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Note

High-performance liquid chromatographic determination of acyclovir in serum

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Topical or systemic administration of acyclovir, a guanine derivative, is reported to exhibit strong antiviral activity toward viruses of the Herpes group [1, 2]. Schaeffer et al. [1] claim that there is essentially no cleavage of acyclovir in vivo and that urinary excretion of the unmetabolized compound is almost quantitative (ca. 99%). Present methods available for quantifying blood or urine acyclovir levels include radioimmunoassay [3], ion-exchange [1, 4] and reversed-phase high-performance liquid chromatography (HPLC) [5]. However, each of these analytical methods are limited by one or more factors such as long analysis time, lack of sensitivity, reduced column life, and lengthy sample preparation. This paper describes a simple, rapid, and highly sensitive reversed-phase HPLC method for the determination of acyclovir in human serum. In this work, sample preparation is simplified using a trifluoroacetic acid (TFA) protein precipitation technique, and enhanced column stability is achieved through the use of a polystyrene divinylbenzene reversed stationary phase. Mobile phase variables such as pH, methanol-to-water ratio, ionic strength, and alkyl sulfonate concentration were investigated and optimized. Using UV absorbance detection at 254 nm, the described method has a minimum detection limit of 12 ng with recoveries at $\geqslant$ 90%.

EXPERIMENTAL

Reagents

Acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine, was provided by the Wellcome Foundation (London, U.K.) and used as received. Methanol was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.) and distilled water was purified via Milli-Q treatment prior to use (Continental Water Systems, El Paso, TX,

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U.S.A.). Freeze-dried QCS normal control serum (Ortho Diagnostic Systems, Raritan, NJ, U.S.A.) was reconstituted with distilled water and used immediately after a standing time of 30 min. TFA and sodium heptanesulfonate (SHS) were purchased from Eastman-Kodak (Rochester, NY, U.S.A.) and all other reagents were analytical-reagent grade and used without further purification.

Standards

Accurately weighed quantities of acyclovir were dissolved and diluted with distilled water in volumetric flasks to obtain a working concentration range of 0.5—12.0 μg/ml.

Chromatography

The HPLC instrumentation used to assay the acyclovir standards and serum samples included a Constametric Model III dual-piston displacement pump (LDC/Milton Roy, Riviera Beach, FL, U.S.A.), a WISP 710 B automated injector (Waters Assoc., Milford, MA, U.S.A.), a Waters Assoc. Model 440 fixed-wavelength UV absorbance detector set at 254 nm and a Spectra-Physics SP4100 computing-integrator (Santa Clara, CA, U.S.A.). Standards and samples (100-μl injection) were chromatographed at ambient temperatures on a 150 mm × 4.2 mm I.D. reversed-phase PRP-1 column (Hamilton, Reno, NV, U.S.A.) with an eluting mobile phase consisting of methanol—0.1 *M* hydrochloric acid—0.02 *M* SHS—0.25 *M* sodium chloride (10:10:20:60). The PRP-1 column, which is a spherical, uniform 10-μm polystyrene divinylbenzene copolymeric adsorbent, was preferred over alkyl-modified silica owing to its high chemical stability with regard to pH (range 1—13) and compatibility with relatively high inorganic salt concentrations (up to 0.5 *M*). This advantage was noticed in initial studies using a Zorbax ODS column in which rapid deterioration (ca. 100 injections) of column performance was observed and, consequently, obviated further investigation with silica-based stationary phases.

Eluents were aspirated prior to use and the pH was measured after the mixing of all mobile phase additives. At a flow-rate of 1.5 ml/min (90 bar) column equilibration with the mobile phase was achieved in approximately 30 min. It is recommended that the column and instrumentation contacting the mobile phase be flushed at the end of each working day with methanol—water (50:50) to prevent possible corrosion due to the high concentration of Cl^- in the mobile phase. The capacity factor, k', was calculated according to $k' = (t_R - t_0)/t_0$ where t_R is the elution time of the retained analyte (acyclovir) and t_0 is the elution time of an unretained analyte (sodium nitrate) at the given eluting conditions. Quantification of serum samples was carried out via external calibration using peak area measurements.

Sample preparation

Accurately weighed quantities of acyclovir were added to blank human serum such that the final concentration was within the working calibration range of the acyclovir standards (i.e. 0.5—12.0 μg/ml). Deproteinization of the serum samples was done by mixing 250 μl of serum with 200 μl of mobile phase solvent and 50 μl of TFA in microcentrifuge tubes and centrifuging for

5 min at 2000 *g*. A 200-μl aliquot of the acidic supernatant was neutralized with an equal fraction of 0.1 *M* sodium hydroxide and subsequently assayed by HPLC. The same extraction procedure was performed on the acyclovir standards prior to generating the calibration curve.

RESULTS AND DISCUSSION

Mobile phase optimization

Several mobile phase variables were investigated in this study. These include pH, SHS concentration, methanol-to-water ratio and ionic strength. The effect of the eluent pH and the concentration of SHS on the retention of acyclovir is shown in Fig. 1. The pH was adjusted using hydrochloric acid while ionic strength was maintained at 0.05 through appropriate addition of sodium chloride. In the presence of SHS, appreciable retention begins to occur once the eluent pH falls below 3.2. This is consistent with the pK_a value for guanine (pK_a = 3.18) of which acyclovir is an analogue. Hence, the ion interaction between the SHS and the acyclovir analyte increases as the equilibrium concentration of positively charged acyclovir increases, i.e., with decreasing pH, the net effect being an increase in the retention factor. An HPLC method [5] previously reported for assaying acyclovir in biofluids employs the same mobile phase counterion, but at an eluent pH of 6.5. Based on data obtained in this study, the use of SHS in the mobile phase at a pH above 3.2 would afford neglible ion interaction with acyclovir, and, subsequently, very little advantage. A sufficiently low eluent pH (i.e. < 3.2) is required whereby the retention

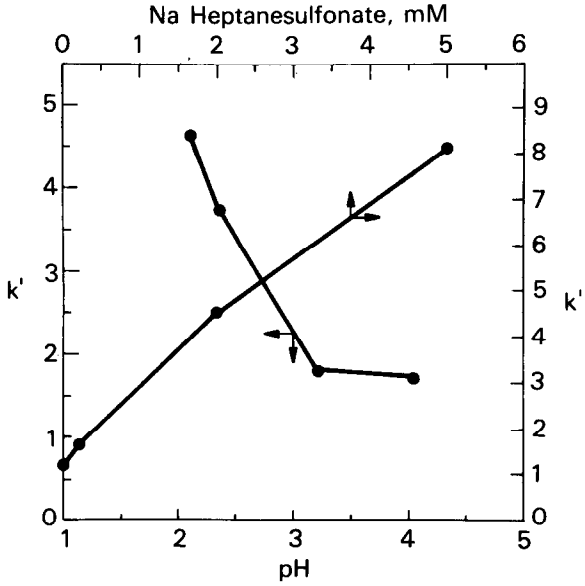

Fig. 1. Retention of acyclovir on PRP-1 as a function of pH and sodium heptane sulfonate (SHS) concentration. Hydrochloric acid was adjusted for the variable-pH study; mobile phase: methanol—water, 0.002 *M* SHS (15:85); ionic strength: 0.05 through addition of sodium chloride; flow-rate: 2.0 ml/min; conditions were the same as above for the variable-SHS study except that 0.01 *M* hydrochloric acid (pH 2.1) was used.

factor of acyclovir increases as the mobile phase concentration of SHS increases (see Fig. 1). Although not shown here, an increase in the percentage methanol and/or ionic strength in the mobile phase decreases the retention of acyclovir, both consistent with that expected from solvophobic [6] and competitive electrolyte effects [7], respectively. The above eluent parameters were optimized based upon speed of analysis, sensitivity, and selectivity from ubiquitous serum interferences, such as guanine and other related purines. These criteria were satisfied with a mobile phase consisting of methanol—0.1 *M* hydrochloric acid—0.02 *M* SHS—0.25 *M* sodium chloride (10:10:20:60) yielding a pH of 2.1 and a total ionic strength of 0.2.

Determination of acyclovir in serum

Recovery experiments were performed on serum samples; results are listed in Table I. The standard linear regression curve used to determine the recovery values is given by the following equation:

$$[\text{Acyclovir}] = 1.96\ (\pm\ 0.06) \cdot 10^{-5}\ \text{peak area} + 7.61\ (\pm\ 22.1) \cdot 10^{-2}$$
$$(n = 8, r = 0.9995, s = 0.155)$$

where n is the number of replicate standards, r is the correlation coefficient and s is the standard deviation (S.D.). The values in parentheses are the 95% confidence intervals. Since the serum protein precipitation procedure dilutes the samples and standards by a factor of four, the effective concentrations of injected samples were 0.12, 0.30, 1.20, and 2.4 μg/ml. While recoveries over this range typically exceed 90%, relative standard deviation is large (ca. 20—30%) at serum concentrations below 1.2 μg/ml (0.30 μg/ml effective). This appears to be associated with the limitations in the PRP-1 column performance at the given eluting conditions. The column efficiencies for acyclovir are rather low (approximately 2000 plates per m) compared to those obtained on PRP-1 (approximately 5000 plates per m) for other purine-related compounds [7]. It should be noted that alkyl-modified silica phases do not offer significant improvement in column efficiency for acyclovir [5]. Above 1.2 μg/ml, S.D. reduces below 10%, which is an acceptable level for most clinical situations.

Chromatograms for a blank serum and serum containing 3.0 μg/ml acyclovir

TABLE I

RECOVERY OF ACYCLOVIR FROM HUMAN SERUM FOLLOWING PROTEIN PRECIPITATION WITH TFA

$n = 4$.

Acyclovir added (μg/ml)	Acyclovir found (mean ± S.D.) (μg/ml)	Percentage recovery (mean ± S.D.)
0.48	0.43 ± 0.15	89.6 ± 31.6
1.20	1.09 ± 0.23	90.8 ± 17.6
4.80	4.80 ± 0.10	100.0 ± 2.2
9.60	8.86 ± 0.92	92.3 ± 9.6
Mean ± S.D.		93.2 ± 15.2

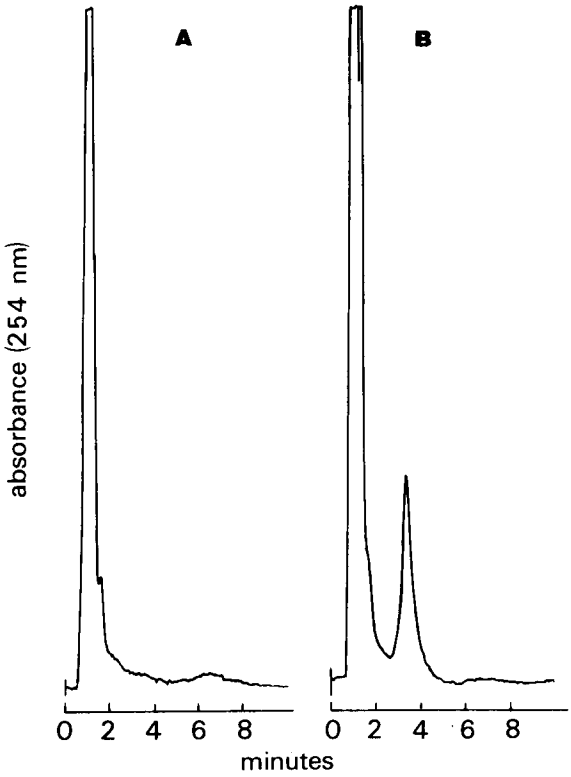

Fig. 2. Typical chromatograms of human QCS normal control serum. (A) Blank; (B) serum containing 3.0 μg/ml acyclovir. Sample volume: 100 μl; column: PRP-1 150 X 4.2 mm I.D.; mobile phase: methanol—0.1 *M* hydrochloric acid—0.02 *M* SHS—0.25 *M* sodium chloride (pH 2.1, ionic strength 0.2) (10:10:20:60), flow-rate: 1.5 ml/min.

(0.75 μg/ml effective) are illustrated in Fig. 2A and B, respectively. The blank is shown to be free of endogenous interferences that could prove to be detrimental to the analysis. Late-eluting peaks were not observed, and column stability was excellent, thus, allowing for high sample through-put. The protein precipitation procedure is rapid and requires only 250 μl of serum, and with proper handling, the serum volume can be redued further if higher detection limits are tolerable.

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