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Development of an Assay for Acyclovir in Pharmaceutical Preparation and Human Plasma by HPLC

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A reversed phase HPLC method was developed to separate acyclovir (antiviral agent) from its major related impurities, guanine and its major metabolite 9-carboxy methoxy methyl guanine (CMMG) base. These agents may participate in a number of interactions including formation of neutral ion-pair and complexation with stationary phase silanols. In optimizing the separation of acyclovir from its degradation product and plasma, stepwise optimization was carried out. The effect of concentration of acetonitrile (ACN), methanol as the organic modifiers, on the retention of acyclovir were examined. The effect of the mobile phase and pH on the retention and peak shape for acyclovir and guanine was also examined and its influence on the capacity factor at constant concentration. The results show the best improvements in peak shape at pH 2.5. The method developed is selective, precise, reproducible and accurate and meets the needs of pharmacokinetics studies.

Key Words: HPLC, Ion-pair, Acyclovir, Guanine.

INTRODUCTION

Acyclovir [9-(2-hydroxyethoxymethyl) guanine is a modified nucleoside which shows strong activity against viruses of the Herpes group[1]. It derives from guanine, which is a constituent of purine. Recently, several methods have been reported for the separation of the nucleoside including high performance liquid chromatography (HPLC) by using ion exchange packed chromatography column materials[2]. However, these methods are generally involved and give poor chromatographic peak shapes.

For clinical samples, following administration of acyclovir when applied to man it was found that the earlier reported HPLC methods for clinical sample analysis lacked selectivity and sensitivity[3]. The problems of sensitivity are compounded by the possibility of the nitrogen-containing basic drugs and quaternary ammonium compounds causing problems in RP-HPLC, where peak tailing and extended or even total, retention is often observed[4]. The peak tailing

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effects are most probably due to the presence of residual (non-reacted) silanol groups on the silica surface, the drug being present in the ionized form.

In order to improve the chromatographic properties of basic solution, a number of alternative methods have been suggested. Among these methods ion pair chromatography is widely used, especially in the bio-analytical area where there is likely to be a range of polarities present in the biological matrix of the drug and its metabolites. Although it is possible to increase the retention simply by decreasing the content of organic solvent in the eluent, the effect on the presence of non-ionic compounds, then an increase in retention to an unacceptable degree. In such cases the retention can be controlled selectively by the addition of an anionic counter ion[5–13].

In this work a reversed phase ion pair HPLC method was developed to separate acyclovir from its major degradation product, guanine and metabolite 9-carboxy-methoxy methyl guanine (CMMG) in pharmaceutical preparation and human plasma.

Experimental

The chromatographic system consisted of a Shimadzu LC 5A pump (Touzart, Matignon, France) connected to a Rheoyden 7520 syringe loading sample injector valve fitted with a 20 μL sample loop, a variable wavelength UV detector (UVSPD 10 A) and a Shimadzu chart recorder. The column was 150 × 4.6 mm i.d. packed with hypersil ODS C18, 3 μm particle size (Touzart, Matignon, France) Acyclovir and Guanine were obtained from Sigma-Aldrich Chemical Company, 9-carboxymethoxy methyl guanine (CMMG) was obtained from Welcome Company, acyclovir (zovirax) tablets were obtained from Welcome Laboratories.

Optimization of mobile phase: Optimization of mobile phase was required to achieve resolution between acyclovir, guanine and 9-carboxymethoxy methyl-guanine, whilst maintaining good peak symmetry and reasonable analysis time. This was accomplished by investigating the effect of counter ion type and concentration, percentage organic modifier, mobile phase pH on capacity factor (K′).

Tablet formulation assay: 20 intact tablets (zovirax) were weighed accurately to obtain the average tablet weight, the tablets were then crushed and triturated in a mortar until a fine powder was obtained. An amount of the powder equivalent to one tablet was weighed accurately and taken in a 100 mL volumetric flask. The powder was dissolved in 25 mL acetonitrile and the solution was diluted to volume with mobile phase: acetonitrile-sodiumoctyl sulphate (2 mM, pH 2.5); KH_2PO_4 (20 mM) (5 : 95 v/v).

The contents of the volumetric flask were shaken vigorously for 3 min before being sonicated for a further 3 min. The mixture was then centrifuged for 5 min and the supernatant filtered under vacuum through a 0.45 millipore filter using an all-glass apparatus. Dilution (1 : 10) from this solution was made and the clear solutions were then ready for injection (10 μL was injected).

Preparation of calibration curves

Standard preparations: A stock solution of acyclovir (200 μg/mL) was prepared by dissolving the powder in the mobile phase. From this, linearity over the range of 1–100 μg/mL of acyclovir was examined.

Plasma samples: Five standard acyclovir solutions were prepared containing 80, 60, 40, 20 and 2 μg/mL. From each solution 250 μL was pipetted and added to 200 μL of plasma. Then the plasma proteins were precipitated with 50 μL of $HClO_4$ (35%) added to each solution to obtain the following final plasma concentration: 40, 30, 20, 10 and 1 μg/mL. The mixture of each solution was shaken on a vortex apparatus and centrifuged at 1500 g for 15 min. The upper layer (20 μL) was injected into the HPLC system.

RESULTS AND DISCUSSION

In setting up the conditions for development of the assay method, the choice of a detection wavelength was based on the scanned absorption spectrum for acyclovir. The spectrum was scanned over the range of 200–400 nm and was obtained by measuring the absorption of 10 μg/mL solution of acyclovir in mobile phase, prepared from a stock solution. The spectrum was obtained by using a 1 cm silica cell and the reference cell contained mobile phase; as a result a wavelength of 253 nm was chosen.

In optimizing the separation of acyclovir from guanine and 9-carboxymethoxy methyl guanine (CCMG), stepwise optimization was carried out. In the first instance the effect of the organic modifier on the retention of acyclovir was examined for both C_{18} and C_8 stationary phases (Fig. 1). This shows the dependence of the capacity factor (k′) of acyclovir on the volume percentage of acetonitrile (ACN); therefore the ODS stationary phase was chosen for this study and 5% ACN was sufficient to give reasonable retention time.

Following this, the effect of the pH of the mobile phase on the retention and peak shape for acyclovir was examined (Fig. 2), where the influence of the pH of the mobile phase on k′ is illustrated. It is clear from this figure that the capacity factor was found to increase on decreasing the pH values for both ACN/H_2O and $MeOH/H_2O$ system. It was also observed that the pH for the optimum conditions is related to the pKa of acyclovir. At $pH < pKa_1$, the acyclovir will be available in its maximum cationic form ($HACN^+$) which may interact with the counter-ion form. At $pH > pKa_1$ the acyclovir will be available in neutral form (HCN). The major limitation is the workable pH range and life time of the column. According to the results and the improvement in peak shape, and retention time, pH 2.5 was chosen for ACN/H_2O system prior to use.

The effect of alkyl type and concentration of the counter-ion on the capacity factor was studied and the results are given in Fig. 3. As can be seen, the k′ was found to increase with increase in alkyl type and concentration of the counter-ion. Therefore $C_8H_{17}SO_4Na$ at 0.002 m was chosen prior to use. The combination of all these parameters is demonstrated in the chromatographic separation and the optimum condition for a particular column resulted in the elution of acyclovir, guanine and 9-carboxy methoxy methyl guanine within approximately 8 min (Fig. 4).

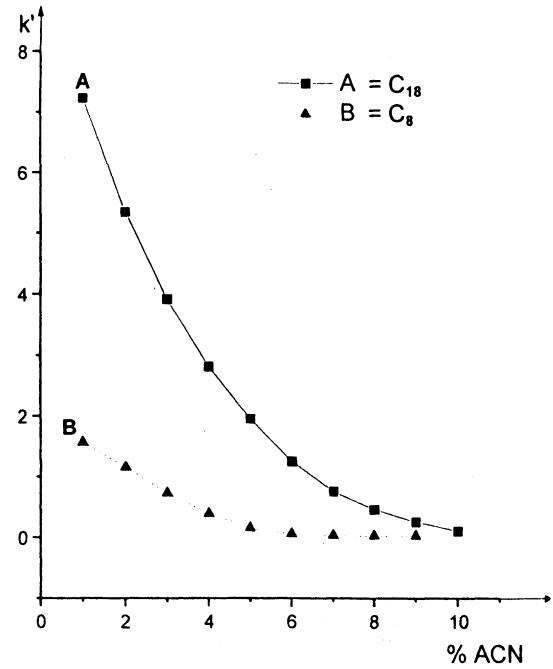

Fig. 1. The effect of organic modifier on the retention time of acyclovir. For chromatographic, see text.

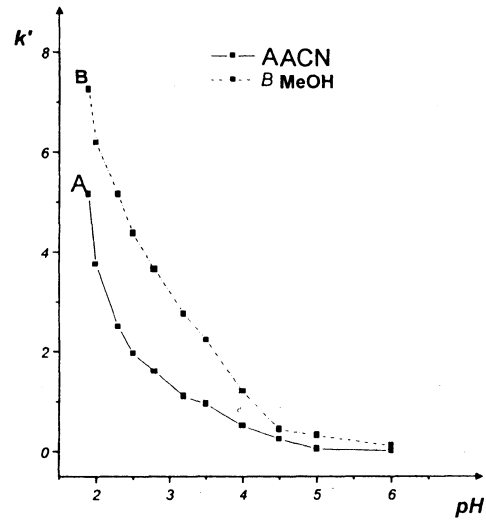

Fig. 2. The effect of pH of the mobile phase on the retention time and peak shape. For chromatographic, see text.

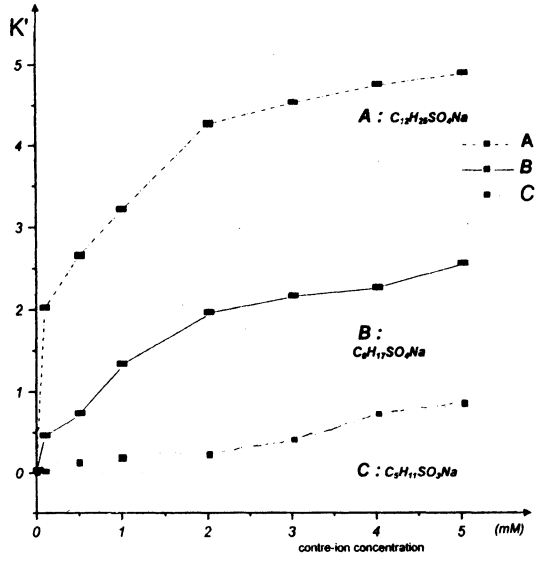

Fig. 3. The effect of alkyl type and concentration of the counter-ion on the capacity factor

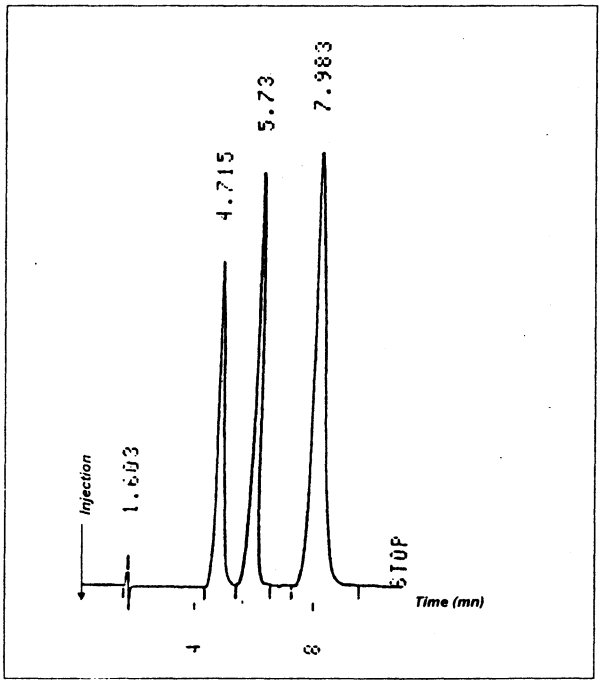

Fig. 4. Separation of (1) acyclovir, (2) 9-carboxy methoxy methyl guanine and (3) guanine

Linearity over the range 1–100 μg/mL of acyclovir was examined and determined by plotting the peak height response *vs.* the concentration. The results showed that the method was linear in accordance with Beer's law over this range and the linearity equation was $y = 2.8232x + 0.01523$ and regression coefficient was $r = 0.99985$ ($n = 11$).

The reproducibility for replicate injection was good (RSD = 0.56% , n = 10) at 30 μg/mL. The absolute limit of detection defined as signal-to-noise of 2 was examined and found to be 3 ng/mL and the limit of quantization (LOQ) was 0.1 μg/mL (20 μL was injected). Reproducibility, which expresses the precision under different conditions, such different laboratories with analyst, using separate instrumentation was also examined. Table-1 shows the results obtained in two different laboratories (France and Algeria) and the RSD was between 2 and 4%.

TABLE-1
REPRODUCIBILITY OF THE METHOD IN TWO DIFFERENT LABORATORIES: SAIDAL (ALGERIA) AND LYON (FRANCE):

Laboratory	N	Cr [μg/mL]	Cm [μg/mL]	SD	RSD (%)
LSA (Lyon, France)	10	10	9.81	0.26	2.65
SAIDAL (Algiers)	10	10	10.26	0.38	3.70

Cr: True value, Cm: mean concentration. For chromatographic, see text.

Accuracy: This expresses the closeness of agreement between the value which is accepted either as conventional true value (in-house standard) or an accepted reference value (international standard, *e.g.*, pharmacopoeial standard) and the value found (mean value) obtained by applying the test procedure a number of times (Table-2). From these results, we can conclude that the method was accurate and precise (RE < 3%).

Recovery: The recovery of extracted acyclovir was estimated either by comparing peak heights obtained from extracted aqueous standard and peak heights obtained from spiked standards or more convenient by comparing the slopes of the equations.

In this case the recovery of acyclovir was 95% ($y_{st} = 2.8232x + 0.01523$ and $y' = 2.68411x' = 0.10304$).

TABLE-2
ACCURACY OF THE METHOD FOR ACYCLOVIR
(for chromatographic, see text)

N	Cr (μg/mL)	The same day				20 days later			
		Cm (μg/mL)	SD	RSD (%)	RE (%)	Cm (μg/mL)	SD	RSD (%)	RE (%)
10	10	10.02	0.076	0.76	0.20	9.93	0.17	1.71	–0.70
10	20	19.89	0.195	0.98	–0.50	20.12	0.26	1.3	0.60

Pharmacokinetics profile

Plasma samples were taken from a healthy female after 400 mg (two tablets of zovirax) was given orally at time 30, 60, 90, 120, 160, 180 and 190 min.

Fig. 5 shows typical plasma concentration of acyclovir against time and the results conclude that the described method satisfactorily meets the needs of human pharmacokinetic studies.

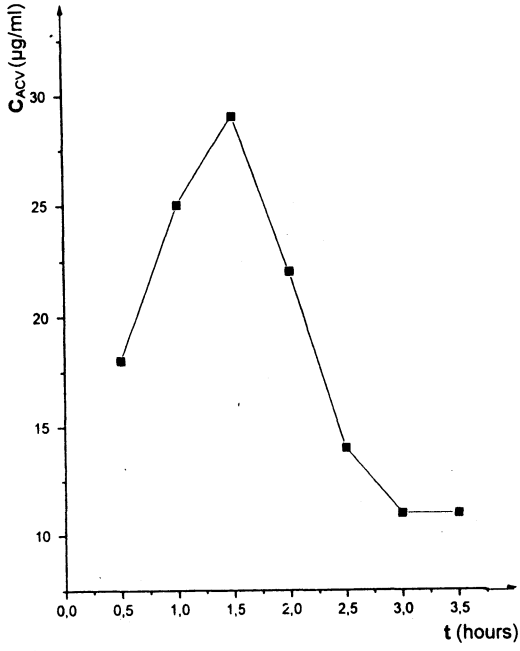

Fig. 5. Typical plasma concentration pharmacokinetics profile of acyclovir

Conclusion

A reversed phase ion pairing chromatography has been developed for the separation and determination of acyclovir, guanine and 9-carboxy methoxy methyl guanine. It has been shown in this work that the method developed is selective, precise, reproducible and accurate and meets the needs of pharmacokinetic studies.

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