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Improved RPLC determination of acyclovir using hexylamine as silanol masking agent ☆

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Abstract

The aim of the present work is to improve the sensitivity in the RPLC determination of acyclovir [9-(2-hydroxy ethoxymethyl) guanine] (ACV) and guanine, the major impurity of the drug synthesis and one of the compounds found in the chemical degradation process of ACV. The method was applied to the quantification of drug in liposomal formulations. The most important problem for RPLC analysis of both compounds are their high pK_a values, mainly guanine, and the interaction with reactive silanol groups in the stationary phase. In order to avoid these problems there are four basic strategies: (i) ionic pair reagents, (ii) deactivated silica columns, (iii) polymeric based columns and (iv) silanol masking agents. A validation protocol was followed to develop the analytical method, using a Spherisorb ODS (250 × 4.6 mm i.d.) analytical column, with a mobile phase of 95% aqueous phosphate buffer (pH 3.0) and 5% HPLC methanol pumped isocratically at 1.3 ml min^{-1}, with ultraviolet detection at 254 nm. The results showed a high reproducibility in retention time value, with R.S.D. of 2.37% for ACV and 0.32% for guanine. The lowest concentration levels assayed, 0.15 μg ml^{-1} for guanine and 1 μg $ml^{-1}$for ACV, showed good R.S.D. in the quantification parameter (peak area) 11.0% (guanine) and 9.64% (ACV)

Keywords: Acyclovir; Liposome; Reversed-phase HPLC

1. Introduction

Acyclovir (ACV) is still the first choice for the treatment of the diseases caused by herpes simplex virus (HSV) and herpes zoster virus (HZV) in humans 13 years after its approval for clinical use. The selectivity and low side effects are due to the mechanism of action of the drug. It acts in the replication phase of the virus. ACV is similar to the nucleoside guanine and it acts by inhibiting the viral DNA synthesis. The drug becomes phosphorilated; the first transformation is carried out by the viral timidinkinase (TK), so that explains the selective distribution of the drug to the infected cells [1].

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One of the main problems of this drug is its low bioavailability when it is administered in current dosage forms, and its brief half life in the plasma (2.3 h) [2]; these two problems make this drug a candidate for the encapsulation in a sustained release system such as liposomal formulations.

This report describes an HPLC method for determination of ACV and presents the results of the validation. For the validation process the following parameters were characterised: Retention time, linearity, precision, accuracy, quantification limits, detection limits.

2. Materials and methods

2.1. Materials

In the validation process standards of ACV and guanine, α-tocopherol hemisuccinate, Sodium deoxycholate (DOC), Cholesterol 90% and the silanol masking agent (hexylamine) were from Sigma–Aldrich (Madrid, Spain). Phospholipids were a gift from Lipoid GmbH (Ludwigshafen, Germany)

Pharmaceutical grade bulk drug used for encapsulation assays was from Heumann pharma.

HPLC water was prepared daily using a milli-Q system and Durapore HV filters were used in mobile phase preparation (Millipore). Merck (Germany) supplied *o*-phosphoric acid, H_2KPO_4 and HPLC methanol gradient grade.

2.2. Instrumentation

The chromatographic system was composed by an autosampler Waters 717 plus, a 626 S pump, and a photodiode array detector (PDA) Waters 996, and a Waters column oven.

Data were processed using the Millennium 2010 software running on a NEC image 466es computer. Spectral scans between 200 and 300 nm were obtained and signal was processed to extract a two-dimension chromatogram at 254 nm. The spectral resolution of the detector was set at 4.8 nm.

The stationary phase was a Spherisorb S5-ODS2 μm (250 × 4.6 mm) (Phenomenex) and the column temperature was set at 40°C.

The mobile phase flow was 1.3 ml min^{-1}, and the sample volume injected was 10 μl in all cases.

As mobile phase a binary mixture composed by methanol HPLC-gradient and monopotasium phosphate buffer (5 mM pH 3.0) 5:95 (v/v) was used. The buffer was made by adding 100 μl l^{-1} of hexylamine (7 mM), finally pH was adjusted at 3.0 by addition of *o*-phosphoric acid.

2.3. Standard solutions

Standard solutions of guanine and ACV were made considering the solubility characteristics of each one of the components [3,4],which are very restrictive for guanine. This has forced to prepare these solutions in a strongly acid medium (HCl 0.1 N), to avoid guanine precipitation. It has been considered the possible hydrolysis of the ACV, although references exists that indicate that under these conditions the degradation is minimal [5], as was tested elsewhere.

The solutions described above were used for preparation of standard curve samples and during the validation of the assay. Samples were diluted to final concentrations of 1, 5, 10, 20, 50, 100 and 150 μg ml^{-1} for ACV. The guanine concentration levels were 0.15, 0.75, 1.50, 3.00, 7.50, 15.00 and 22.50 μg ml^{-1}, based on the idea that the main component would always be ACV, while guanine would only appear as a degradation product or as impurity in bulk drug.

2.4. Sample preparation

Bulk drug samples were analysed prior to the liposome loading process, and were prepared as indicated in Section 2.3.

Liposome samples were prepared as described by Elorza et al. [6]. For the analysis of loaded liposomes the sample preparation process included an additional step, the addition of DOC as surfactant, in order to solubilize the phospholipid bilayer and release the encapsulated drug. The amount of DOC added to the sample is dependent on both, lipidic composition and lipid concentration, and it should be higher than the critical micellar concentration [6].

The concentration in samples was adjusted to reach a final level between 50 and 100 μg ml^{-1} of ACV. Finally samples were filtered through 0.45 μm syringe filters

3. Results and discussion

The physicochemical characteristics (pK_a) of guanine, [7,8] offers problems for the analysis with silica based columns when the usual technique of ionization suppression is used in the pH range supported by these stationary phases. Although this suppression can be obtained working close to neutral pH, the cost of the analyses increases since it is necessary a higher percentage of organic modifier in the mixture used as mobile phase to obtain similar retention times to those reported here. An alternative to the use of silanol maskers is the endcaped columns, but this is a more expensive alternative compared with the traditional C-18 packing. Another possibility would be the use of ionic pair reagents. This is a good choice when the manufacturing process is set, but when samples are unknown or for investigation proposes is difficult to adjust the amount of ionic pair reagent in the mobile phase. The ionic pair reagents present a maximum range of blocking charge activity, and if the analyte concentration is very high the blocking effect can be collapsed.

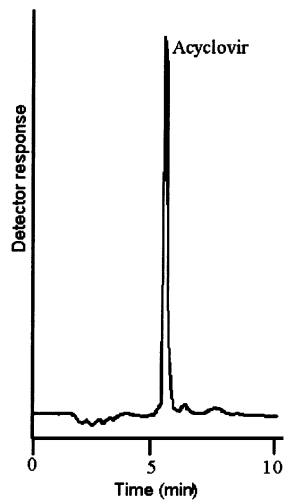

Fig. 1. Chromatogram of ACV in liposome sample.

The silanol effect takes place among charged basic species and silanol groups that show acid behaviour and are accessible to the ionized analyte [9]. This leads to peak broadening, and the overall behaviour does not follow the retention mechanism for hydrophobic interaction between analyte and bonded phase.

Silanol masking agents act in a similar way to the ionic pair reagents; the difference between them is that silanol maskers link to the stationary phase matrix, and ionic pair reagents link to the analyte, rather than to the silanol groups of the stationary phase.

Silanol maskers are usually amines, and there is an important debate on the effectiveness of the different types of amine and the length of the alkilic chain (e.g. hexylamine, trimethylamine) [10]. The silanol effect is difficult to quantify, since diverse types of silanol groups exist, because of differences in accessibility (steric effect), packaging effects and the polarity of the silica used [10,11]. All these phenomenon lead to great batch to batch variations in the chromatographic behaviour of columns.

3.1. Sample preparation

During the process of sample preparation, no modification has been detected in the physicochemical characteristics of ACV and guanine. Studies exist about the chemical stability of ACV [5]. Liposome samples suffered a treatment that did not suppose any chemical attack. The surfactant agent (DOC) did not interfere in the analysis, since it does not elute under our analytic conditions. Due to its hydrophobic character it is strongly retained, as the rest of the components of the liposomal bilayer, by means of the notable increase in the hydrophobic character of the stationary phase that takes place when hexylamine is added to the mobile phase as showed in Fig. 1.

None of the components in the sample, except for both guanine and ACV, absorb at the wavelength registered in the chromatogram (254 nm) [12].

Table 1
Chromatographic characterisitcs of guanine and acyclovir[a]

	RT (min)	K'	N	W_b (min)
Guanine	3.106 ± 0.01	0.635	4926	0.177
Acyclovir	6.736 ± 0.16	2.742	1882	0.621

[a] RT, retention time in min; K', capacity factor; N, number of theoretical plates using USP tangent method; W_b, baseline width.

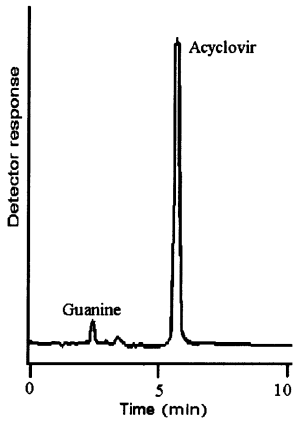

Fig. 2. Chromatogram of bulk drug containing ACV and guanine.

Table 2
Retention time precision for samples of guanine and acyclovir ($n = 63$) injected over a 3-day period

	Mean	S.D.	R.S.D. (%)
Guanine	3.106	0.01	0.32
Acyclovir	6.736	0.16	2.37

3.2. Characteristics of the chromatographic peaks

ACV and guanine eluted in short times, particularly guanine, whose retention time was very close to the void volume of the chromatographic system. Both peaks are well resolved and no interference has been detected as showed in Table 1. In Fig. 2 a typical chromatogram of bulk drug analysis is presented. The precision in retention times was evaluated by injection of 63 samples in a period of 3 consecutive days, the results are described in Table 2, precision of retention time is acceptable for the identification proposes.

3.3. Accuracy

The accuracy of the method assayed for ACV quantification was evaluated using data points from the standard curves, by comparison of the expected amounts and the amounts calculated by linear regression, expressed as deviation in %.

The results are showed in Table 3 and indicate a good accuracy level; for ACV the observed deviation was 20.5% at 1 μg ml^{-1}, and for guanine at 0.15 μg ml^{-1} this value was 50.8%. These results meet the requirements for ACV determination in all the concentrations assayed, for guanine the deviation at 0.15 μg ml^{-1} is extremely high, but this is not very important because of the small concentration assayed, and guanine can be determined in an adequate way starting from 0.75 μg ml^{-1} or higher [13].

3.4. Linearity

The linearity of the assay procedure was determined by calculation of a regression line using the least squares analysis and by investigating the accuracy of the assayed method.

The results of the regression analysis were linear in the whole concentration range for ACV (1–150 μg ml^{-1}) and guanine (0.15–22.5 μg ml^{-1}), as indicates the results of the residual distribution (runs test) [14].

Determination coefficient (r^2) values were between 0.9990 and 0.9999 ($n = 21$) for ACV and guanine respectively.

3.5. Precision

During the process of validation within-day variations (Table 4) have been studied analysing three replicates of standards each day for 3 days [15], the result indicate an acceptable precision for all concentrations assayed in measures made in the same day.

Between-day variation (Table 5) has been studied for analyses made during 3 consecutive days,

the results for the lowest concentrations expressed as R.S.D. were 9.9 (ACV 1 μg ml^{-1}) and 9.6 (guanine 0.15 μg ml^{-1}). This procedure is not as precise for low concentrations as some referred by the bibliography, but most of them use variable wavelength UV detectors instead of PDA, and the concentration range assayed is wider.

3.6. Quantification limits

To determine the detection limit and quantification limit many methods are used, as the study of the signal–noise ratio, or the study of the ordinate in the origin of the regression line. The quantification limit is defined as the lowest con-

Table 3
Accuracy of area values ($n = 63$) for acyclovir and guanine

Acyclovir		Guanine	
Concentration (μg ml^{-1})	Deviation (%)	Concentration (μg ml^{-1})	Deviation (%)
1	20.5	0.15	50.8
5	4.1	0.75	5.7
10	3.4	1.50	3.8
20	1.8	3.00	1.1
50	2.8	7.50	3.5
100	1.3	15.00	1.2
150	0.6	22.50	0.9

Table 4
Mean within-day variability ($n = 3$) for determination of acyclovir and guanine

Acyclovir		Guanine	
Concentration (μg ml^{-1})	R.S.D. (%)	Concentration (μg ml^{-1})	R.S.D. (%)
1	2.2	0.15	5.3
5	1.2	0.75	2.4
10	1.4	1.50	0.6
20	1.9	3.00	0.7
50	1.9	7.50	0.7
100	1.4	15.00	1.7
150	0.7	22.50	1.0

Table 5
Between-day variability ($n = 9$) for determination of acyclovir and guanine

Acyclovir		Guanine	
Concentration (μg ml^{-1})	R.S.D. (%)	Concentration (μg ml^{-1})	R.S.D. (%)
1	9.9	0.15	9.6
5	5.2	0.75	8.4
10	7.0	1.50	8.2
20	6.3	3.00	9.9
50	6.2	7.50	8.0
100	6.3	15.00	8.8
150	4.2	22.50	7.0

centration that can be measured with adequate accuracy [13]; in this case ACV can be detected and quantified under these conditions at concentrations of 1 μg ml^{-1}, while guanine can be detected at 0.15 μg ml^{-1}, but as indicated in Section 3.5, quantification is possible at concentrations of 0.75 μg ml^{-1} or higher.

3.7. Selectivity

The selectivity of the method for the two substances analysed is adequate, since the peaks come out very separate. For the rest of components that can be found in liposome samples (phospholipids cholesterol, α-tocopherol hemisuccinate, and DOC) no interference has been detected with guanine or ACV.

This behaviour of the lipidic compounds can be explained by the great increase in the hydrophobic character of the column when the silanol masker is present, making very difficult the elution of these lipophilic compounds with a mobile phase that contains only 5% of organic modifier.

This information was obtained comparing the spectral data associated to each chromatographic peak, comparing the second derivative of the spectrum and studying the purity angle.

4. Conclusions

An improved HPLC assay procedure for determination of ACV in presence of guanine and its validation, as well as its application to liposomal samples is described. This assay was found to be linear and suitable for quantification of ACV in the manufacture process of liposomes loaded with ACV and for bulk drug control. The limit of quantification was 1 μg ml^{-1} for ACV and 0.75 μg ml^{-1} for guanine. Area precision and accuracy of the predicted values were acceptable for quantification of ACV and guanine in liposomal formulations.

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References

[1] A. Goodman Gilman (Ed.), The Pharmacological Bases of Therapy, 9th edn, McGraw-Hill Interamericana, Mexico City, 1996.
[2] M. Yamazaki, S. Itoh, M. Sawanoi, M. Kobayashi, S. Suzuky, T. Komatsu, K. Tanabe, J. Pharm. Pharmacol. 42 (1990) 441–443.
[3] Handbook of Physics and Chemistry, 73rd edn, CRC Press, Boca Raton, FL, 1993.
[4] The Index Merck, 12th edn, Merck, New York, 1997.
[5] G. Giamona, G. Puglisi, G. Cavallaro, A. Spadaro, G. Pitarresi, J. Control. Release 33 (1995) 261–271.
[6] M.A. Elorza, B. Elorza, J.R. Chantres, Int. J. Pharm. 158 (1997) 173–183.
[7] A. Kristl, A. Mrahr, F. Kozjek, Int. J. Pharm. 99 (1993) 79–82.
[8] A. Kristl, S. Srcic, F. Vrecer, B. Sustar, D. Bojnovic, Int. J. Pharm. 139 (1996) 231–235.
[9] D. Chan Leach, M.A. Stadalius, J.S. Berus, L.R. Snyder, LC·GC 6 (1988) 494.
[10] A.I. Gaso-Lopez, A. Santos-Montes, R. Izquierdo-Hornillos, J. Chromatogr. Sci. 93 (1996) 253–265.
[11] D.W. Hill, A.J. Kind, J. Liquid Chromatogr. 16 (18) (1993) 3941–3964.
[12] Avanti Polar Lipids Inc. Catalog, 1994.
[13] C. Hartmann, J. Smeyers-Verbeke, D.L. Massart, R.D. McDowall, J. Pharm. Biomed. Anal. 17 (1998) 193–218.
[14] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, 2nd edn., Ellis Horwood, London, 1988.
[15] P.S. Vinod, K.K. Midha, S. Dighe, et al., J. Pharm. Sci. 81 (1992) 309–312.