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Short communication

High-throughput HPLC assay of acyclovir and its major impurity guanine using a monolithic column and a flow gradient approach

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Abstract

Acyclovir and its major impurity guanine are determined rapidly by the incorporation of a monolithic column (100 mm × 4.6 mm i.d., Merck) to an automated HPLC system. A simple flow gradient protocol was adopted in order to accelerate the separation–detection cycle. Using 0.2% CH_3COOH (pH 3.1) as the mobile phase and detection at 254 nm, guanine was effectively separated from the system peak (t_R = 1.25 min), while the retention time of acyclovir was 2.35 min. Linearity of the assay was validated in the range 0.1–1.0% guanine and 80–120% acyclovir (n = 5). The accuracy and within- and day-to-day precision of the method were also validated, while the limits of detection and quantitation of both analytes were determined. The proposed method was successfully applied to the quality control of acyclovir raw material and the quality and stability control of acyclovir-containing pharmaceutical creams (Hagevir® 5%, w/w, Cosmopharm Ltd., Korinthos, Greece).

Keywords: Acyclovir; Guanine; HPLC assay; Monolithic column; Pharmaceutical analysis

1. Introduction

Acyclovir is an antiviral drug found in both tablets and creams that slows the growth and spread of the herpes virus so that the body can fight off the infection. Acyclovir does not cure herpes. It reduces pain and helps the sores caused by the infection to heal faster. Mild common side-effects may be diarrhea, nausea, vomiting, itching and skin rash, while more serious side-effects include hematuria, encephalopathic changes and elevation of serum creatinine [1–3].

Several methods have been reported for the determination of acyclovir in pharmaceutical formulations, based on various analytical techniques. Flow injection analysis with either chemiluminescence [4] or electro-chemiluminescence [5] detection schemes, a non-destructive method based on FT-Raman spectroscopy [6], and a derivative spectrophotometric assay developed by Daabees [7]. Separation techniques are usually the techniques of choice in pharmaceutical analysis and quality control mainly due to the possibility of multi-analytes determination. Baeyens et al. reported a fluorimetric HPLC assay for the analysis of acyclovir in pharmaceuticals using a micellar mobile phase (t_R of acyclovir ca. 8 min) [8]. Neubert et al. used micellar electrokinetic chromatography (MEKC) with UV detection (t_M of acyclovir ca. 4 min, 250 nm) [9] and Zhang et al. capillary electrophoresis (CE) with amperometric detection (t_M of acyclovir ca. 9 min) [10]. Although in methods [8] and [10] the authors report separation of guanine and acyclovir peaks, no data on the quantitative determination of guanine are included [8–10]. Methods based on separation techniques reporting the simultaneous determination of acyclovir and guanine in pharmaceutical formulations, include HPLC [11,12], ion-pairing chromatography [13,14] and capillary electrophoresis (CE) [12]. HPLC assays employ conventional particulate-based columns with various dimensions and particle sizes, namely SB-CN 150 mm × 4.6 mm i.d. × 3.5 μm [11] and CLC-ODS 150 mm × 6 mm i.d. × 10 μm [12]. Using flow rates of 1.0 ml min^{-1} in both cases, the retention times of guanine and acyclovir were 4 and 6 min [Fig. 7 of Ref. 11] and 5 and 13 min [Fig. 1 of Ref. 12], respectively. The CE assay also developed in [12] enables the simultaneous determination of guanine and acyclovir with migration times of 6 and 9 min [Fig. 1 of Ref. 12], respectively. A disadvantage of CE compared

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to HPLC is that washing of the capillary for several minutes is required between injections. The drawbacks of ion-pairing chromatography [13,14] are more or less known and include prolonged equilibration times, difficult regeneration of the column by removing the ion-pairing reagents and irreproducible migration times.

Monolithic materials offer a very interesting alternative to particulate-based HPLC columns, since very efficient separations can be carried out at high flow rates within minimum backpressure [15–18]. An additional advantage of monolithic columns, is the possibility of improving separations using flow programming or flow rate gradient elution. By continuous or stepwise increase of the flow rate of the mobile phase (while keeping its composition unaltered) faster separations can be achieved. Compared to solvent gradient elution, there is no need for time consuming, post-run re-equilibration of the analytical column [19].

The aim of this work was to develop and validate a rapid HPLC assay for the simultaneous determination of acyclovir and its major impurity guanine. Taking into advantage of the ability of a 100 mm × 4.6 mm i.d., Chromolith® column (Merck) to operate at elevated flow rates with low backpressures, a simple flow gradient approach was adopted in order to reduce analysis time. To the best of our knowledge this is the first study reporting the application of a monolithic column to the determination of acyclovir and guanine by HPLC. Under the selected HPLC conditions, the separation–detection cycle was completed in 180 s, which is considerably faster compared to acyclovir/guanine assays mentioned above [11,12]. The assay was validated in terms of detection and quantification limits, linearity, accuracy and within- and day-to-day precision. The developed HPLC method was successfully applied to the simultaneous determination of acyclovir and guanine in pharmaceutical creams during long-term stability tests and to the quality control of acyclovir raw materials.

2. Experimental

2.1. Materials

The mobile phase consisted of 0.2% (v/v) CH_3COOH (pH 3.1). HPLC-grade water was used throughout this work (Merck, Germany). All other reagents were of analytical grade and were also provided by Merck, unless stated otherwise. The mobile phase was always filtered under vacuum (0.45 μm, Schleicher and Schuell) and degassed ultrasonically for 30 min prior to use.

Acyclovir (lot no. ACP/WS/001/03, Assay = 99.9%) and guanine (lot no. GUN/WS/001/03, Assay = 99.6%) reference standards (Fig. 1) were provided by Matrix Laboratories Ltd. (India). A 1000 mg l^{-1} acyclovir standard stock solution was prepared by dissolution of an accurately weighed amount (50.0 mg) in 50 ml of 0.01 M NaOH. A 100 mg l^{-1} guanine standard stock solution was prepared by ultrasonically dissolving an accurately weighed amount (10.0 mg) in 100 ml of 0.01 M NaOH. The stock solutions were stable for at least 1 week if kept refrigerated and protected from light. Working solutions of acyclovir (80–120%) and guanine (0.1–1.0%) were prepared by appropriate dilutions of the stocks in 0.01 M NaOH. It should be noted that the reported acyclovir concentrations are calculated on an anhydrous basis. Since acyclovir may contain up to 6% of water, it is necessary to determine its exact content by Karl–Fischer titration and make the appropriate corrections.

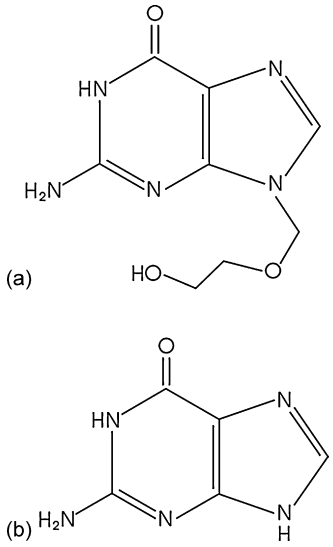

Fig. 1. Chemical structures of acyclovir (a) and guanine (b).

Pharmaceutical excipients for the preparation of a placebo cream (all excipients excluding the active ingredient) used in accuracy studies (propylene glycol, sodium lauryl sulfate, paraffin soft white, paraffin liquid, cetostearyl alcohol and poloxamer 407) were purchased from domestic suppliers.

2.2. Instrumentation

An automated HP 1100 HPLC instrument (Hewlett Packard) was used throughout this study. It comprised a quaternary pump, a vacuum degasser, a column thermostat, an autosampler and a DAD spectrophotometric detector. Chromatograms and chromatographic parameters (peak areas, retention times, theoretical plates, etc.) were recorded and calculated, respectively, via the Chem Station® software. A RP-18e monolithic column Chromolith® Performance (100 mm × 4.6 mm i.d., Merck) was used for separation of the analytes.

A vacuum filtration system (Schleicher and Schuell) and 0.45 μm membrane filters (RC 55, Schleicher and Schuell) were used for the filtration of the mobile phase.

A model DL-18 Karl Fischer titrator (Mettler-Tolledo) was used for the determination of the water content of acyclovir in both the working standard and the raw material.

2.3. HPLC procedure

The standards and/or samples (20 μl in all cases) were injected in the monolithic column via the autosampler of the HPLC instrument. Separation of acyclovir and guanine was achieved with 0.2% (v/v) CH_3COOH as mobile phase and detection at 254 nm. A simple flow gradient was adopted in order to accelerate the elution of acyclovir (2.0 ml min^{-1} in the range

0–1.5 min and 5.0 ml min^{-1} in the range 1.51–3.0 min). The column temperature was set at 25 °C. Under the above-mentioned conditions the separation cycle was completed in 180 s. Peak areas were used for signals evaluation, while all standards and samples were injected in triplicate. In order to evaluate the reliability of the method during real samples analyses, the purity of acyclovir and guanine peaks was monitored at every injection using the Chem Station® software. In all cases, peak purity was within the threshold limits.

2.4. Analysis of raw materials and pharmaceutical creams

Accurately weighed amounts of acyclovir raw material (three samples of ca. 50 mg each) were dissolved (ultrasonically for 10 min) in 50.0 ml of 0.01 M NaOH. The samples were 10-fold diluted in 0.01 M NaOH and injected in the HPLC system in triplicate (three samples × three injections).

Accurately weighed amounts of the pharmaceutical creams (three samples of ca. 100 mg each of Hagevir® cream, Cosmopharm Ltd., Greece) were dispersed in 50.0 ml of 0.01 M NaOH. The samples were stirred mechanically for 15 min and ultrasonically for another 15 min until complete dissolution of the cream. Portions (ca. 5 ml) of the resulting suspensions were filtered through disposable syringe filters (0.45 μm pore size, Whatman) and injected in triplicate in the HPLC system (three samples × three injections).

3. Results and discussion

3.1. Study of chromatographic conditions

The HPLC conditions were studied using a mixture of acyclovir (at the 100% level, corresponding to 100 mg l^{-1}) and its major impurity guanine (at the 0.5% level, corresponding to 0.5 mg l^{-1}). The starting mobile phase was 0.1% (v/v) CH_3COOH at a flow rate of 2.0 ml min^{-1}. The injection volume and column temperature were fixed at 20 μl and 25 °C, respectively, and kept constant throughout this study. Under the above-mentioned conditions the retention times of guanine and acyclovir were 1.33 and 3.77 min, respectively.

The effect the volume fraction of CH_3COOH in the mobile phase was studied in the range of 0.1–1.0% (v/v). Increase of the CH_3COOH volume fraction caused a decrease in the retention times of both guanine and acyclovir. However, for values above 0.2% guanine was co-eluted with the "system peak". Additionally, at 0.2% CH_3COOH the best symmetry for both peaks was obtained (symmetry factors of 1.06 ± 0.10 and 1.22 ± 0.14 for guanine and acyclovir, respectively, mean of three injections ± standard deviation). This CH_3COOH volume fraction was selected for further experiments.

The potential effect of addition of an organic solvent in the mobile phase was studied using HPLC-grade ACN. The experiments showed that ACN has a marking effect on the retention of the analytes even at a volume ratio of 99.5:0.5 to the aqueous CH_3COOH solution. By adding ACN in the mobile phase, guanine could not be resolved from the "system peak", making quantitative determinations inaccurate. For this reason, it was chosen not to add any organic solvent in the mobile phase.

Using 0.2% (v/v) CH_3COOH as the mobile phase at a flow rate of 2.0 ml min^{-1} ($P = 53 \pm 1$ bar), the retention time of guanine was 1.25 min and of acyclovir 3.51 min. In order to further decrease the time needed for completion of the separation–detection cycle and thus increase the samples throughput, the possibility of applying a simple flow gradient was examined. The flow rate was kept at 2.0 ml min^{-1} until complete elution of guanine (0–1.5 min) and then increased at 3.0, 4.0, 5.0 and 6.0 ml min^{-1} (at 1.51 min until the end of the separation–detection cycle). The application of the flow gradient resulted in – as expected – faster elution of acyclovir. Its respective retention times were 2.87, 2.54, 2.35 and 2.22 min corresponding to the flow rates mentioned above. A flow rate of 5.0 ml min^{-1} was adopted for further experiments. The main chromatographic parameters of the system are presented in Table 1.

Table 1
Chromatographic parameters of the system

HPLC parameters[a]	Guanine	Acyclovir
Retention time (min)	1.25	2.35
Width at half peak (min)	0.030	0.042
Symmetry	1.06	1.22
Theoretical plates	9,586	17,260
Resolution	–	17.95

[a] As calculated by the Chem Station® software for a guanine/acyclovir mixture (concentration level of 0.5%/100%, respectively), mean of three injections.

3.2. Validation of the HPLC assay

The developed HPLC assay was validated in terms of linearity, limit of detection and quantitation, within-day and day-to-day precision and accuracy.

3.2.1. Linearity, limits of detection and quantitation

An acyclovir mass concentration of 100 mg l^{-1} was established as the 100% level. Linearity of acyclovir was therefore studied in the range of 80–120% (80–120 mg l^{-1}), using five standard solutions ($n = 5$). Since the maximum allowed limit of guanine in acyclovir-containing formulations is 0.7% [20], its linearity was tested in the range of 0.1–1.0% (0.1–1.0 mg l^{-1}, $n = 5$). Both analytes showed excellent linearity in the studied ranges. Peak areas (A) versus analytes mass concentrations (γ in mg l^{-1}) obeyed the regression equations:

$$A = 28.289(\pm 0.198) \times \gamma(\text{acyclovir}) + 63.7(\pm 20.0),$$

$$A = 40.454(\pm 0.262) \times \gamma(\text{guanine}) + 7.16(\pm 0.16)$$

The regression coefficients were 0.9999 in both cases. Validation of the regression lines was performed by the response factor (r.f.) test [21]. The deviation of the r.f. of each point of the calibration curve must be within ±3% of the experimental slope and is given

Table 2
Accuracy of the HPLC assay

Synthetic sample	Guanine/acyclovir (%)	Placebo added (mg ml^{-1})	Recovery (±S.D.) (%)	
			Guanine	Acyclovir
Day I/analyst I				
S1	0.2/80.0	3.0	97.3 (±1.2)	99.5 (±1.0)
S2	0.5/100.0	3.0	99.4 (±1.0)	99.2 (±0.8)
S3	1.0/120.0	3.0	100.6 (±1.5)	99.5 (±1.4)
Day II/analyst II				
S4	0.2/80.0	3.0	98.9 (± 0.9)	101.6 (±0.9)
S5	0.5/100.0	3.0	101.2 (±0.9)	99.3 (±0.7)
S6	1.0/120.0	3.0	98.7 (±1.3)	101.4 (±1.4)

Table 3
Analysis of acyclovir raw material

Sample (lot no.)	Assay (±S.D.) (%)		Certified values (%)	
	Acyclovir	Guanine	Acyclovir	Guanine
ACP 0220802	99.2 (±1.3)	0.26 (±0.03)	99.9	0.29
ACP 1131203	100.8 (±0.6)	0.30 (±0.04)	100.2	0.27

by the equation:

$$\text{r.f.} = \frac{\text{peak area} - \text{intercept}}{\gamma[\text{Analyte}]}$$

The experimental results conform to the above-mentioned limit (±3%). The deviations of all points of the calibration curve of acyclovir were in the range of −2.1 to +1.3% of the slope of the corresponding regression equation, while the respective values for the calibration curve of guanine were −1.4 to +1.9%.

The detection (LOD) and quantitation limits (LOQ) of the assay were determined based on the S/N criteria. The LOD's were 0.05 and 0.02 mg l^{-1} for acyclovir and guanine, respectively (S/N = 3), and the LOQ's 0.15 and 0.07 mg l^{-1} (S/N = 10), respectively.

3.2.2. Within- and day-to-day precision

The within-day precision of the HPLC assay was validated by calculation of the relative standard deviation of six consecutive injections of a mixture of guanine (0.5% level) and acyclovir (100% level) at the beginning, middle and end of a working day. The calculated R.S.D.s were 1.3% for guanine and 0.5% for acyclovir ($n = 6 \times 3$).

The day-to-day precision was validated by constructing six consecutive calibration curves (0.1–1.0% guanine, 80–100% acyclovir, 6 calibration curves × 5 concentration levels). The experimental results verified the day-to-day precision of the assay. The relative standard deviation of the slopes for guanine was 2.9% and for acyclovir 2.1% ($n = 6$).

It should also be noted that the precision of the retention times of both analytes was excellent. The R.S.D. values both inter- and intra-day were <0.5% in all cases.

3.2.3. Accuracy studies

In order to validate the within- and day-to-day accuracy of the HPLC assay, synthetic samples were constructed independently at three concentration levels (guanine/acyclovir mixtures: S1 and S4 = 0.2%/80%, S2 and S5 = 0.5%/100% and S3 and S6 = 1.0%/120%, respectively). A placebo cream (all excipients excluding the active ingredient) was prepared according to the manufacturing protocol of the formulation. The placebo cream consisted of 40.0% propylene glycol, 0.75% sodium lauryl sulfate, 12.5% paraffin soft white, 5.0% paraffin liquid, 6.75% cetostearyl alcohol and 1.0% poloxamer 407. Accurately weighed amounts of the placebo cream were dispersed in the standard guanine/acyclovir solutions at the concentration levels mentioned above. The placebo concentration in the synthetic samples was fixed at 3.0 mg ml^{-1} (the theoretically expected concentration of the placebo in real samples is ca. 2.0 mg ml^{-1}). Both within- and day-to-day accuracies were evaluated by performing the analyses on two consecutive days by different analysts. The experimental results are presented in Table 2. The percent recoveries were satisfactory in all cases ranging between 97.3 and 101.2% for guanine and 99.2–101.6% for acyclovir. All samples were injected in triplicate.

3.3. Applications to real samples

The developed HPLC assay was applied to the quality control of two batches of acyclovir raw material (Matrix Laborato-

Table 4
Analysis of pharmaceutical creams

Sample	Assay (±S.D.) (%)	
	Acyclovir	Guanine
Production (0 month)	100.3 (±0.8)	0.33 (±0.05)
3 months (ACS)	101.9 (±1.5)	0.43 (±0.03)
6 months (ACS)	101.4 (±1.5)	0.21 (±0.04)
3 months (LTS)	100.9 (±1.1)	0.28 (±0.03)
6 months (LTS)	102.9 (±0.9)	0.25 (±0.03)
9 months (LTS)	101.8 (±1.3)	0.28 (±0.02)
12 months (LTS)	102.5 (±1.5)	0.21 (±0.03)

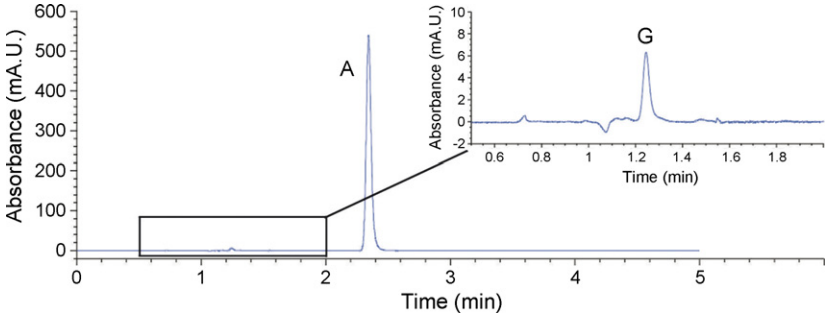

Fig. 2. Typical chromatogram of an acyclovir-containing pharmaceutical cream; A: acyclovir and G: guanine.

ries Ltd., lot nos. ACP 0220802 and ACP 1131203) and the accelerated (ACS) and long-term stability (LTS) control of a pharmaceutical cream (Hagevir® 5.0%, w/w, Cosmopharm Ltd., Korinthos, Greece). The results are tabulated in Tables 3 and 4, respectively, while a representative chromatogram of the cream is depicted in Fig. 2. Table 3 also includes the certified values of the raw materials for comparative reasons. Long-term stability control of the formulation involves storage at 25 °C/60% relative humidity (R.H.), while the respective conditions for accelerated stability control are 40 °C/75% R.H.

4. Conclusions

Based on the unique properties of silica-based monolithic columns to operate at elevated flow rates with low backpressures, a flow gradient HPLC assay was developed and validated for the simultaneous determination of acyclovir and its major impurity guanine. Separation and detection of the analytes was completed in 180 s enabling high-throughput analyses. This feature is of particular interest and significance in industrial pharmaceutical analyses and quality control, especially when a lot of samples have to be analyzed during new product development and manufacturing process validation. The HPLC assay was applied to the quality control of acyclovir raw material and the long-term stability control of a pharmaceutical cream.

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