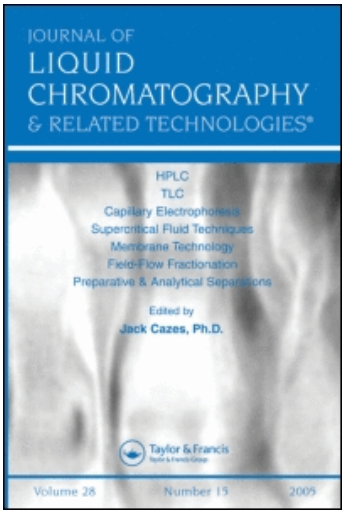

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REVERSE-PHASE HPLC ANALYSIS OF THE ANTIVIRAL DRUG, 9-[1,3-DIHYDROXY-2-PROPOXY METHYL]-GUANINE (DHPG), WITH AMPEROMETRIC DETECTION

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ABSTRACT

The electrochemical activity of purine derivatives forms the basis for a sensitive and selective LCEC assay for an antiviral analog of deoxyguanosine. This report describes a reverse-phase chromatographic method with amperometric detection and an electroactive internal standard (tyrosine) for analyzing 9-[1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG) in a parenteral dosage form. Using a glassy carbon electrode at +1.2 V vs. Ag/AgCl and a 25-cm 5 μm C18 column, a linear current-concentration dependence was obtained for DHPG between 25 ng/mL and 250 ng/mL, with a detection limit below 0.2 ng (S/N = 2). Statistical validation of the method showed good recovery

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efficiency and reproducibility. Parallel analyses of partially degraded DHPG solution samples by LCEC and by a cation-exchange chromatographic system with 254-nm detection demonstrated statistically equivalent estimations of the DHPG degradation rate constant. The general utility of the electrochemical detection technique was further demonstrated by the response linearity for another antiviral nucleoside, acyclovir. Our results demonstrate the versatility of reverse-phase HPLC with electrochemical detection for accurate, specific, and reliable analysis of purine-related therapeutic agents.

INTRODUCTION

The synthesis of therapeutic agents that combine specific activity against viral targets and reduced toxicity to the host, is a major goal of current drug research (1-5). The recent development of the acyclic deoxyguanosine analogues DHPG[1] and acyclovir[2], represents a major step toward this goal. DHPG is a broad-spectrum antiviral agent with activity against herpes simplex virus types 1 and 2, as well as cytomegalovirus and Epstein-Barr virus (6,7,8,9). The formulation development, stability, and clinical studies needed for registration of DHPG required a sensitive and selective technique for analyzing the drug in various formulations. Sensitive analysis of purine bases and nucleosides are routinely performed by reverse-phase, partition, and ion-exchange chromatography with UV detection (10-13). In most applications the ultraviolet extinction coefficients of these N-heterocycles ($\varepsilon \simeq 10{,}000\ M^{-1}cm^{-1}$) are sufficient for both *in vivo* and *in vitro* drug monitoring. However there are instances where the on-line detection and identification of therapeutic agents like DHPG, by a different type of detection system may prove useful. Analyses invoking alternative detection methods may be implemented, for example to conclusively establish peak purity, and/or the identity, and structural elements of drug degradation products and metabolites.

Coupling HPLC with electrochemical detectors (LCEC) is now a well-established method for analyzing oxidizable and reducible species. The success of LCEC results from the excellent sensitivity, linear dynamic range and response specificity obtained with amperometric detectors. The electrochemistry of purines has been extensively investigated in both aqueous and nonaqueous solutions.[15-17] Although only adenine is easily reducible at mercury electrodes, most other purine bases and nucleosides are oxidizable at carbon electrodes.[18,19]

The objectives of our investigation, were to: (1) develop a reverse-phase HPLC method with electrochemical detection for determining DHPG in a parenteral dosage form, (2) quantify the DHPG degradation rate constant at elevated temperature by the LCEC method, (3) compare the LCEC degradation rate, with the same constant determined by cation-exchange chromatography with UV detection (LCUV), and (4) ascertain the flexibility of the LCEC system by examining the response linearity for acyclovir.

MATERIALS AND METHODS

Reagents

Purine (Aldrich) and tyrosine (Sigma) were reagent grade and used as received. DHPG and acyclovir were provided by Syntex Institutes of Bio-organic Chemistry and Organic Chemistry. Tetraethylammonium perchlorate and sodium perchlorate (GFS Chemicals) were electroanalytical grade. Buffers were prepared from reagent grade or ammonium phosphate (Sigma). Mobile-phase solvents were HPLC grade (Burdick & Jackson or J. T. Baker).

Instrumentation

An IBM Instruments Model EC-225 Voltammetric analyzer was used for cyclic voltammetry experiments. A Houston Instruments Model 100 X-Y recorder was

used to plot current-potential data. Voltammetric experiments were conducted in a thermostated cell at 25°C. A glassy carbon working electrode with 19-mm^2 surface area was used; a platinum coil served as the counter electrode. Potentials were recorded versus an aqueous Ag/AgCl reference electrode. Sensitivity was 100 μA full scale. HPLC separations were performed on a Spectra Physics Model SP-8100 solvent delivery system equipped with a Valco 6-port injection valve. A BAS Model LC-4B potentiostat was used for amperometric detection, with a glassy carbon working electrode set at a potential of +1.2 V vs. the Ag/AgCl reference electrode. Sensitivity was 100 nA/V for the stability experiment and 10 nA/V for the detection limit experiment. The LCUV analyses were performed on the aforementioned chromatograph using a Spectra Physics Model SP-8300 detector at 254 nm. The detector signals were integrated with a Shimadzu Model CR-1A computing integrator. Peak areas were used for analytical determinations.

Mobile Phase

LCEC analysis employed a Whatman Partisil-5 ODS-3 25-cm x 4.2 mm i.d. column. The mobile phase was 97% 0.1 M sodium perchlorate in 0.01 M ammonium phosphate adjusted to pH 3 with phosphoric acid, and 3% methanol. At a 1.0 mL/min flow rate the backpressure was approximately 3000 psig. The DHPG retention time on the above system was 7.2 min. The LCUV method used a Whatman Partisil-10 25-cm x 4.2 mm i.d. strong cation-exchange column with 72% 0.01 M ammonium phosphate, and 28% methanol; the flow rate was 1.0 mL/min (1700 psig), and DHPG retention time was 8.7 min.

Analytical Procedures

Five milliliters of a 1.0 mg/mL DHPG solution and 5 mL of 1.0 mg/mL tyrosine solution (internal standard) were diluted to 100 mL with water (adjusted to pH 11 with NaOH), and 0.2 mL of this solution was further diluted to 10 mL with mobile phase. The injection volume was 50 μL; 50 ng each of tyrosine and DHPG was injected.

For cation-exchange HPLC, 6 mL of the DHPG solution was first diluted to 25 mL with the pH 11 adjusted water. A 9-mL aliquot of this solution plus 5 mL of a 0.9 mg/mL purine solution (internal standard) was diluted to 50 mL with mobile phase. The injection volume was 10 μL. The amount of DHPG injected was 432 nG, and 900 nG of purine was injected.

Accelerated Degradation Study

Fifty, 10-mL samples of 1.0 mg/mL DHPG solution were sealed under argon in glass vials and stored in the dark at 80°C. At timed intervals over a 2-mo period, vials were withdrawn and the DHPG remaining was measured by the LCEC and by the LCUV methods.

RESULTS AND DISCUSSION

Figure 1 shows a representative cyclic voltammogram obtained for DHPG at a glassy carbon electrode in pH 3, 0.1 M sodium perchlorate/ammonium phosphate 0.01 M. The electrochemical oxidation of DHPG was irreversible at all scan rates, and the peak potential observed at pH 3 (+1.2 V vs. Ag/AgCl) was selected as the operating potential in subsequent LCEC determinations.

The primary advantages of electrochemical detection are its high sensitivity and response selectivity. For DHPG analysis by LCEC, 150 pg was the conservative (see Figure 2) limit of detection under the established operating conditions. The LCEC detection limit is approximately 5 times lower than that for LC with spectrophotometric detection at 254 nm (800 pg).

This relatively modest decrease in detection limit for DHPG with electrochemical detection may be due to the high operating potential needed for diffusion-limited oxidation to occur. Thus, high background and noise levels encountered at +1.2 V prevented using high gain settings.

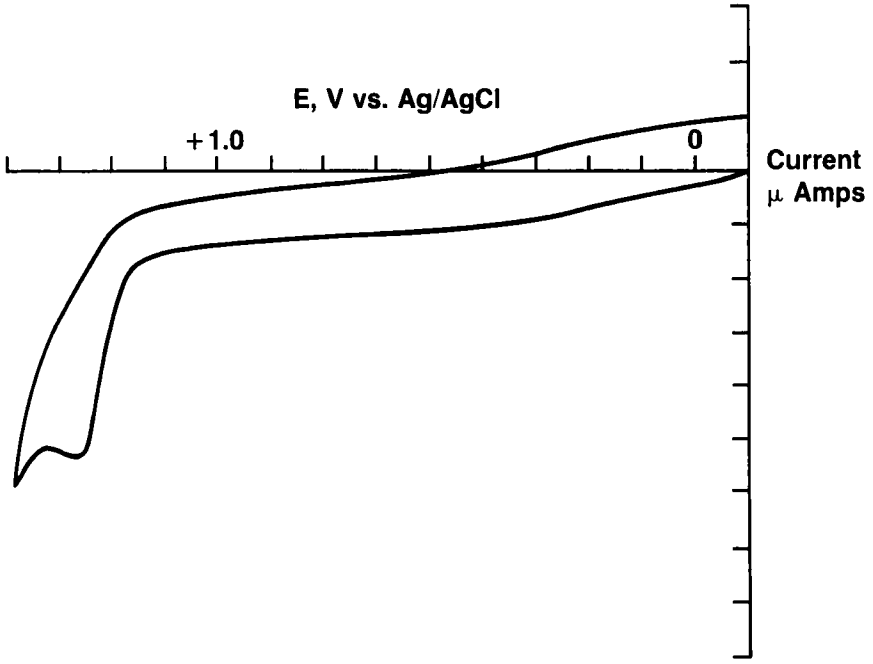

FIGURE 1. Cyclic Voltammogram of 1 x 10^{-3}M DHPG in 0.1M sodium perchlorate pH 3. (Glassy carbon working electrode, Ag/AgCl reference, Pt auxillary, scan rate = 100 mV/s).

Additionally, electrode responsiveness and reproducibility of currents were found to vary after continual use.

In order to compensate for variability in electrode response after extended use at high potential, an electroactive internal standard (tyrosine) was added. Polar phenolic compounds like tyrosine are easily separated from DHPG by reverse-phase chromatography. Under the defined chromatographic conditions employed for DHPG analysis, tyrosine eluted approximately 2 min after and completely resolved from DHPG (see Figure 3). At the operating potential (+1.2 V) in the LCEC system, tyrosine oxidation is diffusion controlled, and

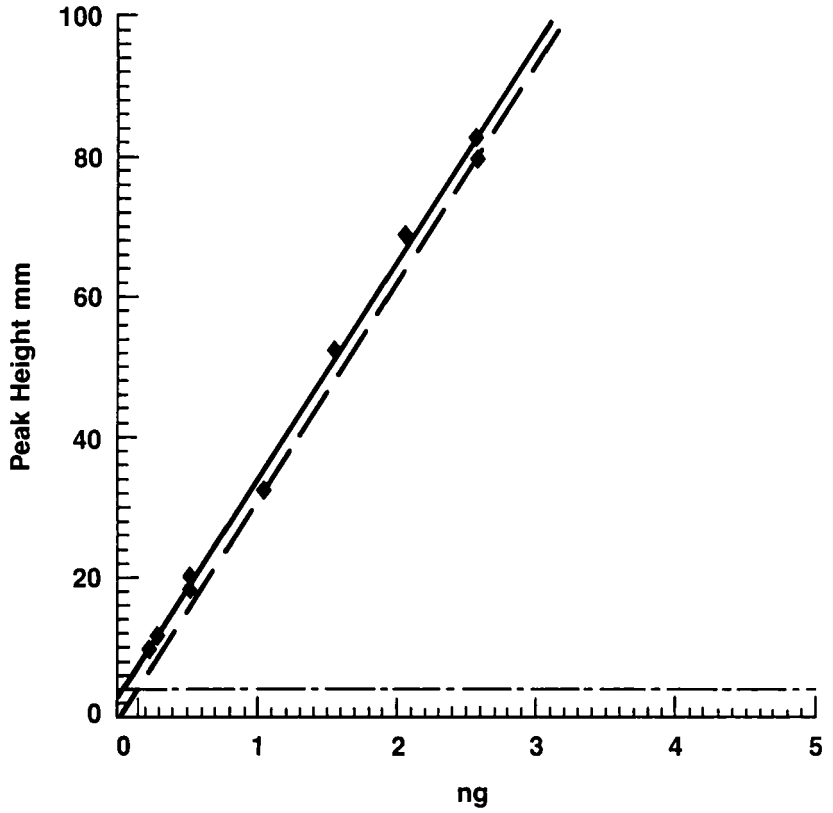

FIGURE 2. Lower limit of detection for DHPG as determined by LCEC analysis. — — — — — represents lower 95% confidence interval. —·—·—·—·— S/N = 2.

thus allows comparable concentrations of DHPG and tyrosine in the analyses (20).

To assess the LCEC method performance, we generated a DHPG calibration curve for the 0.7 to 1.2 µg/mL range. Least-squares linear regression analysis of the DHPG injected vs DHPG found data gave: slope = 1.02 ± 0.028, intercept = -1.36 ± 2.52, and correlation coefficient = 0.9986. The LCEC method recovery

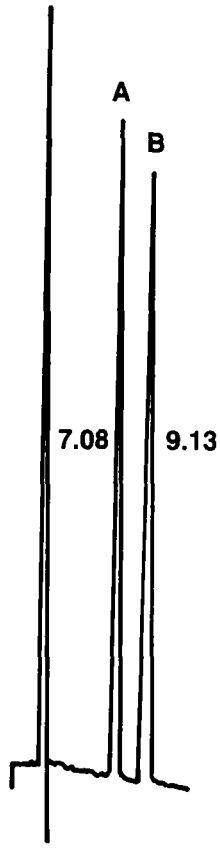

FIGURE 3. Chromatogram of reverse-phase separation and electrochemical detection of DHPG (A) and internal standard tyrosine (B). On column injections represent 50 ng of each compound. Retention times in minutes.

efficiency was determined by assaying duplicate samples of DHPG (n = 12) spiked at 0.7 to 1.2 μg/mL, and calculating expected concentrations using response factors from standards encompassing the same range. The average recovery of DHPG was 100 ± 2.54%. These data indicate that the amperometric detection of DHPG is both linear and accurate.

We investigated degradation product effects on the LCEC analysis of DHPG to establish the specificity of the method. In LCEC, the selectivity of the system can be controlled almost as well by the applied potential as by the chromatographic separation. In the case of DHPG analysis, however, some of the selectivity inherent in electrochemical detection was sacrificed by operating at a relatively high potential. Consequently, it was important to prove that the system resolves DHPG from its degradation products, and that the current response for DHPG could be attributed to only unchanged drug.

Therefore parallel analyses were performed on degraded DHPG samples by both the LCEC system described and a strong-cation-exchange separation method with

TABLE I

Degradation rate constants (kd) for DHPG at 80°C, pH 10.5 as determined by reverse-phase HPLC with electrochemical detection (LCEC) and by cation-exchange HPLC with spectrophotometric detection (LCUV).

Method	% DHPG Remaining [a] After t = Days					K_d, mo^{-1}	Correlation Coefficient
t =	0	2	13	21	35		
LCEC	100	94.5	86.5	76.0	70.5	0.314 ± 0.085	0.981
LCUV	100	93.0	86.5	76.5	68	0.327 ± 0.033	0.995

a. Values indicated are mean of duplicate determinations, and are precise to within ± 2%.

b. Determined by linear least squares regression according to the equation: $\ln\{(DHPG)_t/(DHPG)_0\} = -k_d t$. Error limits are ± 95% confidence intervals.

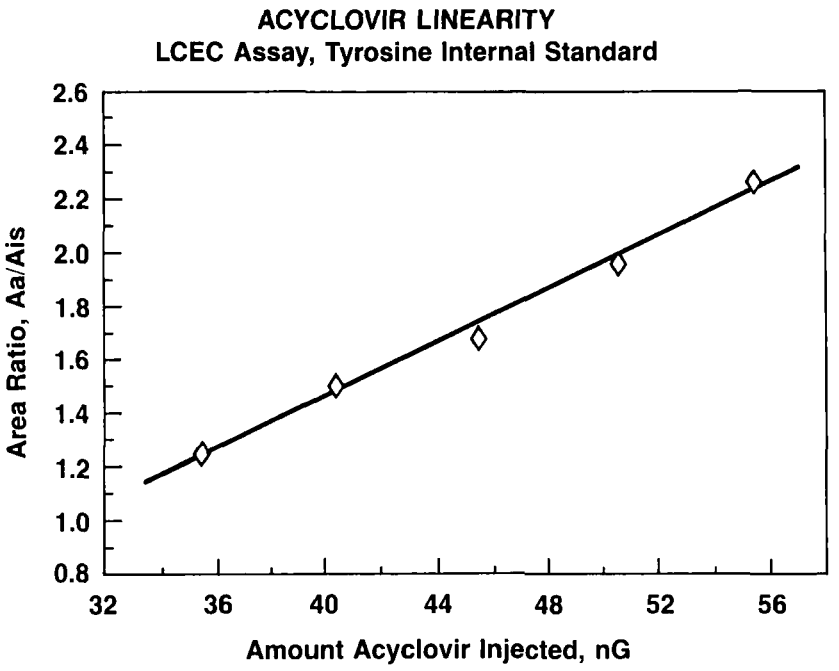

FIGURE 4. Calibration curve for the acyclic antiviral nucleoside Acyclovir, as determined by LCEC. Response is plotted as a unitless ratio of peak currents (area integrations) for DHPG/Tyrosine.

spectrophotometric detection. By employing a second analytical system with a distinctively different mode of separation and detection, the stability specificity of the LCEC methodology for DHPG could be established.

Table I presents a comparison of the average percent recovery for DHPG from 1.0 mg/mL solutions stored at 80°C over a 2-mo period. The data indicate that there are no statistical differences in the amounts of DHPG remaining as determined by either analytical system. Furthermore the corresponding pseudo-first-order rate constants calculated from these data (Table I) were not statistically different.

The general utility of the LCEC system developed for DHPG separation and analysis, was further demonstrated for a related drug, acyclovir. Again, using tyrosine as internal standard and an applied potential of +1.2 V, a linear current-concentration profile for acyclovir 9-(2-hydroxyethoxymethyl)-guanine was obtained (see Figure 4).

It is thus evident that the electrochemical activity of purines can be useful in the analyses of these compounds in various dosage forms, and may find further application in biological media.

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