LC methods for acyclovir and related impurities determination

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Abstract

Acyclovir, guanine, and impurity A have been baseline separated with isocratic conditions at pH = 3.0 and run time under 15 min by employing a SB-CN column from Agilent (150 mm × 4.6 mm and 3.5 μm). Moreover, when run time was increased to 40 min six impurities (guanine, impurities A, F, G, Vir 3/4 and N[7]) plus acyclovir were separated in the same conditions. The mobile phase consisted of buffer A/acetonitrile 96:4 (v/v), being buffer A:25 mM H_3PO_4 (Milli-Q H_2O) brought to pH 3.0 with KOH.

The same column provided separation for all the seven impurities described in pharmacopoeia, including impurity C, which coeluted with acyclovir in the previous conditions with a mobile phase prepared with 25 mM phosphoric acid (pH = 1.8)/acetonitrile 96:4 (v/v).

The method has been validated following ICH guidelines and it has demonstrated to be reliable for acyclovir and its impurities determination.
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Keywords: HPLC; Acyclovir; Cyano stationary phase

1. Introduction

Acyclovir is a synthetic purine nucleoside analogue with in vitro and in vivo inhibitory activity against herpes simplex virus and varicella-zoster virus. It is a white to off-white crystalline powder with molecular weight of 225.21 and solubility in water below 2.5 mg/ml at 37 °C. Acyclovir pK_a are 2.27 and 9.25 [1].

Acyclovir determination in pharmaceuticals has been performed spectrophotometrically. A visible spectrophotometric method, not very sensitive [2], a derivative spectrophotometric [3] and a differential spectrophotometric method [4] have been proposed.

The more common technique is HPLC with UV detection [5–7] or coupled to MS [8,9]. Nevertheless, none of these methods include related compounds more than guanine.

The related compounds to be determined following the Pharmacopeias are guanine and impurity A (acetate form of acyclovir), but the bulk material can present at least seven

impurities all of them with similar structures as can be seen in Fig. 1. The presence of these impurities can be important not only for quality control, but also in enforcing patent rights because they can be route indicative of the synthesis of acyclovir.

Only one method in literature includes three of these impurities plus acyclovir [10]. Authors say that it is difficult to quantitate, because it employs liquid extraction and HPLC with fraction collection with further analysis by mass spectrometry.

As guanine and acyclovir are very polar and easily ionizable, they are usually separated in reverse phase with ion-pairing chromatography at acidic pH using decanosulfonate [11]. The drawbacks of ion-pairing chromatography are generally known: a long equilibration time; as the ion pairing compounds interact strongly with the stationary phase, they are difficult to eliminate from the column after their use, and they provide poor migration time reproducibility. The last is a very undesirable point during a stability indicating assay.

The availability of new stationary phases with new selectivities in reverse phase HPLC provided us with the possibility of new separations avoiding these drawbacks. There-

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Acyclovir

Guanine

Impurity C

Impurity A

Impurity F
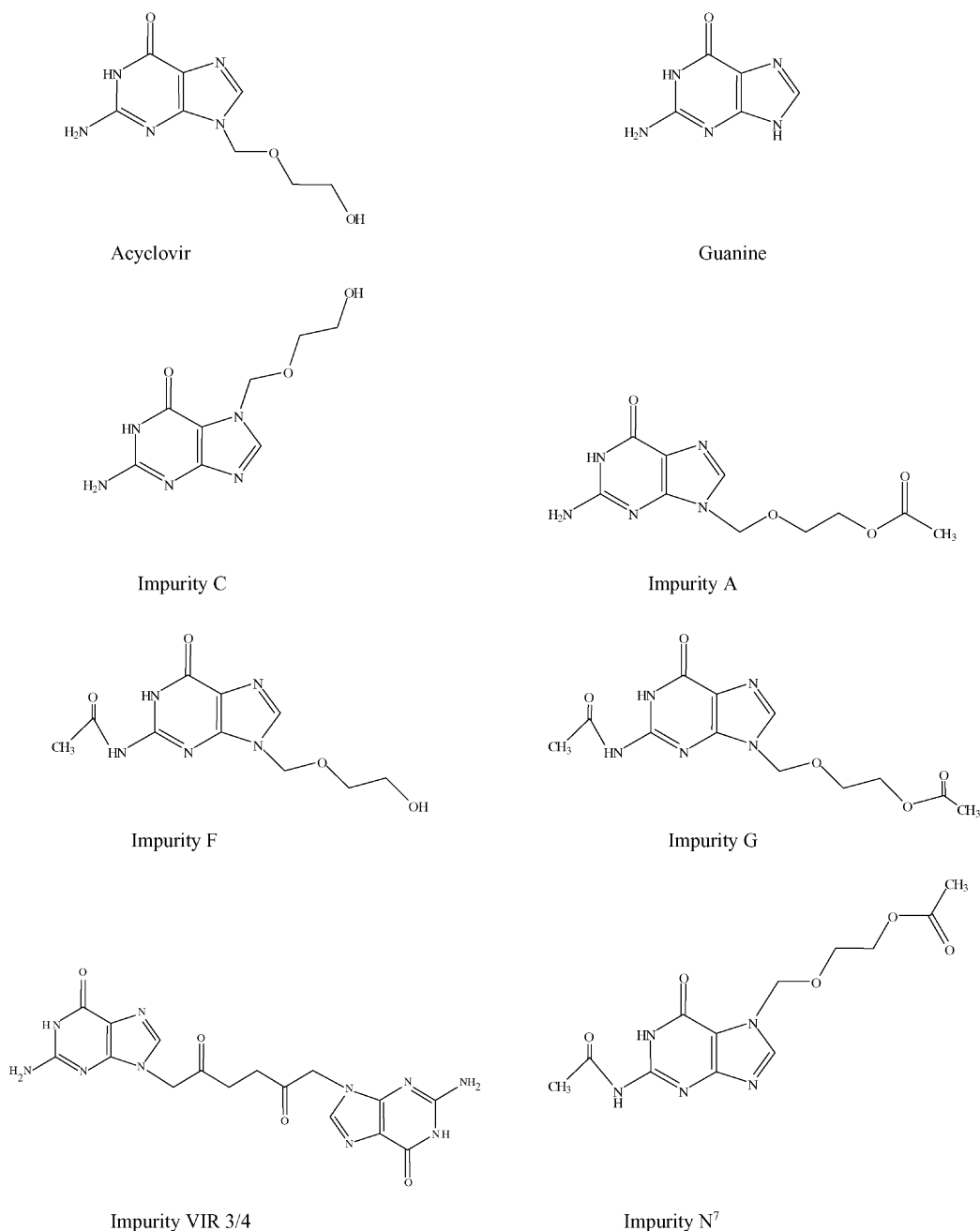
Impurity G

Impurity VIR 3/4

Impurity N[7]

Fig. 1. Chemical structures of the assayed compounds.

fore, the objective of this work was the exploration of HPLC columns for the separation of acyclovir impurities avoiding ion-pairing reagents.

2. Experimental

2.1. Apparatus

LaChrom Elite HPLC equipment from VWR consisted of quaternary pump, an automatic injector, a single wavelength detector and a column oven. Different columns and

mobile phases were tested. Finally, the method was validated with a SB-CN column from Agilent (150 mm × 4.6 mm and 3.5 μm). It provided baseline separation with isocratic conditions at pH = 3.0 and run time under 15 min for guanine, acyclovir and impurity A. Moreover, when run time was increased to 40 min six impurities (guanine, impurities A, F, G, Vir 3/4 and N[7]) plus acyclovir could be separated in the same conditions. The mobile phase consisted of buffer A/acetonitrile 96:4 (v/v), being buffer A:25 mM H_3PO_4 brought to pH 3.0 with KOH. The oven temperature was 35 °C and UV detection was performed at 254 nm.

The same column provided separation for all the seven impurities, including impurity C, which coeluted with acyclovir in the previous conditions with a mobile phase prepared with 25 mM phosphoric acid (pH = 1.8)/acetonitrile 96:4 (v/v).

2.2. Chemicals

Standard of acyclovir and impurities (impurity A, impurity C, impurity F, impurity G, impurity Vir 3/4, impurity N[7]) as well as tablets and excipients (silicon dioxide, talc, magnesium stearate, povidone®, avicel®, primogel®) were kindly provided by CINFA S.A. (Pamplona, Spain). Guanine was purchased from Sigma–Aldrich (Steinheim, Germany), NaOH (>99%) was from Panreac (Barcelona, Spain), H_3PO_4 (85%) and CH_3CN (HPLC grade) were from Merck (Darmstadt, Germany) and water was purified with a Milli-Q plus system from Millipore (Bedford, MA, USA).

2.3. Standard solutions and sample preparation

In all cases the solvent solution (SS) for standards and samples was the mobile phase phosphate buffer 25 mM pH 3.0/acetonitrile 96:4 (v/v). Acyclovir standard solution was prepared with 40.0 mg of acyclovir exactly weighed and dissolved with SS in a 100 ml volumetric flask.

All the impurities were individually prepared with 10 mg exactly weighed and dissolved in 250 ml volumetric flasks with SS, except for guanine and impurity Vir 3/4, which needed 25 ml of NaOH (0.1N). An intermediate solution containing all the impurities was prepared with 0.2 ml of each impurity stock solution, all together, made up 10 ml with SS.

For quantification 53.73 mg of the pulverised tablets were made up to 100 ml with SS. After bath sonication for around 5 min samples were filtered with 0.45 μm nylon filters prior to the injection.

2.4. Validation

The selectivity was tested by running solutions containing the excipients of the speciality in the same quantities and conditions that the samples to show that there was no peak at the retention times corresponding to the analytes. Moreover, solutions of the standards with the identified impurities at the 0.2% level added were also run to show the resolution and selectivity of the method.

Validation parameters were tested in two ranges: quantification range and impurities range. The linearity was tested for the high range by preparing standard solutions at five concentration levels, from 75% to 120% of the target analyte concentration. In this case, acyclovir concentrations were from 0.3 mg/ml to 0.48 mg/ml. They were prepared in 100 ml volumetric flasks by weighting the exact amount of acyclovir (from 30 mg to 48 mg) and completed the total volume with SS. Each point was analysed three times.

For the low range, acyclovir concentrations ranged from 0.2 μg/ml to 4 μg/ml while guanine concentrations were from 0.2 μg/ml to 20 μg/ml, they were prepared in 10 ml volumetric flasks by adding the corresponding volumes of acyclovir and guanine stock standard (0.050–1.00 ml for acyclovir and 0.050–2.5 ml for guanine) and completed the total volume with SS.

For pharmaceutical studies in drug products one method for determining accuracy is the application of the analytical procedure to synthetic mixtures of the drug components to which known quantities of the drug substances to be analysed have been added. It was tested in triplicate at three levels (80, 100 and 110%) and in parallel with the linearity assay for the two main components. The percent recovery and the R.S.D were then calculated.

For accuracy in the low range, 7.0 mg of excipients were weighted in 50 ml volumetric flasks and the corresponding volumes of acyclovir stock solution were added to obtain 0.05, 0.1, 0.2, 0.5 and 1.0% and 0.05, 0.2, 0.5, 1.0 and 5.0% for guanine. These ranges included from the limit of declaration to a value over the limit of acceptance.

Intra-assay precision data were obtained by repeatedly analysing, in one laboratory on 1 day, 10 aliquots of an homogeneous sample (for the high range) and six aliquots (for the low range), each of which were independently prepared according to the method procedure and the corresponding standards. Data for intermediate precision were obtained by repeating the intra-assay experiment on a different day with newly prepared solutions.

The detection limit (LOD) was evaluated by measuring the baseline noise and by calculating the analyte concentration that gives S/N = 3, while the limit of quantification (LOQ) was established for the analyte concentration that gives S/N = 10. Nevertheless, that is an approach, because the actual LOQ was established by validating the method to the lower level.

Response factors for impurities A, F, G, Vir 3/4, and N[7], relative to acyclovir, were calculated by running ten times a mixture containing the impurities plus acyclovir at 0.2%.

Standard stability was tested by running the same sample corresponding to the medium point in the linearity assay for 0–3 days and with the same mobile phase. Between runs, solutions were stored at ambient temperature. The initial area was considered 100% and recoveries in the following days were evaluated.

3. Results and discussion

In order to obtain an adequate resolution in isocratic conditions without employing ion-pairing reagents several stationary phases and pHs in the mobile phase were tested. New stationary phases compatible with 100% aqueous mobile phases and polar reverse phase stationary phases, such as polyethyleneglycol or cyano may help to avoid using gradient elution when separating a mixture of compounds with widely varying concentrations. They provide a balance of retention for polar and non-polar compounds in reversed-phase chromatography with higher retention for more po-
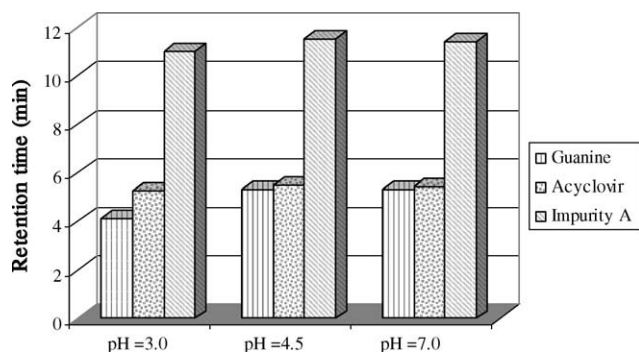
Fig. 2. Influence of mobile phase pH in the separation of acyclovir, guanine and impurity A. The column was SB-CN Agilent 150 mm × 4.6 mm, 3.5 μm. Mobile phase phosphate buffer 25 mM/acetonitrile 95:5 (v/v). UV detection at 254 nm.

lar compounds while lower retention to more hydrophobic compounds. Therefore, different columns were tested in this line: Atlantis™ dC18 (Waters) 5 μm, 4.6 mm × 250 mm, a silica-based line of difunctionally bonded C18 columns; Zorbax SB C18 Aq 5 μm, 3.0 mm × 150 mm and a Discovery cyanopropyl 5 μm, 4.6 mm × 250 mm (Supelco), the bonded phase is –O–Si(CH$_3$)$_2$CH$_2$CH$_2$CH$_2$CN, it is endcapped and has a pore size of 180 Å. Some of the characteristics according to the manufacturer are the low hydrophobicity for rapid elution of hydrophobic molecules, the retention and separation of strongly basic analytes, the compatibility with highly percent of aqueous mobile phases and its excellent stability and reproducibility. It has been previously described that the properties of the CN columns are governed by the type of cyano phase (trifunctional or monofunctional) on the silica gel support and endcapping [12,13]. Therefore, a very different column was tested. Agilent Zorbax SB-CN is described by the manufacturer as a densely covered, sterically protected, diisopropyl cyanopropyl stationary phase, not endcapped, with pore size of 80 Å. This column was assayed in two sizes: the classical 5 μm, 4.6 mm × 250 mm column, and another one with lower particle diameter and length, considered equiv-

alent and named by manufacturers as "solvent saver" with 3.5 μm, 4.6 mm × 150 mm. Finally, polyethyleneglycol PEG (Supelco) 5 μm, 4.6 mm × 250 mm, was also tested.

All the columns assayed can be considered equivalent in dimensions, although working at a different flow rates: 1 ml/min for 5 μm, 4.6 mm × 250 mm columns and 0.6 ml/min for 3.5 μm, 4.6 mm × 150 mm columns.

The first assay was to study the effect of mobile phase pH. Different columns were checked at 90:10 and 95:5 (v/v) 25 mM phosphate buffer/acetonitrile. Phosphate buffer was employed at pH = 3.0, 4.5, and 7.0. Preliminary assays were developed only with guanine, acyclovir and impurity A, which are those included in Pharmacopoeia methods. Fig. 2 shows the result for Agilent Zorbax SB-CN, and similar trends were observed in most of the columns, that is resolution increases at pH = 3.0 because guanine retention decreases slightly. It can be justified with the pK_a = 3.30 for guanine [14], because at lower pH the nitrogen gets a proton and the ionised form is less retained, meanwhile the equivalent nitrogen in acyclovir has a pK_a = 2.01.

The results for 95:5 (v/v) 25 mM phosphate buffer pH 3.0/acetonitrile are shown in Fig. 3. Polyethyleneglycol PEG and CN columns from Supelco were discarded because they did not provide enough resolution in these conditions. Zorbax SB-Aq and C18 from Agilent were also discarded because guanine was too near to the dead volume. Atlantis column from Waters showed good resolution, but too high retention time for impurity A, and therefore, a gradient seemed necessary. The best ratio and values in migration times was for SB-CN columns from Agilent. Moreover, it was experimentally confirmed that the two dimensions 5 μm, 4.6 mm × 250 mm and 3.5 μm, 4.6 mm × 150 mm provided almost the same retention times, but working at 1.0 ml/min and 0.6 ml/min respectively, and therefore, the waste of a higher solvent amount is useless.

All the impurities were then injected in the SB-CN from Agilent. Fig. 4 shows the separation obtained for all the standards at 0.2% level. As can be seen, impurity C and acyclovir
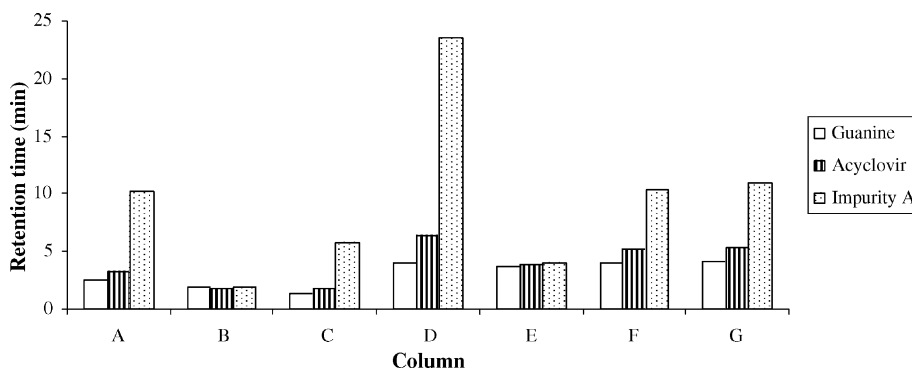

Fig. 3. Comparative behaviour of acyclovir, guanine and impurity A on different stationary phases. Chromatographic conditions: phosphate buffer 25 mM pH = 3.0/acetonitrile 95:5 (v/v). UV detection at 254 nm. Column: (A) Agilent Zorbax Eclipse XDB C$_{18}$ 150 mm × 4.6 mm, 3.5 μm, flow rate 0.6 ml/min; (B) Supelco PEG 150 mm × 4.6 mm, 5 μm, flow rate 1.0 ml/min; (C) Agilent Zorbax SB-Aq 150 mm × 3 mm, 5 μm, flow rate 1.0 ml/min; (D) Waters Atlantis 250 mm × 4.6 mm, 5 μm, flow rate 1.0 ml/min; (E) Supelco CN 250 mm × 4.6 mm, 5 μm, flow rate 1.0 ml/min; (F) Agilent CN 250 mm × 4.6 mm, 5 μm, flow rate 1.0 ml/min; (G) Agilent CN 150 mm × 4.6 mm, 3.5 μm, flow rate 0.6 ml/min.
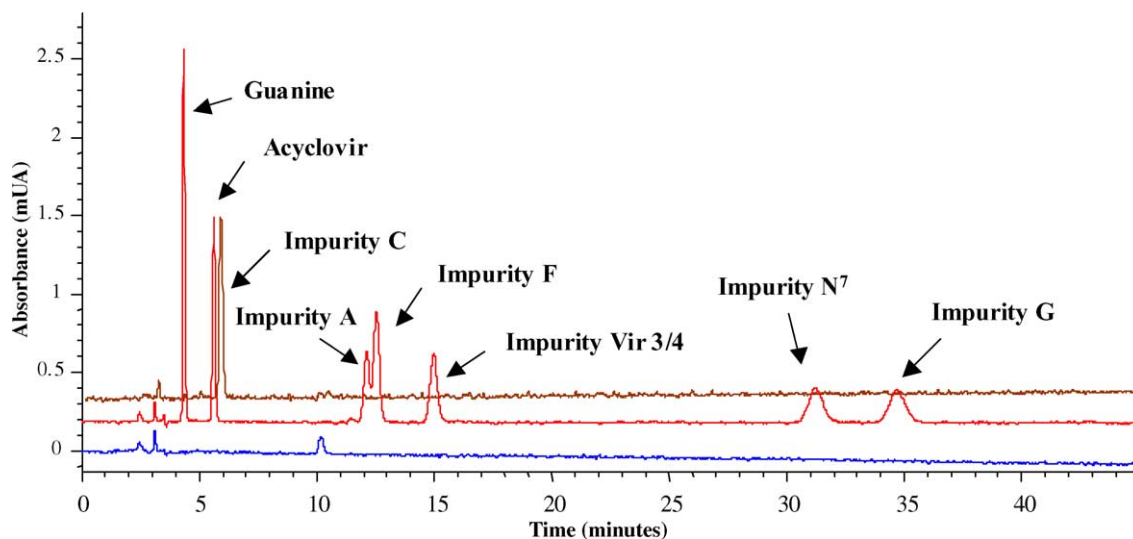
Fig. 4. Chromatogram showing acyclovir (0.4 mg/ml) and related impurities (all of them 0.2% of the main peak) separation. Agilent Cyano column, 3.5 μm particle size, 150 mm × 4.6 mm kept at 35 °C. The mobile phase consisted of phosphate buffer 25 mM (pH = 3.0)/acetonitrile 96:4 (v/v). Flow rate, 0.6 ml/min and UV detection, 254 nm. The lower line corresponds to the excipients.

coeluted in these conditions and increasing the water amount to 100% did not improve the result.

Looking at the pK_a values it could be thought that under pH 2 a new protonation of a nitrogen could occur with the corresponding change in retention times that could produce some differentiation. Therefore, we tested 96:4 (v/v) 25 mM phosphoric acid (pH 1.8)/acetonitrile with SB-CN column,

which is described as stable in these conditions. Fig. 5 shows the separation obtained with impurity C separated from acy-clovir and impurity Vir 3/4 eluting at shorter retention time. Nevertheless, as impurity C comes from the synthesis, but it is not a degradation product, we decided to discard its pres-ence in the raw material at pH 1.8, as can be seen in Fig. 6, but to validate the method at pH 3.0, which is less aggressive for
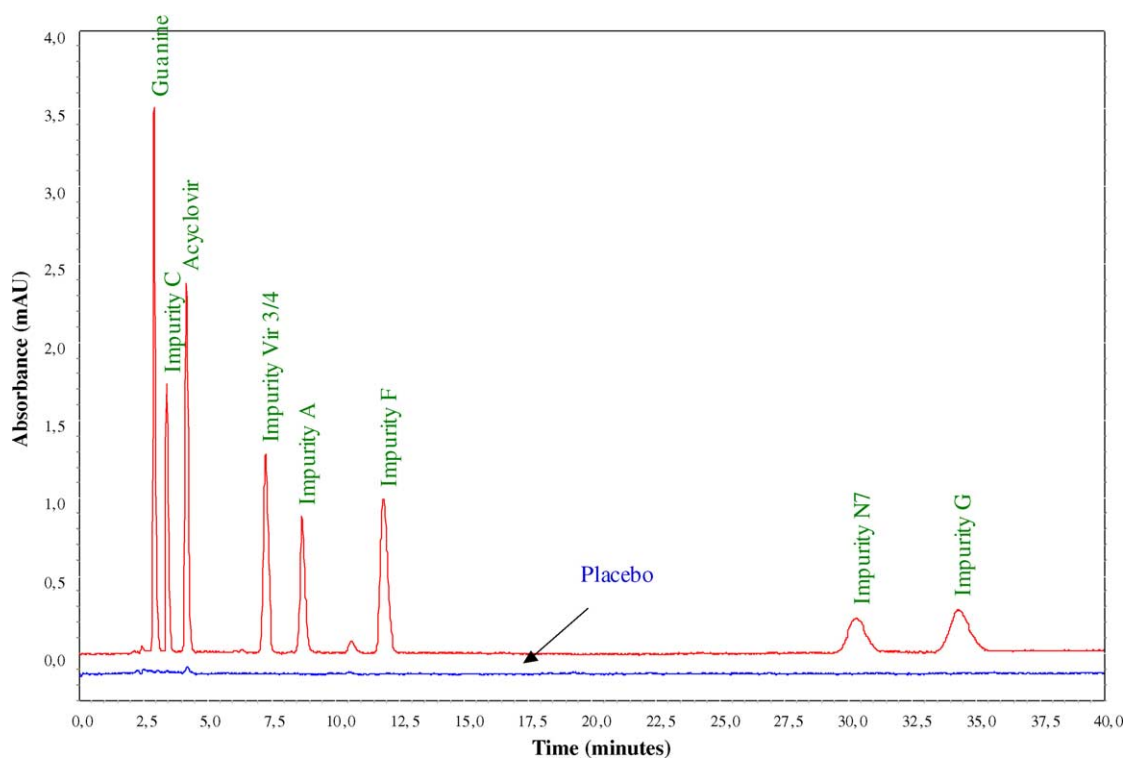

Fig. 5. Chromatogram of acyclovir (0.4 mg/ml) and related impurities, all of them at 0.2%. Column SB-CN Agilent, 3.5 μm, 150 mm × 4.6 mm. Mobile phase phosphate buffer 25 mM (pH = 1.8)/acetonitrile 96:4 (v/v). Flow rate, 0.6 ml/min, UV detection at 254 nm.
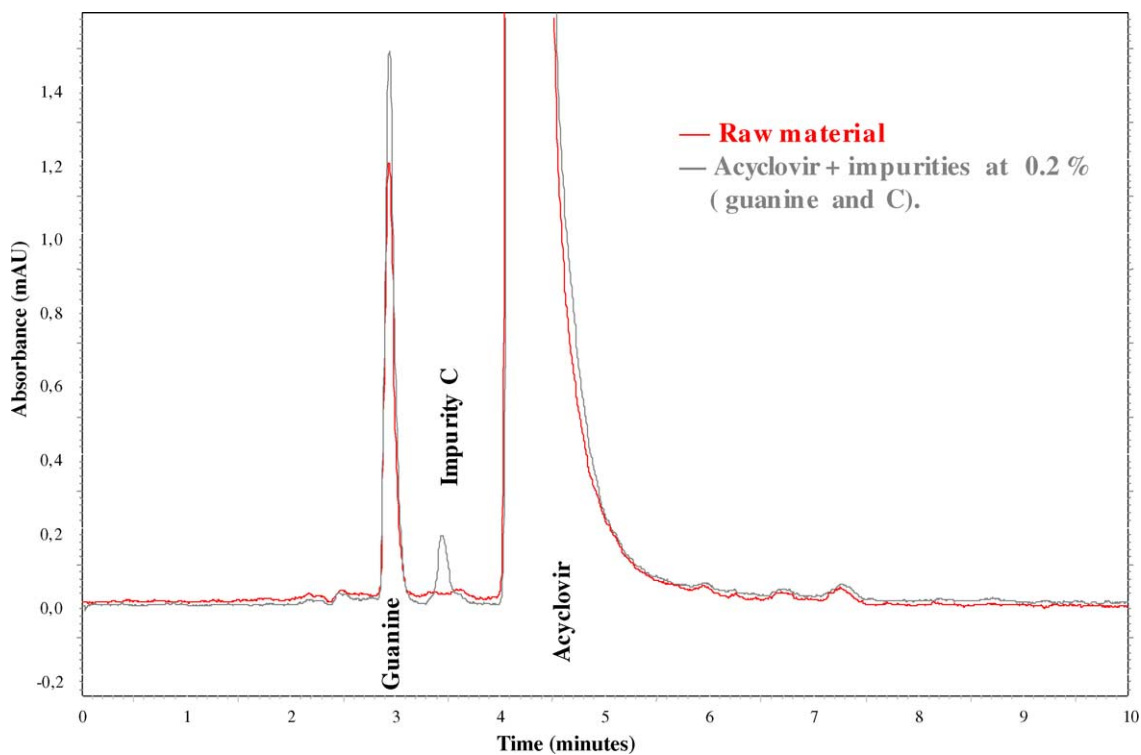
Fig. 6. Chromatograms corresponding to: (a) acyclovir and impurities at 0.2% (guanine and C); (b) Raw material at 100%.
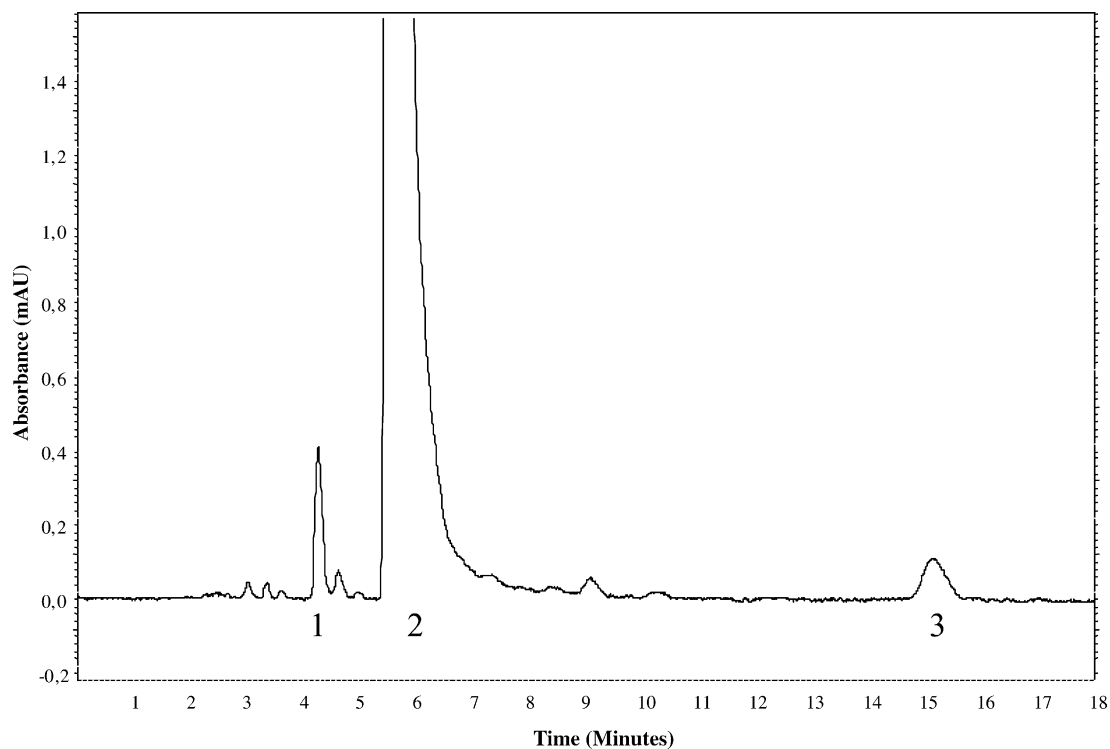

Fig. 7. Chromatogram corresponding to a tablet of acyclovir at the quantification level. Peak identification 1: guanine, 2: acyclovir, 3: impurity Vir 3/4. Conditions: Agilent Cyano column, 3.5 μm particle size, 150 mm × 4.6 mm kept at 35 °C. The mobile phase consisted of phosphate buffer 25 mM (pH = 3.0)/acetonitrile 96:4 (v/v). Flow rate, 0.6 ml/min and UV detection, 254 nm.

Table 1
Main validation parameters for acyclovir and guanine

	Quantification range		Impurities range	
	Acyclovir		Acyclovir	Guanine
Standards linearity				
Intercept	-82 ± 135		0.37 ± 0.19	0.28 ± 0.34
Slope	12762 ± 337		12804 ± 95	19019 ± 37
r	0.9996		0.99999	0.999998
Range (mg/ml)	0.3040–0.4800		0.0002–0.0040	0.0002–0.0200
Sample linearity				
Intercept	-41 ± 93		0.29 ± 0.13	0.11 ± 0.29
Slope	12632 ± 137		12795 ± 63	18763 ± 31
r	0.999		0.99997	0.999996
Accuracy % recovery				
Standard	99.99		99.4	98.4
R.S.D. (%)	0.47		1.8	3.4
Sample	99.82		98.7	96.4
R.S.D. (%)	0.18		1.2	6.7
Standards precision instrumental				
Intra-assay				
Mean (mg/ml)	0.39	$n = 10$	0.001 $n = 6$	0.002 $n = 6$
R.S.D. (%)	0.30		0.61	0.23
Intermediate				
Mean (mg/ml)	0.39	$n = 19$	0.001 $n = 12$	0.0002 $n = 12$
R.S.D. (%)	0.74		1.3	0.42
Sample precision instrumental				
Intra-assay				
Mean (mg/ml)	0.41	$n = 10$	0.001 $n = 6$	0.002 $n = 6$
R.S.D. (%)	0.23		0.69	0.51
Intermediate				
Mean (mg/ml)	0.41	$n = 20$	0.30 $n = 12$	0.002 $n = 12$
R.S.D. (%)	0.26		0.88	0.62
Precision methods				
Intra-assay				
Mean (mg/ml)	0.40	$n = 10$	–	–
R.S.D. (%)	0.67		–	–
Intermediate				
Mean (mg/ml)	0.40	$n = 20$	–	–
R.S.D. (%)	0.82		–	–

routine work. The final chromatogram of an acyclovir tablet at working conditions is shown in Fig. 7.

Validation has been performed following ICH guidelines [15–18] with standards and acyclovir tablets. Validation parameters are summarised in Table 1. Standards showed a good linearity as much for acyclovir in the quantification range as for acyclovir and guanine in the low range, with correlation coefficients over 0.999. No bias was found in the regression lines, because the intercepts with their limits of confidence include the zero value. It must be pointed out that the objective of validating the method at impurity level for acyclovir was to use a dilution of the standard to quantify all the impurities different from guanine with the corresponding response factors, because these impurities are not usually available.

R.S.D. values were low enough to consider the method precise as much for standards as for acyclovir samples. Recoveries do not statistically differ from 100% (*t*-test, $p < 0.05$) in any case. Response factors are included in Table 2 for the different impurities related to acyclovir. They were very near

Table 2
Response factors for impurities relative to acyclovir

Analyte	Response factor
Impurity A	0.6974
Impurity F	1.1732
Impurity Vir 3/4	0.9053
Impurity N^7-diacetilacyclovir	0.9459
Impurity G	0.9681

to 1, as the chromophore is very similar in all cases. Real limit of quantification of the method is the lower concentration value where it has been validated and therefore, it is 0.05% for all the compounds. The mathematical approach gave 0.004%. Limits of detection were 0.001% for both acyclovir and guanine, which are under the necessary values for the method.

Finally, for stability of the standard the recovery after 3 days was 100.0% with a R.S.D. value of 0.83%. Therefore, the standard can be considered stable for at least 72 h kept in solution at room temperature.

Although a formal robustness assay has not been achieved, this method has been applied over 6 months in a pharmaceutical formulations and it has always passed the system suitability test.

4. Conclusions

Agilent CN column has provided the development of a reversed phase HPLC method with UV detection. This column offers the separation of acyclovir, guanine and impurity A, under isocratic conditions and avoiding the employ of ion-pairing reagents, in less than 15 min of total run and less than 40 min if four additional impurities are included in the measurement. Furthermore the geometry of the column employed presents lower dimensions that allowed a 40% saving of solvents when compared with classical columns.

The method has been validated and it has been shown that it is reliable, being linear, accurate and precise both in upper and lower concentration range. Therefore, it can be applied for quantification of the active compound and its impurities.

At last, the employment of a mobile phase adjusted at pH 1.8, compatible with this kind of columns, permitted us also to evaluate the absence of impurity C that only appears in the synthetic process, assuring the selectivity of the method under lower aggressive conditions for the routine analysis.

References

[1] K. Balon, B.U. Riebesehl, B.W. Muller, Pharm. Res. 16 (1999) 882–888.
[2] K. Basavaiah, H.C. Prameela, Farmaco 57 (2002) 443–449.
[3] H.G. Daabees, Anal. Lett. 31 (1998) 1509–1522.
[4] M.S. Mahrous, M.M. Abdel Khalek, H.G. Daabees, Y.A. Beltagy, Anal. Lett. 31 (1992) 1491–1501.
[5] G.C.K. Battermann, S. Heizenroeder, D. Lubda, Labor Praxis 30 (1998) 32–34.
[6] Y. Pramar, V. Das Gupta, T. Zerai, Drug Dev. Ind. Pharm. 16 (1990) 1687–1695.
[7] S.S. Dubhashi, P.R. Vavia, Indian Drugs 37 (2000) 464–468.
[8] E. Kourany Lefoll, T.D. Cyr, Can. J. Appl. Spectrosc. 40 (1995) 155–159.
[9] A.M. Kamel, P.R. Brown, B. Munson, Anal. Chem. 71 (1999) 5481–5492.
[10] D.S. Ashton, A. Ray, Anal. Proc. 30 (1993) 44–46.
[11] The Official Compendia of Standards, U.S. Pharmacopeia National Formulary, 2004.
[12] I. Chappel, LC GC Int. 7 (1994) 282.
[13] K. Okusa, H. Tanaka, M. Ohira, J. Chromatogr. A 869 (2000) 143–149.
[14] W. Pleiderer, Ann. Chem. 647 (1961) 167.
[15] ICH, ICH Harmonised Tripartite Guideline, 1995, p. 1.
[16] ICH, ICH Harmonised Tripartite Guideline, 1996.
[17] ICH, ICH Harmonised Tripartite Guideline, 1996.
[18] ICH, ICH Harmonised Tripartite Guideline ICH topic Q2B, 1996.