 15.9 min.

Animal study

Six male Sprague–Dawley rats (Harlan Labs.) weighing 150–250 g were fasted 12–18 h before the study. On the day of the study, the rats were randomly selected and placed in two groups of three rats each. One group was given a single intravenous dose of PMEA at 30 mg/kg, and the other group was given a single oral dose at 30 mg/kg. The urine from each rat was collected in 0–24 and 24–48 h intervals at dry ice temperature. The samples were stored frozen until analyzed for PMEA at which time they were thawed, the volume measured, and an aliquot removed for analysis as described above. The oral bioavailability of PMEA was estimated from the ratio of the percentage of the dose excreted in urine after oral administration to that excreted after intravenous administration.

RESULTS AND DISCUSSION

Typical chromatograms of blank, spiked and rat study urine samples are shown in Fig. 2. No endogenous interferences were observed in the blanks. The retention time of the 1,N^6-etheno derivative of PMEA was 15.9 min.

Quantification was based on the peak height of the PMEA derivative. Over the range 2.0–200 μg/ml PMEA, the standard curve did not deviate significantly from

TABLE I

ACCURACY AND PRECISION OF THE METHOD

Test	*n*	Concentration (μg/ml)		C.V. (%)	d.f.
		Theoretical	Found		
Intra-assay	10	8.0	7.5	1.3	9
	10	140.0	133.8	1.4	9
Inter-assay	6	8.0	7.6	1.1	5
	6	8.0	7.6	2.7	5
	6	8.0	7.6	2.0	5
	6	8.0	7.4	1.4	5
Pooled	24	8.0	7.6	1.9	20
Combined	24	8.0	7.6	3.3	3
	6	140.0	147.9	1.6	5
	6	140.0	130.5	1.7	5
	6	140.0	130.4	0.9	5
	6	140.0	133.5	1.7	5
Pooled	24	140.0	136.6	5.6	20
Combined	24	140.0	135.6	15.0	3

linearity. Unknowns were determined from a linear least-squares fit of the peak height of the standards *versus* the corresponding theoretical concentration of PMEA, which was typically described by the equation: peak $\text{height}_{\text{PMEA}} = 1826 (\pm 8.5) \times (\text{concentration}, \mu\text{g/ml}) + 2388(\pm 880)$ with a correlation coefficient of 0.999.

The data for the accuracy and precision of the method are tabulated in Table I. The intra-assay accuracy was 93.2 ± 0.9 and 95.5 ± 1.0% at the 95% confidence level for concentrations of PMEA in the urine of 8 and 140 μg/ml, respectively. The coefficient of variation ranged from 1.3% (8 μg/ml) to 1.4% (140 μg/ml). The inter-assay accuracy was 94.3 ± 1.9 and 94.0 ± 1.7% (95% confidence level) at concentrations of 8 and 140 μg/ml, respectively. The inter-assay coefficient of variation (combined) was 3.3% (8 μg/ml) and 15% (140 μg/ml). These data show the method to be precise and reproducible from day to day. The method was successfully used to provide urinary excretion data for PMEA in the rat. The mean amount of PMEA excreted in the urine after a 30 mg/kg intravenous and oral dose was 3880 and 303 μg, respectively. The oral bioavailability based on the urine excretion data is 7.8%, which is comparable to that obtained after measuring plasma levels of PMEA [11].

An assay which is suitable for analysis of PMEA in rat urine has been developed. The method is simple, has a determination limit of 2 μg/ml and requires minimal sample preparation. The method may be applicable to the analysis of other acyclic phosphonate adenine nucleotide analogues in biological fluids from other species.

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