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Note

Determination of acyclovir in plasma by column liquid chromatography with fluorescence detection

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Acyclovir, 9-(2-hydroxyethoxymethyl)guanine (I, Fig. 1), is an acyclic analogue of guanosine that exhibits strong antiviral activity against viruses of the herpes group [1]. For the determination of acyclovir in biological samples a radioimmunoassay method [2] and high-performance liquid chromatographic (HPLC) techniques using reversed-phase [3] or styrene–divinylbenzene [4] column packings have been described. For detection UV absorption has been used (detection limit ca. 10 ng), but when complex biological fluids were chromatographed the unequivocal identification of acyclovir was difficult.

Börresen [5–7], Udenfriend and co-workers [8,9] and others [10–15] found that purines do not exhibit fluorescence in the non-ionized state. This is ascribed to efficient intersystem crossing from an $n\pi^*$ excited state, which lies below the lowest $\pi\pi^*$ singlet state, to the triplet state. Protonation of the molecule increases the energy of the lowest $n\pi^*$ state, leaving the $\pi\pi^*$ state as the lowest singlet level. Fluorescence then occurs on return of a molecule from the $\pi\pi^*$ singlet state to the ground state. Based on this observation, Assenza and Brown [16] developed a method for determination of purines by reversed-phase liquid chromatography with a post-column reactor for the protonation or deprotonation of purine molecules. The low pH needed for the fluorescence detection of purines precluded the use of an acidic mobile phase for chromatography because of the instability of the silicon–carbon bonds of the packing.

Borák and Smrž [17] and Jandera et al. [18], for the chromatographic separation of nucleosides and bases, used as a column packing Spheron, which is a semi-rigid hydroxyethyl methacrylate gel. This polymeric material is hydrolytically very stable in acidic media. Using the new microparticulate form (Spheron Micro) a sufficiently high separation efficiency can be achieved.

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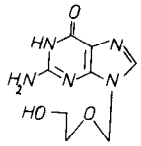

I

Fig. 1. Structure of acyclovir.

In this paper, a method for the determination of acyclovir in plasma is described, based on the separation of acyclovir on Spheron Micro 300 at pH 1.8 with fluorescence detection.

EXPERIMENTAL

Chemicals

Acyclovir (99.5% pure by HPLC) was obtained from Lachema (Brno, Czechoslovakia). All other chemicals were of analytical-reagent grade.

Equipment

Chromatographic separations were performed on a Spectra-Physics Model SP 8100 liquid chromatograph with a Model SP 8110 autosampler (Spectra-Physics, Darmstadt, F.R.G.), fitted with a 10-μl loop, and a Schoeffel Instruments Model FS 970 fluorescence detector (Kratos, Trappenkamp, F.R.G.) with a 5-μl flow cell. The excitation wavelength was set at 285 nm (plus 7-54 filter) and fluorescence was measured by using a 370-nm cut-off emission filter. An analytical cartridge glass column (15×0.32 cm I.D.) was packed with Spheron Micro 300, particle diameter 12.5 μm (Lachema). For its protection a guard column (2×0.46 cm I.D.) packed with Spheron Micro 300, particle diameter 16 μm, was used. Chromatographic data were evaluated by using a Spectra-Physics 4000 data system. In some analyses, peak heights were also measured in addition to peak areas. The fluorescence spectrum of acyclovir was obtained on an Aminco-Bowman spectrofluorimeter.

Mobile phase

Mixtures of phosphate buffer of various pH, sodium sulphate of various concentrations and organic solvents were used. The final mobile phase was a solution of 0.1 *M* phosphoric acid and 0.1 *M* sodium sulphate (pH 1.8). The flow-rate of the mobile phase was 1 ml/min. All chromatographic separations were carried out at 45 °C. Before entering the detector the effluent was cooled to 2 °C.

Samples

Heparinized blood from a dog treated with acyclovir was centrifuged for 15 min at 16 000 *g*. Samples that could not be chromatographed immediately after collection were stored at −12 °C (there were no changes in acyclovir content up to

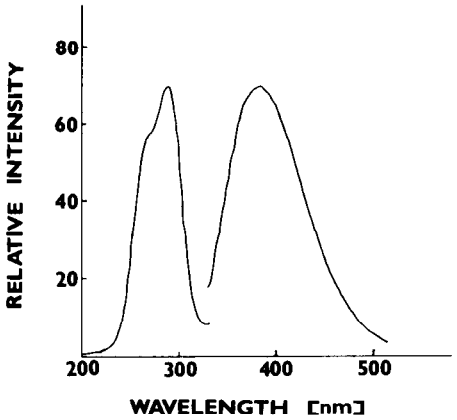

Fig. 2. Fluorescence excitation and emission spectra of acyclovir recorded at pH 2.0.

seven days). Standard samples containing 20 ng of acyclovir were prepared by mixing 0.9 ml of blank plasma with 0.1 ml of an aqueous solution of acyclovir.

The detection limit (LOD) was calculated according to Foley and Dorsey [19] from the equation

$$\mathrm{LOD} = 3s_B/S$$

where s_B is the standard deviation of the noise (baseline fluctuations; s_B can be estimated from one fifth of the peak-to-peak noise) and S is the analytical sensitivity (slope of the calibration curve – signal output per unit amount of acyclovir). Moreover, the standardized chromatographic detection limit was calculated for chromatographic reference state $k_{ref} = 4$, $N_{ref} = 10\,000$ and $V_{M,\,ref} = 1.5$ ml [19].

RESULTS AND DISCUSSION

Fluorescence characteristics

The origin of the fluorescence was assumed to be the same for acyclovir as for other purines. Fig. 2 shows the fluorescence spectrum of acyclovir at pH 2.0. The excitation maximum is at 285 nm and the emission maximum at 380 nm. In alkaline solution the intensity of fluorescence was much lower than in acidic media, where acyclovir was in the protonated form.

Effect of various parameters on fluorescence response

Influence of pH. In agreement with Börresen's results, the dependence has the form of a titration curve. In Fig. 3 the relative fluorescence is plotted versus pH. As the final mobile phase for the chromatographic separation a solution of 0.1 *M* phosphoric acid and 0.1 *M* sodium sulphate (pH 1.8) was used. Although the fluorescence response at this pH is not the maximum, it is sufficient for monitoring the level of acyclovir in plasma.

Influence of temperature. The intensity of fluorescence usually decreases with increasing temperature owing to a greater probability of the occurrence of other mechanisms of the deactivation of excited molecules. The temperature coeffi-

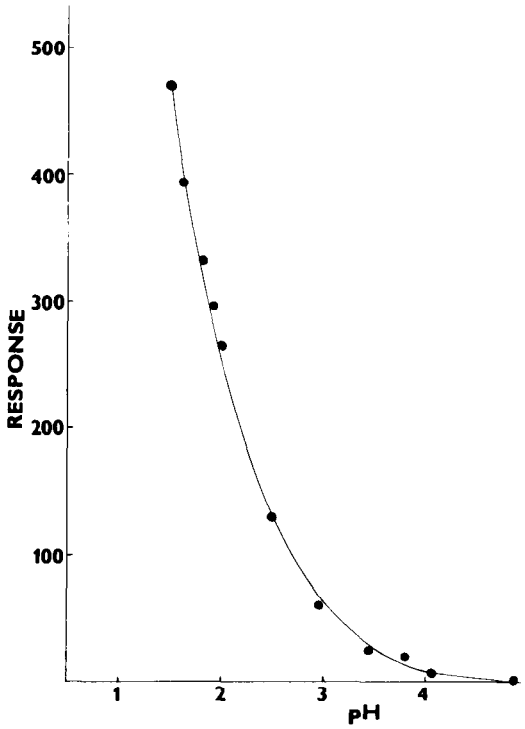

Fig. 3. Relative fluorescence intensity of acyclovir as a function of pH of mobile phase. For other conditions see Experimental.

cients are usually in the range 1.0–1.2 relative fluorescence units per 1 °C. Fig. 4 shows the dependence of fluorescence intensity on the temperature of the eluent entering the detector cell. The dependence is linear. To obtain reproducible results the effluent was cooled before entering the detector to a constant 2 °C.

Influence of organic solvents, oxygen and sodium sulphate. In some instances, the fluorescence can be enhanced by the presence of an organic modifier or by

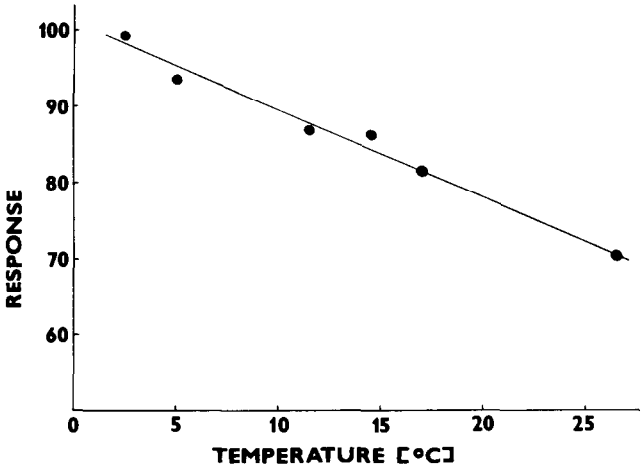

Fig. 4. Dependence of relative fluorescence intensity on temperature of eluent entering the detector. For other conditions see Experimental.

the absence of oxygen in the mobile phase. The influence of these factors was tested by addition of methanol, acetonitrile and dimethylformamide to the mobile phase. Organic solvents, the presence of oxygen and sodium sulphate (up to 1.0 *M*) did not change the fluorescence response significantly.

Separation

Spheron Micro 300 was found to be an advantageous column packing for the separation of acyclovir from other components of plasma samples. As Spheron hydroxyethyl methacrylate gels are very stable in acidic media, the packing can be utilized for separations under optimum conditions for fluorescence detection. In 0.1 *M* phosphoric acid the resolution (R_s) of acyclovir from guanine was 1.5, whereas other fluorescent compounds were eluted after acyclovir (see Fig. 5). The column efficiency for acyclovir was 6000 theoretical plates per metre.

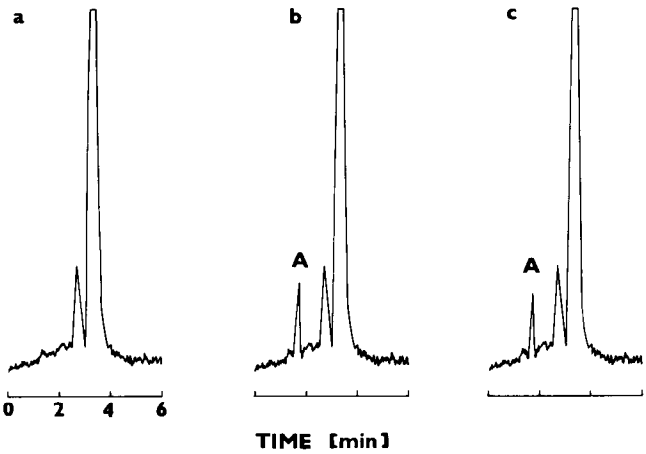

Fig. 5. Chromatograms of plasma of a dog treated with acyclovir (A). (a) Blank plasma; (b) plasma spiked with acyclovir (concentration 2.0 μg/ml); (c) plasma sample collected 4 h after administration of 5 mg/kg acyclovir (concentration found, 1.9 μg/ml).

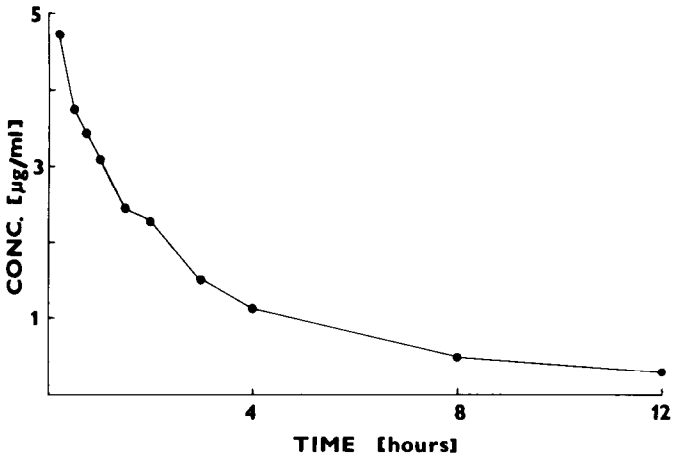

Fig. 6. Time dependence of acyclovir level in plasma of a dog after a single intravenous dose of 5 mg/kg acyclovir.

Application of the method

The method was used for monitoring the concentration of acyclovir in plasma. The chromatogram of a plasma sample collected 4 h after the application of 5 mg/kg acyclovir is shown in Fig. 5. In Fig. 6 the time dependence of acyclovir level in a dog after a single intravenous dose of acyclovir is shown.

A linear relationship between peak height and amount of acyclovir was observed up to 2000 ng. Coefficients of variation of less than 2.5% were found for amounts of acyclovir, ranging from 20 ng to the upper limit of linearity. Using this fluorescence method, the limit of detection in plasma was 1.0 ng. The standardized absolute limit of detection was 0.5 ng.

CONCLUSIONS

The proposed method for the determination of acyclovir in biological fluids has several advantages over other methods: (i) fluorescence detection is more selective and sensitive than UV detection; (ii) the Spheron Micro column packing has a much higher chemical stability in acidic media than silica-based reversed-phase column packings, so a sufficiently acidic mobile phase can be used and no post-column acidification of the effluent is needed; (iii) Spheron gels are only mildly hydrophobic so that no protein denaturation on their surface occurs, so the column life is increased when working with plasma samples.

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