Development and Validation of an RP-HPLC Method to Quantitate Acyclovir in Cross-Linked Chitosan Microspheres Produced by Spray Drying

Hellen Karine Stulzer[1,2,*], Monika Piazzon Tagliari[2], Fábio S. Murakami[2], Marcos A.S. Silva[2], and **Mauro C.M. Laranjeira[1]**

[1]Laboratório Quitech, Departamento de Química, Universidade Federal de Santa Catarina; [2]Laboratório de Controle de Qualidade, Departamento de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, Campus Universitário Trindade, bloco K, 3º andar, Brasil, Florianópolis, SC, CEP 88040-900

Abstract

An accurate, simple, reproducible, and sensitive liquid chromatographic method is developed and validated to quantitate acyclovir (ACV) in cross-linked chitosan microspheres produced by spray drying. The analysis is carried out using a reversed-phase C_{18} column with UV–vis detection at 254 nm. The mobile phase is diluted with pure water and acetonitrile (95:5 v/v) at a flow-rate of 0.8 mL/min. The parameters used in the validation process are: linearity, range, quantitation limit, detection limit, accuracy, specificity precision, and ruggedness. The retention time of acyclovir is approximately 3.5 min with symmetrical peaks. The linearity in the range of 1–10 µg/mL presents a correlation coefficient of 0.9999. The chitosan and the tripolyphosphate in the formulation do not interfere with the analysis, and the recovery is quantitative. Results are satisfactory, and the method proves to be suitable to quantitate ACV in cross-linked chitosan microspheres.

Introduction

Acyclovir (ACV) (Figure 1) is a synthetic analogue of 2-deoxyguanosine with antiviral activity. It is a highly potent inhibitor of herpes simplex virus (HSV) types 1 and 2 and varicella zoster virus, which remain as common viral infections in humans. Over the past decade, the incidence and severity of infections caused by HSV have increased due to the growth in number of immuno-compromized patients, produced by aggressive chemotherapy regimens, expanded organ transplantation, and greater occurrence of human immunodeficiency virus infections (1,2).

ACV owes its antiherpetic selectivity to preferential phosphorylation by the virus encoded thymidine kinase (TK), which confines further action to the virus-infected cell (3,4). According to the type of infection, it can be administered by intravenous infusion, as the sodium salt, in a final concentration not greater than 5 mg/mL, by oral administration in a dose of 200 mg five times daily, or by topical administration as ointment or cream containing 5% of drug, to be applied 5–6 times daily. ACV has quite a short lifetime in plasma and when administered orally it is scarcely absorbed by the gastrointestinal tract, which are responsible for its innumerous administration daily (5). Thus, it presents a low oral bioavailability (20%) and a short circulation half-life ($t_{½}$ 2.5 h) (6). Therefore, limited solubility of the drug, both in water and lipids, is a reason for its quite low bioavailability, especially in oral dosage forms.

In this way, the development of a delivery system for oral administration of ACV, able to provide a sustained release and improved bioavailability of the drug, is desirable. ACV was entrapped in chitosan microspheres prepared by the spray drying method, whereby different concentrations of tripolyphosphate cross linked were used to promote different release profile of ACV.

Spray-drying is a technique widely used in the pharmaceutical field to dry materials susceptible to heat, to improve the drug solubility, or the flowability of particular excipients, and several others applications. Recently, this technique has received considerable interest as a microencapsulation process to obtain a controlled delivery system. The method may offer, in comparison with the usual coating techniques, the advantage of realizing the micro-encapsulation process in one step (7,8,9).

Chitosan is currently gaining a great deal of attention for

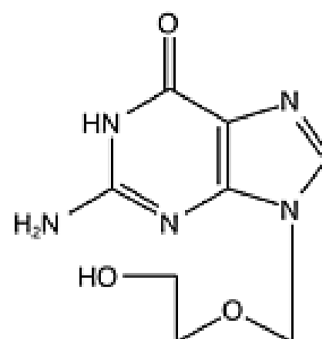

Figure 1. Chemical structure of acyclovir.

* Author to whom correspondence should be addressed: email hellen.stulzer@gmail.com.

Reproduction (photocopying) of editorial content of this journal is prohibited without publisher's permission.

pharmaceutical applications as well as for the controlled release of drugs. The successes of chitosan microspheres as carriers is due the following features: (*i*) they can dissolve poorly soluble drugs and, thus, increase their bioavailability; (*ii*) they can stay in the body (in the blood) long enough time to provide gradual accumulation in the required area; (*iii*) their size permits them to accumulate in body regions with leaky vasculature; (*iv*) they can be tailored to achieve targeting or other desired properties by attachment of a specific ligand to the outer surface; (*v*) they have low toxicity and a high loading capacity, as well as minimizing drug degradation and loss; and (*vi*) they can be easily produced in large quantities (10–14).

The therapeutic importance of ACV has prompted the development of many formulations and methods for its assay. Several methods have been reported for ACV determination in pharmaceutical formulations, based on various analytical techniques. Huidobro and co-workers reported a liquid chromatography (LC) method to quantitate ACV and related impurities avoiding ion-pairing reagents (t_R of ACV 5.8 min). Baeyens et al. presented a fluorimetric HPLC assay for ACV analysis in pharmaceuticals formulations using a micellar mobile phase (t_R of ACV 8.0 min). Neubert et al. described micellar electrokinetic chromatography with UV detection (t_M of ACV 4.0 min), and Zhang et al. used capillary electrophoresis with amperometric detection (t_M of ACV 9.0 min) (15–18).

For routine quality control, the development of a simple, rapid, and sensitive method is highly desirable. LC assays for determination of ACV in cross-linked chitosan microspheres produced by spray drying have not been reported. Since our research involves development of cross-linked chitosan microspheres containing ACV, the present paper describes a rapid, simple, and precise LC method for ACV determination in these pharmaceutical formulations. The main advantage of the proposed method is the simplicity of the mobile phase (acetonitrile–water) and short time of analysis.

Table I. The Recovery Test Performed

Fortified solution	Sample solution	Standard solution
3 µg/mL	2 µg/mL	1 µg/mL
7 µg/mL	2 µg/mL	5 µg/mL
10 µg/mL	2 µg/mL	8 µg/mL

Table II. The Linearity, Quantitation, and Detection Limits

Range of Linearity (µg/mL)	Peak area (mean and RSD)*
1	103036 ± 0.013
3	306289 ± 0.068
5	503997 ± 1.120
7	716928 ± 0.711
10	1014936 ± 0.441
Equation	$y = 101532x + 1069$
r^2	0.9999
QL	0.85 µg/mL
DL	0.48 µg/mL

* Mean ± RSD (n = 3).

Experimental

Reagents and Standard

The ACV raw material was received from Shenyang Fine Chemical Co., China. The reference standard with stated purity of 100% was obtained from Brazilian Pharmacopoeia (Brazil). Ultrapure water was obtained from a Milli-Q purification system by Millipore. Acetonitrile of HPLC grade was purchased from Vetec (Brazil). Other solvents and reagents used were analytical grade.

Development of acyclovir cross-linked chitosan microspheres

ACV (200 mg) and chitosan (2 g) were dissolved in 200 mL of acetic acid 0.1M solution, with constant stirring at 5000 rpm for 1 h. Different volumes (1, 3, and 5 mL) of cross-linked tripolyphosphate (TPP) 2% aqueous solutions were added dropwise into the acetic acid–chitosan–drug solution with constant stirring. Thus, the different chitosan–TPP–drug solutions were spray dried to obtain the cross-linked chitosan microspheres loaded with ACV. The process was carried out using laboratory scale spray dryer Buchi (model B-191, Switzerland) under the following set conditions: inlet temperature, 180°C; outlet temperature, 92°C; feed rate, 7 mL/min; airflow rate, 500 L/h; and aspirator, 100%.

Instrumentation and chromatography

The HPLC analysis were performed on a Shimadzu LC-10 system (Kyoto, Japan) equipped with a LC-10AD pump, SPD-10AV UV detector (set at 254 nm), SCL-10Avp controller unit, and the sample injection was performed via a Rheodyne 7125 valve with a 20 µL loop. A reversed-phase C_{18} Phenomenex column (150 mm × 4, 6 mm i.d., 5 µm particle size) was employed. The mobile phase was diluted with pure water and acetonitrile (95:5 v/v) at a flow-rate of 0.8 mL/min. The HPLC system was operated at 40 ± 1°C temperature. The volume of injection was 20 µL for all standards and samples. Data acquisition was performed using CLASS-VP software with measurement of detected peak areas.

Table III. Results From the Precision Assays

Sample solution (5 µg/mL)	Mean peak area ± RSD*	Sample solution (5 µg/mL)	Mean peak area ± RSD*
Day 1	526873 ± 0.17	Morning	526873 ± 0.17
Day 2	527322 ± 0.44	Afternoon	528611 ± 0.13
Day 3	528361 ± 0.11	Night	528395 ± 0.12
Inter-day	527518 ± 0.24	Same day	527959 ± 0.14

* Mean ± RSD (n = 6).

Standard and sample preparation

Standard preparation

A stock standard solution of 50 µg/mL was prepared by dissolving 10 mg of the ACV reference standard, accurately weighed, in 10 mL of sodium hydroxide 0.1N in a 200-mL volumetric flask and stirred in an ultrasonic bath for 10 min. The volume was completed with the mobile phase.

Sample preparation

A cross-linked microspheres sample loaded with 10 mg of ACV was accurately weight and dissolved in 10 mL of 90% ethanol in a 200-mL volumetric flask and stirred in an ultrasonic bath for 15 min to extract the drug from the microspheres. The volume was completed with the mobile phase. A volume (5 mL) of this solution was diluted with mobile phase in a 50-mL volumetric flask (5 µg/mL). The sample used for specificity assay was prepared in the same form, but the volume taken was 6 mL, and the final concentration was 6 µg/mL.

Method validation

The method was validated to quantitate the ACV in cross-linked chitosan microspheres based on International Conference on Harmonization guidelines Q2 (R1) (19), by the determination of the following parameters: linearity, range, quantitation and detection limits, accuracy, specificity, precision, and ruggedness.

Linearity and range

The linearity of response was assessed in the range of 1–10 µg/mL. The appropriate amounts of stock solution were diluted with mobile phase, yielding concentrations of 1, 3, 5, 7, and 10 µg/mL. Triplicate injections of each sample were carried out. Peak area ratios of standard compounds were plotted against theoretical concentrations of standards. The linearity was expressed as a correlation coefficient by linear regression analysis.

Table IV. Results from the Recovery Test

Fortified solution	Mean peak area ± RSD	Mean recovery ± RSD*
3 µg/mL	306289 ± 0.06	99.5 ± 0.05
7 µg/mL	716828 ± 0.71	101.2 ± 0.68
10 µg/mL	1014936 ± 0.44	100.3 ± 1.16

* Mean ± RSD ($n = 3$).

Quantitation limit and detection limit

The quantitation limit (QL) was based on the standard deviation of the response and the slope of the constructed calibration curve. The QL may be expressed as:

$$QL = 10\sigma/S$$

and the DL was expressed by the following equation:

$$DL = 3.3\sigma/S$$

where σ is the standard deviation of the response and S is the slope of the calibration curve.

Specificity

The specificity was evaluated by the analysis of placebo microspheres (microspheres without ACV). Triplicate injections of each sample were made.

Precision

The repeatability of the analytical method was evaluated by analyzing six samples of ACV 5 µg/mL, during the same day and under the same experimental conditions. Intermediate precision was evaluated by the analysis of solutions on 3 different days. Peak areas were determined and compared. Precision was expressed as a percentage relative standard deviation (RSD).

Accuracy

The accuracy of the developed method was evaluated by a recovery test. An ACV sample of 2 µg/mL was fortified with 3 known concentrations of reference standards at 3 different levels: lower, medium, and upper concentrations (Table I). The recovery of added standard was determined in triplicate analysis and calculated with the use of the formula:

$$R\% = (Fs - St/Ss) \times 100$$

in which R is the recovery, Fs is the fortified solution, Ss is the sample solution, and St is the standard solution.

Ruggedness

The method ruggedness was established by introducing small changes in these parameters: flow rate (0.8 mL/min ± 0.2), pH of mobile phase (2.5 ± 0.5), and wavelength (254 nm ± 2). These parameters were studied using triplicate injections at a concentration level of 5 µg/mL. Data were treated to calculate %RSD.

Table V. Results from the Ruggedness Test

Flow rate		pH		Wavelength	
Sample solution (5 µg/mL)	Mean peak area ± RSD*	Sample solution (5 µg/mL)	Mean peak area ± RSD*	Sample solution (5 µg/mL)	Mean peak area ± RSD*
0.6 mL/min	505422 ± 0.44	2.0	520562 ± 0.36	252 nm	530582 ± 0.35
0.8 mL/min	510512 ± 0.64	2.5	510822 ± 0.81	254 nm	529212 ± 0.25
1.0 mL/min	509513 ± 0.85	3.0	500562 ± 0.78	256 nm	519582 ± 0.53

* Mean ± RSD ($n = 3$).

Results and Discussion

Validation of analytical methods for quality control in pharmaceutical formulations is essential. The proposed method uses a simple mobile phase and is considered more useful than LC methods cited in the literature. The physical-chemical prop-

erties of ACV, such as solubility, polarity, and UV absorption, influenced the chromatographic conditions. The mobile phase was chosen after several trials with water and acetonitrile. The optimum mobile phase was: water–acetonitrile (95:5 v/v) using a reversed-phase C_{18} column. The UV spectrum of acyclovir shows an intense absorption at 254 nm, and this wavelength was chosen for the analysis.

Linearity of the detector response was determined by the preparation of calibrations graphs. The linearity of the peak area responses versus concentration was studied from 1 to 10 µg/mL. The representative linear equation for ACV was: $y = 101532x + 1069$. The quantitation and the detection limits calculated were 0.85 µg/mL and 0.48 µg/mL, respectively, which demonstrated the adequate sensitivity of the method. The results are shown in Table II.

The specificity of the method was evaluated by the analysis of cross-linked chitosan microspheres samples without ACV (placebo). The chromatograms presented in Figure 2 showed that the method is specific. The corresponding peak of ACV loaded in cross-linked chitosan microspheres (Figure 2B) was confirmed by the comparison of retention time and the obtained area from the reference standard (Figure 2A). The placebo chromatogram given in Figure 2C evidenced no interference or overlaps of the chitosan and tripolyphosphate with the ACV response at the detection wavelength of 254 nm.

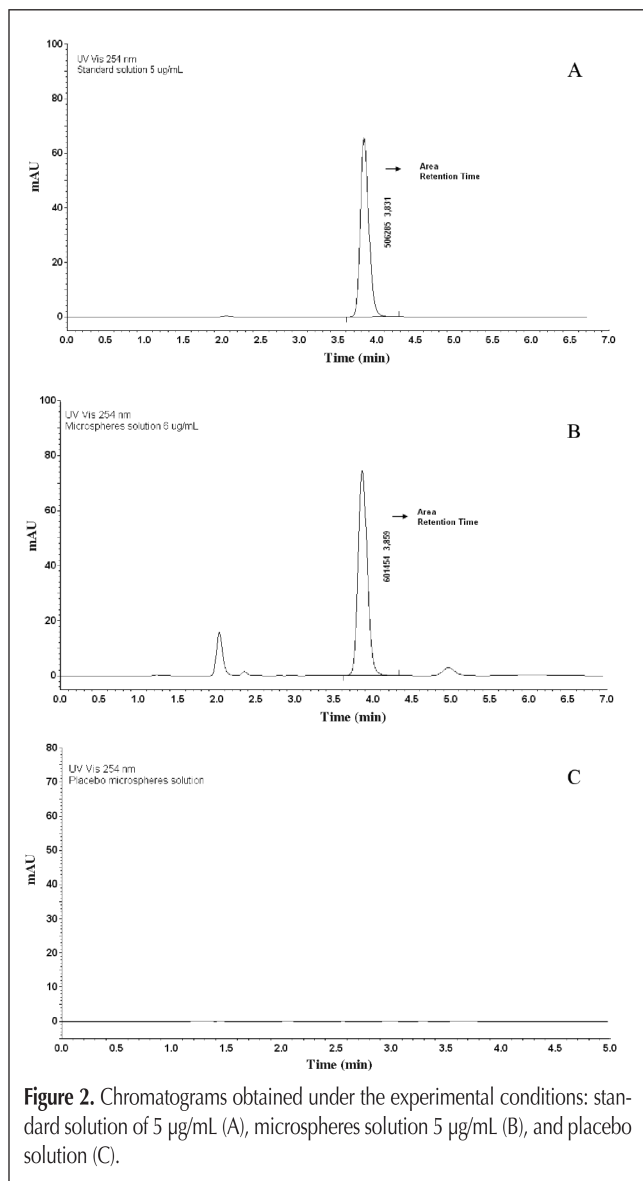

Figure 2. Chromatograms obtained under the experimental conditions: standard solution of 5 µg/mL (A), microspheres solution 5 µg/mL (B), and placebo solution (C).

The repeatability and intermediate precision of the method was evaluated on the same day (morning, afternoon, and night) and three different days. It was performed by replicate injections (n = 6) of a 5 µg/mL ACV sample solution. The mean peak area and the relative standard deviation are reported in Table III. The obtained results indicate acceptable precision.

The accuracy was determined via a recovery test by fortifying the samples with ACV standard at 3 levels and assayed by the developed method. The percentage recovery was calculated from the differences between the peak areas obtained for fortified and sample solutions (Table IV).

Deliberate variation of the method conditions (wavelength, flow rate, and pH of mobile phase) had no significant effect on the assay results or on the chromatographic performance, indicating the ruggedness of method and its suitability for transfer to other laboratories and equipments. The results from the ruggedness testing are presented in Table V.

Conclusions

The LC method developed in this study has the advantage of simplicity, precision, accuracy, and convenience. The method uses simple reagents and minimal sample preparation procedures. The results demonstrated that this method is useful for routine quality control analysis of ACV in cross-linked chitosan microspheres. The method described a short analysis time and a simple mobile phase, which was an important advantage in relation with other methods reported in the literature.

Acknowledgments

The authors acknowledge to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for fellowships and Conselho Nacional de Desenvolvimento Científico and Tecnológico (CNPq) for the financial support.

References

1. G. Giammona, G. Puglishi, G. Cavallaro, A. Spadaro, and G. Pitarresi. Chemical stability and biovailability of acyclovir coupled to α,β-poly (N-2-hydroxyethyl)- DL-aspartamide. *J. Controlled Release* **33:** 261–71(1995).
2. E.G. Jalón, M.J. Blanco-Príeto, P. Ygartua, and S. Santoyo. Increased efficacy of acyclovir-loaded microparticles against herpes simples virus type 1 in cell culture. *Eur J. Pharmaceut. Biopharmaceut.* **56:** 183–87 (2003).
3. M. Suzuki, T. Okuda, and K. Shiraki. Synergistic antiviral activity of acyclovir and vidarabine against herpes simplex virus types 1 and 2 and varicella-zoster vírus. *Antiviral Res.* **72:** 157–61 (2006).

4. B. Golankiewicz and T. Ostrowski. Tricyclic nucleoside analogues as antiherpes agents. *Antiviral Res.* **71:** 134–40 (2006).
5. B. Conti, C. Bucolo, C. Giannavola, G. Puglishe, P. Giunchedi, and U. Conte. Biodegradable microspheres for the intravitreal administration of acyclovir: in vitro/in vivo evaluation. *Eur. J. Pharmaceut. Biopharmaceut.* **5:** 287–93 (1997).
6. Y. Jin, L. Tong, P. Ai, M. Li, and X. Hou. Self-assembled drug delivery systems: 1. Properties and in vitro/in vivo behavior of acyclovir self-assembled nanoparticles (SAN). *Int. J. Pharm.* **309:** 199–207 (2006).
7. R. Bodmeier and H. Kranz. A novel in situ forming drug delivery system for controlled parenteral drug delivery. *Int. J. Pharm.* **332:** 107–14 (2007).
8. S.K. Broadhead, E. Rouan, and C.T. Rhodes.The spray drying of pharmaceuticals. *Drug Dev. Ind. Pharm.* **18:** 1169–1206 (1992).
9. P. Dangprasirt and G.C. Ritthidej. Development of diclofenac sodium controlled release solid dispersion by spray drying using optimization strategy I. Powder formulation. *Drug Dev. Ind. Pharm.* **21:** 2323–37 (1995).
10. R. Hejazi and M. Amiji. Chitosan-based gastrointestinal delivery systems. *Controlled Release* **89:** 151–63 (2003).
11. S.L. Kosaraju, L. D'ath, and A. Lawrence. Preparation and characterisation of chitosan microspheres for antioxidant delivery. *Carbohydrate Polymers* **64:** 163–67 (2006).
12. J.C. Kim, H.Y. Lee, M.H. Kim, H.J. Lee, H.Y. Kang, and S.M, Kim. Preparation and characterization of chitosan/gelatin microcapsules containing triclosan. *Colloids and Surf B.* **52:** 52–56 (2006).
13. V.R. Sinha, A.K. Singla, S.Wadhawan, R. Kaushik, R. Kumria, K. Bansal, and S. Dhawan. Chitosan microspheres as a potencial carries for dugs. *Int. J. Pharm.* **274:** 1–33 (2004).
14. U. Guliyeva, F. Oner, S. Ozsoy, and R. Haziroglu. Chitosan microparticles containing plasmid DNA as potential oral gene delivery system. *Eur. J. Pharm. Biopharm.* **62:** 17–25 (2006).
15. A.L. Huidobro, F.J. Ruperez, and C. Barbas, J. LC methods for acyclovir and related impurities determination. *Pharm. Biomed. Anal.* **37:** 687–94 (2005).
16. W.R.G. Baeyens, G. van der Weken, Y. Zhao, and A.M. Garcia-Campana. Extended abstracts from Xth International Symposium on Luminescence Spectrometry. *Luminescence* **17:** 210–13 (2002).
17. R.H.H. Neubert, Y. Mrestani, M. Schwarz, and B. Colin. Application of micellar electrokinetic chromatography for analyzing antiviral drugs in pharmaceutical semisolid formulations. *J. Pharm. Biomed. Anal.* **16:** 893–97 (1998).
18. S.S. Zhang, Z.B. Yuan, H.X. Liu, H. Zou, H. Xiong, and Y.J. Wu. Analysis of acyclovir by high performance capillary electrophoresis with on-column amperometric detection. *Electrophoresis* **21:** 2995–98 (2000).
19. ICH International Conference on Harmonization. Validation of Analytical Procedures: ethodology, 1996, Geneva.

Manuscript received February 8, 2007;
revision received September 11, 2007.