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Development and validation of a rapid reversed-phase HPLC method for the determination of the non-nucleoside reverse transcriptase inhibitor dapivirine from polymeric nanoparticles

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ABSTRACT

The objective of this work was to develop and validate a rapid reversed-phase (RP) high-performance liquid chromatography (HPLC) method for the *in vitro* pharmaceutical characterization of dapivirine-loaded polymeric nanoparticles. Chromatographic runs were performed on a RP C18 column with a mobile phase comprising acetonitrile–0.5% (w/v) triethanolamine solution in isocratic mode (80:20, v/v) at a flow rate of 1 ml/min. Dapivirine was detected at a wavelength of 290 nm. The method was shown to be specific, linear in the range of 1–50 μg/ml (R^2 = 0.9998), precise at the intra-day and inter-day levels as reflected by the relative standard deviation values (less than 0.85%), accurate (recovery rate of 100.17 ± 0.35%), and robust to changes in the mobile phase and column brand. The detection and quantitation limits were 0.08 and 0.24 μg/ml, respectively. The method was successfully used to determine the loading capacity and association efficiency of dapivirine in poly(lactic-*co*-glycolic acid)-based nanoparticles and its *in vitro* release.

1. Introduction

Dapivirine (TMC120; R147681; 4-[[4-[2,4,6-trimethylphenyl) amino]-2-pyrimidinyl]-amino]-benzonitrile) (Fig. 1) is a highly potent non-nucleoside reverse transcriptase inhibitor from the diarylpyrimidine (DAPY) class currently being tested as a candidate microbicide drug [1]. Both *in vitro* [2,3] and animal *in vivo* [4] testing demonstrated that dapivirine presents enough potential to be tested in the prevention of vaginal HIV-1 transmission, either alone or in association, combining it with a favorable local safety profile and advantageous pharmacokinetic properties [5]. Presently, this microbicide drug is undergoing clinical testing with early trials showing encouraging results [6,7].

The eventual success of microbicides is thought to be strongly dependent on adequate formulation of candidate drugs and is intimately related with the particularities of the vaginal route. Up to now, dapivirine has been formulated as semisolid preparations (e.g. polymeric hydrophilic gels) [4,5] or vaginal rings [8,9] but other novel dosage forms or delivery systems may also present potentially advantageous properties that justify their investigation for the vaginal administration of dapivirine. Particularly, polymeric drug nanocarriers have been suggested recently as an interesting strategy for the development of next-generation microbicides [10]. Some of the advantageous features presented by antiretroviral-loaded nanoparticles for microbicides formulation are the ability to modulate drug release, the capacity to penetrate epithelial linings, and the possibility of providing specific drug targeting to HIV-target cells.

In order to fully characterize dapivirine candidate formulations or delivery systems such as polymeric nanoparticles, suitable and validated quantification methods are required to assess critical pharmaceutical parameters such as drug content, release or stability. A few HPLC [8,9] and a brief reference to one UPLC [11] methods for dapivirine have been described in the literature, mainly with the objective of measuring drug release from different vaginal rings. However, no data on the optimization and validation of these chromatographic methods has been reported, being characterization studies usually limited to linearity. Thus, the objective of this work is to develop and validate a fast, simple and optimized reversed-phase HPLC method to quantify the content and *in vitro* release of dapivirine incorporated in poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles.

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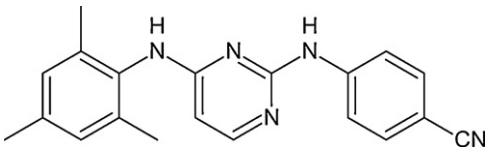

Fig. 1. Chemical structure of dapivirine.

2. Materials and methods

2.1. Materials

Dapivirine (99.8%, w/w, HPLC) was kindly provided by the International Partnership for Microbicides and used as received. HPLC grade acetonitrile was purchased from Fisher Scientifics UK Limited (Loughborough, UK) and triethanolamine (TEA) 99% was acquired from Guinama S.L.U. (Valencia, Spain). Acid terminated PLGA in a 50/50 molar ratio and an inherent viscosity of 0.40 dl/g (Purasorb PDLG 5004A) was a kind offer from Purac Biomaterials (Gorinchem, The Netherlands). Poly(vinyl alcohol) (PVA) 87–90% hydrolyzed (MW: 30,000–70,000) was purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade water was prepared in-house by using Simplicity® UV Ultrapure Water System (Millipore Corporation, Billerica, USA). All other reagents and solvents were of analytic or HPLC grade. Prior to use, mobile phase solvents were degassed in an ultrasonic bath for 15 min.

2.2. Equipment

All HPLC runs were performed using an Agilent 1100 Series Liquid Chromatography apparatus (Agilent Technologies, Santa Clara, USA), equipped with a G1322A vacuum degasser, a G1311A quaternary pump, a G1313A autosampler, and a G1314A variable UV–vis wavelength detector. Results were acquired and processed with the Agilent ChemStation data system software (Rev. A.07.01; Agilent Technologies, Santa Clara, CA, USA). HPLC analysis was conducted by using a RP C18 column (TR-010004 Mediterranea Sea_{18}; Teknokroma, Barcelona, Spain), with 5 μm particle size, 4.6 mm internal diameter, and 150 mm length.

2.3. Chromatographic conditions

Chromatographic analysis was performed in isocratic mode. Mobile phase consisted of acetonitrile–0.5% (w/v) TEA solution (80:20, v/v), being pumped at a flow rate of 1 ml/min. Sample injection volume was 20 μl and detection wavelength was 290 nm. Total run time was 7 min. All experiments were performed at room temperature and the total area of peak was used to quantify dapivirine.

2.4. Preparation of standard and sample solutions

A stock standard solution of 1 mg/ml of dapivirine was prepared by accurately weighing approximately 100 mg of dapivirine into a 100 ml volumetric flask and making up to volume with acetonitrile. Two working standard solutions (100 and 10 μg/ml) were obtained by further dilution of the stock standard solution with acetonitrile. Seven standard solutions (1, 5, 10, 20, 30, 40 and 50 μg/ml) were obtained by measuring the required amount of 100 μg/ml working standard solution, mixing with a sufficient quantity of acetonitrile (up to 5 ml) and making up to 10 ml with water. Similarly, six standard solutions (0.3, 0.4, 0.6, 0.8, 1.0 and 1.5 μg/ml) were obtained from the 10 μg/ml working standard solution, in order to determine the detection limit (DL) and quantitation limit (QL) of the method.

The various aqueous samples to be tested were directly mixed with the same amount of acetonitrile before chromatographic analysis. In cases where results were above the method linearity range, previous dilution with HPLC grade water was performed.

2.5. Method validation

The HPLC method was validated according to the International Conference on Harmonization (ICH) guidelines [12]. The following characteristics were considered for validation: specificity, linearity, range, accuracy, precision, detection limit (DL), quantitation limit (QL), and robustness. Specificity was evaluated by comparison of representative chromatograms of samples containing possible interfering substances (e.g. excipient solutions used in nanoparticles production, dissolution media components, supernatants resulting from the production of blank nanoparticles) and samples containing dapivirine. Additionally, specificity was demonstrated by performing stress studies. Linearity was determined by calculation of a regression line from the peak area vs. concentration plot for seven standard solutions (1, 5, 10, 20, 30, 40 and 50 μg/ml) using linear least squares methodology, and by analysis of the respective response factors (i.e. peak area divided by concentration of each standard sample). Accuracy was tested by calculating the percentage recoveries of the mean concentration of dapivirine at three different concentrations and by determining the relative standard deviation (RSD). Precision was assessed at different levels – repeatability (system repeatability by testing three different standard solutions, 10 times each, in the same day; and analysis repeatability by testing three different samples for three different standard solutions in the same day) and intermediate precision (by analyzing three different standard samples in three different days) – and reported as the standard deviation (SD) and RSD. Range was derived from linearity, accuracy, and precision studies. Detection limit (DL) and quantitation limit (QL) were determined based on the response and slope of a specific calibration curve obtained from six standard solutions (0.3, 0.4, 0.6, 0.8, 1.0 and 1.5 μg/ml) that where in proximity of these limit concentration values. The following expressions were used [12]:

$$\mathrm{DL} = \frac{3.3\sigma}{S}$$

$$\mathrm{QL} = \frac{10\sigma}{S}$$

where σ is the standard deviation of the response and S is the slope of the calibration curve. Robustness was evaluated by deliberately varying the concentration of the 0.5% (w/v) TEA solution used to compose the mobile phase and by using a different but similar column (Waters Spherisorb ODS2, RP C18 column, 5 μm particle size, 4.6 mm internal diameter, and 150 mm length; Waters, Milford, USA) from the one described in Sub-Section 2.2. Supernatants were previously mixed with the same amount of acetonitrile before chromatographic analysis. The influence of not performing the dilution of aqueous samples of dapivirine with equal amounts of acetonitrile was also evaluated by comparing the recovery percentage of both procedures for supernatants recovered from nanoparticles preparation. All samples were analyzed in triplicate, except when indicated otherwise.

2.6. Method applicability

2.6.1. Preparation of dapivirine-loaded PLGA nanoparticles

Dapivirine-loaded PLGA nanoparticles were obtained by single emulsion/evaporation technique [13]. Briefly, 50 mg of PLGA and 1 mg of dapivirine were dissolved in 4 ml of ethyl acetate and mixed with 10 ml of 3% (w/v) PVA aqueous solution. An oil-in-water emulsion was then obtained by sonication for 1 min (VibraCell model VCX 130 equipped with a 6 mm probe, Sonics & Materials, Inc., New-

town, CT, USA). The emulsion was immediately transferred to 20 ml of 0.3% (w/v) PVA aqueous solution and stirred overnight under magnetic stirring (250 rpm) to allow organic solvent evaporation. Finally, nanoparticles were centrifuged (45,000 × *g* for 30 min, at 4 °C), the resulting supernatant was collected for dosing, and the pellet was re-suspended in 30 ml of water in order to wash non-incorporated dapivirine and PVA. This last procedure was repeated twice.

2.6.2. Evaluation of drug content and in vitro release from PLGA nanoparticles

Dapivirine content of nanoparticles was assessed indirectly from supernatants by determining the association efficiency (AE) and loading capacity (LC) according to the following equations:

$$\mathrm{AE} = \frac{\text{total amount of drug} - \text{drug recovered from supernatant}}{\text{total amount of dapivirine}} \times 100$$

$$\mathrm{LC} = \frac{\text{total amount of drug} - \text{drug recovered from supernatant}}{\text{total weight of nanoparticles}} \times 100$$

The *in vitro* release profile of dapivirine from PLGA nanoparticles was determined in an adequate dissolution medium (phosphate buffered saline (PBS) pH 7.4 + 2% (w/v) polysorbate 80). Nanoparticles were placed in screw capped bottles containing 20 ml of dissolution media and placed in a horizontal shaking water bath (150 strokes/min) at 37.0 ± 0.1 °C. Aliquots of 250 μl were collected at appropriate time intervals without reposition of volume and filtered by 0.22 μm membranes to remove nanoparticles in suspension. The amount of dapivirine in the resulting samples was determined by the described HPLC method.

3. Results and discussion

3.1. Method development and optimization

Prior to the validation step, the hereby proposed method was developed in order to provide a simple and optimized procedure, with reduced time and cost of analysis. Therefore, several chromatographic performance parameters were considered, namely peak symmetry (as described by the tailing factor, *T*), number of theoretical plates (*N*), and retention factor (*k*′). Initial runs were performed using similar mobile phases to the ones previously described by others [8,9], mainly consisting of acetonitrile (with or without the addition of 0.1% (v/v) trifluoroacetic acid) mixed with an acidic aqueous solution ((0.1%, v/v) trifluoroacetic acid solution or 50 mM pH 3.0 acetate buffer) in various proportions, ranging from 90:10 to 50:50 acetonitrile/acidic aqueous solution. Noticeable tailing and irregular shaping of dapivirine peak was observed at all conditions, as indicated by poor and variable tailing factor values, which can compromise accuracy and precision of HPLC methods. Also, increasing tailing factor values were observed as further runs were performed (e.g., from around 1.4 to 2.0 for acetonitrile/0.1% (v/v) trifluoroacetic acid at a ratio of 70:30). These events can probably be explained by the establishment of interactions between amine groups of dapivirine and silanol groups of the stationary phase, as it is commonly observed for molecules containing these chemical groups [14]. Additionally, our observations suggest that the affinity of dapivirine towards active centers of silica surface is high, since the column used for chromatographic runs possesses a low level of silanol activity, being known for improving symmetry results for several drugs that strongly interact with silanol groups (e.g. amitriptyline) [15]. These problems were solved by substituting the acidic aqueous component of the mobile phase by 0.5% (w/v) TEA solution, which radically changes the pH value and provides a competing amine that can also strongly bond to free silanol groups, thus inhibiting, or at least reducing, dapivirine interaction with these groups. Resulting tailing factor values were kept stable around 1.1–1.2, which are acceptable values indicating good symmetry of the method. Additionally, the influence of different ratios of acetonitrile and 0.5% (w/v) TEA solution in the overall performance of the method was also assessed, with an 80:20 ratio of acetonitrile/TEA solution showing to be the most acceptable combination for the mobile phase. The resulting chromatographic performance parameters of the chosen setup for validation, as previously described, are presented in Table 1.

Table 1
Chromatographic performance parameters for chosen setup as described in Section 2.

Chromatographic parameters	Result[a]	Acceptance criteria
Dapivirine retention time (min)	4.06 ± 0.01	–
Solvent retention time (min)	0.91 ± 0.03	–
Retention factor, *k*′	3.45 ± 0.10	$2 < k' < 10$
Tailing factor, *T*	1.17 ± 0.13	0.8–1.5
Number of theoretical plates, *N*	9981 ± 79	$N > 2000$

[a] Presented as mean value ± SD.

3.2. Method validation

3.2.1. Specificity

Specificity of the method was evaluated by comparing the chromatograms of dapivirine and those of potential interfering formulation components. A 3% (w/v) aqueous solution of polyvinyl alcohol and the supernatant from the production of empty nanoparticles (i.e. obtained without the incorporation of the active drug) were analyzed by the described HPLC method and compared with the chromatogram of dapivirine standard samples (Fig. 2). The observed peak at around 1.3–1.5 min is consistent with the maximum UV absorption peak of PVA at 280 nm [16]. Possible interference by substances present in the dissolution medium was also tested by comparison of chromatograms. No peaks at the retention time of dapivirine were observed, being any potential interfering compounds washed from the column alongside the solvent. Also, tests were performed under four stress conditions (temperature, UV light, pH and oxidation) in order to detect the occurrence of possible interfering peaks at 290 nm resulting from the degradation of dapivirine. Furthermore, these tests are regarded as helpful tools in establishing degradation pathways and the inherent stability of the molecule, and help validating the power of the proposed method for studying the stability of dapivirine. Obtained results are presented in Table 2, showing no alterations in dapivirine retention times. Also, percentage recovery for stress conditions revealed that dapivirine was not affected, except for low pH values and when exposed to oxygen peroxide plus acetonitrile. The presence of one degradation peak at 290 nm was only observed under oxidation conditions in the presence of acetonitrile but it was completely resolved from the peak of dapivirine as indicated by the calculated resolution value ($R = 6.9$). This observation is probably due to the increase in oxidative degradation of tertiary and aromatic amines by hydrogen peroxide in the presence of acetonitrile, as previously described [17]. Overall, obtained data provides evidence that the method can be regarded as specific since no potential interfering peak was observed.

3.2.2. Linearity

Linearity was studied in the concentration range of 1–50 μg/ml by visual inspection of a calibration curve plotting (Fig. 3) and by calculating the regression equation and the correlation coefficient (R^2) by the method of least squares:

$$A = 87.813(\pm 0.329) \times C - 5.5629(\pm 9.2359) \qquad R^2 = 0.9998$$

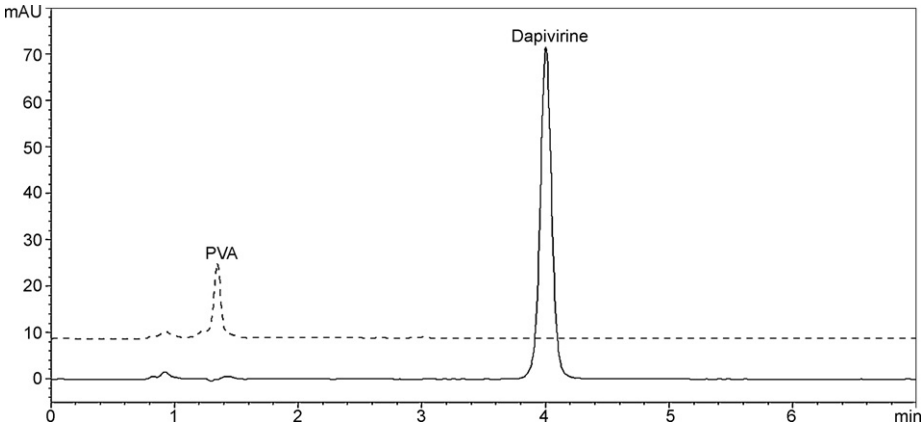

Fig. 2. Representative chromatograms of 5 μg/ml dapivirine standard solution (solid line) and supernatant from the production of empty nanoparticles (dashed line).

Table 2
Assay results for 48 μg/ml dapivirine standard solution under different stress conditions.

Stress conditions	Sample treatment	Unexpected peaks (t_R; R)	Dapivirine t_R [a]	Percentage recovery (±SD)
Reference	None (freshly prepared)	–	4.06	99.69 ± 0.18
Temperature	40 °C for 5 days	No	3.96	100.52 ± 0.20
UV light	UV light for 24 h	No	4.03	100.16 ± 0.17
pH	1 M HCl for 24 h	No	3.96	94.21 ± 0.31
	1 M NaOH for 24 h	No	4.03	99.55 ± 0.28
Oxidation	3% (v/v) H_2O_2 for 24 h	No	4.03	99.70 ± 0.18
	3% (v/v) H_2O_2 + acetonitrile for 24 h	Yes (2.1; 6.9)	3.92	93.84 ± 0.62

[a] SD ≤ 0.01 in all cases; t_R: retention time in min; R: resolution.

where A is the peak area, C is the standard solution concentration in μg/ml, and standard deviation values for A and C are indicated in brackets. The R^2 is higher than 0.999, as frequently advocated [18], indicating a good linearity in the proposed range. Although this parameter is often used to evaluate the quality in linear regression analysis, it is not a true measure of linearity and should be reinforced by other linearity assessment methods [19]. Thus, an analysis of the response factors (i.e. peak area divided by concentration of each standard sample) for the proposed range was performed. The visual inspection and linear regression by the method of least squares of the plot of the response factors (peak area divided by concentration at each standard sample concentration level) vs. standard sample concentrations (Fig. 3) revealed a near zero slope (−0.0234) and RSD of 1.6% across all standard concentration levels, thus reinforcing the evaluation of the method as linear [20].

3.2.3. Range

The method working range was defined as to fulfill the required versatility, proving to be linear, accurate and precise between 1 and 50 μg/ml (see respective sections). Therefore, samples presenting these concentration levels may be suitably assayed by the proposed HPLC method.

3.2.4. Accuracy

Accuracy indicates the agreement between obtained results and those accepted as true, and can be assessed by calculating the percentage recoveries of the mean concentration of the analyte at three different concentrations and the relative standard deviation (RSD). Three standard solutions (3, 16 and 48 μg/ml) were carefully prepared in triplicate and analyzed by the proposed method. The overall recovery was found to be 100.17 ± 0.35%, thus showing strong agreement between experimental and theoretical values. Detailed results for the three tested concentration levels are presented in Table 3.

3.2.5. Precision

Precision expresses the importance that random errors have on the method performance and can be expressed at different levels. In the case of the developed method, precision has been validated for intra-day (system repeatability and analysis repeatability) and inter-day (intermediate precision) variations at three different concentration levels. Results are presented in Table 4. Relative standard variation for system repeatability and analysis repeatability was ≤0.53% and ≤0.38%, respectively; as for inter-day variation, RSD was ≤0.85%. These results indicate that the proposed method presents good precision [21].

3.2.6. Detection limit (DL) and quantitation limit (QL)

The lowest concentration at which an analyte can be detected (DL) or quantified with acceptable precision and accuracy (QL) can be determined by different methodologies. In the present study, these parameters have been calculated from the SD of the response and slope of a specific calibration curve obtained from standard samples (0.3, 0.4, 0.6, 0.8, 1.0 and 1.5 μg/ml) in the low end region of the proposed range [12]. The DL and QL were found to be 0.08 and 0.24 μg/ml, respectively. It is also noteworthy that the simple analysis of the R^2 (0.998) of the regression equation in the range of the DL and QL, as determined by the method of least squares, falsely indicated an acceptable linearity [19,20]. However, complementary study of the linear regression for the response factors at concentration levels between 0.3 and 1.5 μg/ml indicated poor linearity (linear regression slope: −9.9982; RSD: 7.89%). This fact recommends caution when using HPLC assay methods where linearity has

Table 3
Accuracy results for different levels of dapivirine in standard solutions.

Standard solution (μg/ml)	Recovery (%)	RSD (%)
3	100.30	0.35
16	100.07	0.22
48	100.12	0.17

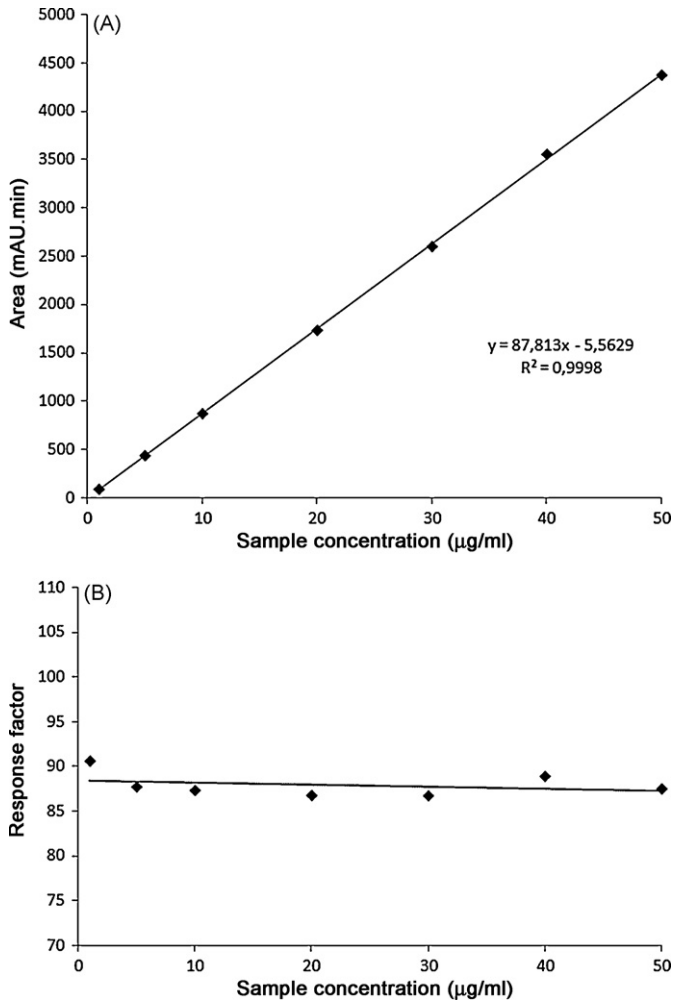

Fig. 3. Linearity studies for the proposed HPLC method: (A) calibration curve obtained with dapivirine standard solutions ($n = 21$), and (B) response factor versus dapivirine standard solutions concentration ($n = 21$).

Table 4
Precision results for different levels of dapivirine in standard solutions.

Day	Standard solution (μg/ml)	Measured concentration (μg/ml)	SD	RSD (%)
System repeatability ($n = 10$)				
1	5	4.97	0.03	0.53
	20	20.02	0.06	0.29
	50	49.91	0.20	0.39
Analysis repeatability ($n = 3$)				
1	5	5.03	0.02	0.37
		4.99	0.01	0.29
		5.03	0.02	0.38
	20	20.00	0.06	0.28
		20.05	0.03	0.17
		20.00	0.04	0.21
	50	50.02	0.11	0.21
		50.06	0.07	0.15
		50.11	0.07	0.14
Intermediate precision ($n = 3$)				
1	5	4.99	0.04	0.80
	20	19.99	0.07	0.36
	50	49.95	0.13	0.25
2	5	5.01	0.04	0.85
	20	19.95	0.06	0.32
	50	49.78	0.17	0.33
3	5	5.03	0.01	0.14
	20	20.08	0.09	0.43
	50	50.20	0.14	0.28

only considered R^2 values, particularly at low drug concentration levels.

3.2.7. Robustness

Robustness testing is useful in order to prove that typical variations to the method are negligible in the procedure outcome, being usually studied by deliberately changing critical parameters and monitor possible alterations. A method is said to be robust when these alterations produce no significant changes in its results. The evaluation of robustness was based on the percentage recovery and RSD values obtained for different concentrations of TEA present in the mobile phase and for two different columns (Table 5). In the case of the tested variations for TEA concentration, obtained recovery percentages were near 100% and similar to those for the original method, while RSD were lower than 0.7%. No other variations were observed for chromatographic descriptors, namely dapivirine retention factor, peak symmetry and efficiency (as described by the number of theoretical plates). Additionally, pH variations between different TEA solution batches at different concentrations (0.4–0.6%) were in the range of 9.6–10.0. Thus, the method showed to be robust concerning small but expectable variations of the amount of TEA in the mobile phase. As for the variation of the used column, the observed differences in recovery and RSD were relatively low and may be considered acceptable. However, distinct peak tailing was observed for the Waters Spherisorb ODS2, as expressed by elevated values for the tailing factor (1.39 ± 0.03, $n = 9$). Although peak symmetry is still acceptable these values are significantly higher than those obtained with the original column. This behavior may be justified by the higher silanol activity of the Waters Spherisorb ODS2, which cannot be completely hindered by used levels of the competing amine, i.e. TEA. Thus these results reinforce the claim that dapivirine is able to strongly interact with silanol groups and requires columns with low silanol activity.

The influence of not diluting the aqueous samples with the same amount of acetonitrile before chromatographic evaluation was also assessed by comparing the results of the concentration of dapivirine in supernatant samples of different nanoparticles batches ($n = 3$) prepared independently. When compared to the standard sample preparation procedure (i.e. by diluting with acetonitrile), the recovery percentage was of 99.70 ± 0.85 (%), thus indicating that the acetonitrile dilution step may be skipped. Besides the obvious advantages of faster analysis and acetonitrile savings, this fact may be of particular importance when assaying samples with low concentrations of dapivirine, around the lower limit of the range.

3.3. Method applicability

The proposed method was used to study the content of dapivirine in PLGA nanoparticles presenting diameter of around 120 nm and zeta potential of −21 mV. AE and LC for dapivirine were determined to be 88.3 ± 0.8% and 2.1 ± 0.1%, respectively ($n = 3$), indicating a high degree of encapsulation of dapivirine into PLGA nanoparticles.

Dapivirine poor solubility in water required the addition of a surfactant (i.e. polysorbate 80) to the PBS, in order to maintain sink conditions. Also, the pH of the dissolution medium was chosen to be 7.4 and not 4.2 (normal vaginal pH) since previous studies showed the ability of PLGA nanoparticles to rapidly permeate and accumulate into the deepest layers of the vaginal epithelium [10,22]. The *in vitro* release profile of dapivirine from PLGA nanoparticles is depicted in Fig. 4. An initial burst effect was observed in the first hours, with approximately 50% of the drug content being released within 4 h. By the end of the first 12 h, drug release rate remained constant, reaching a stable plateau at around 168 h with nearly 70% of the total dapivirine content being released by 336 h. Taken

Table 5
Robustness results for different concentrations of TEA in the mobile phase and different columns.

Changes to original method[a]	Recovery percentage ± RSD (%)			
	3 μg/ml	16 μg/ml	48 μg/ml	Mean
None	100.65 ± 0.38	99.99 ± 0.21	100.03 ± 0.21	100.22 ± 0.40
0.4% (w/v) TEA solution	101.57 ± 0.25	101.37 ± 0.66	100.33 ± 0.35	101.09 ± 0.69
0.6% (w/v) TEA solution	101.55 ± 0.27	100.79 ± 0.38	100.46 ± 0.21	100.93 ± 0.54
Waters Spherisorb ODS2	98.88 ± 0.78	97.17 ± 2.59	99.12 ± 0.71	98.39 ± 1.67

[a] 0.5% (w/v) TEA solution and Teknokroma TR-010004 Mediterranea Sea_{18}.

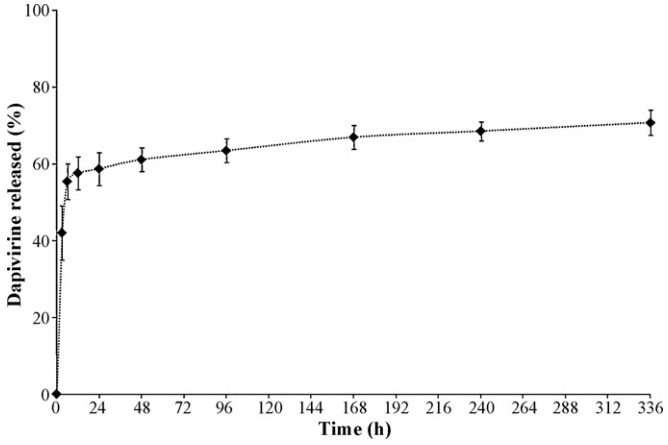

Fig. 4. Cumulative *in vitro* release profile of dapivirine from PLGA nanoparticles for 14 days ($n = 3$; vertical bars represent SD).

together, *in vitro* drug release data suggests that PLGA nanoparticles may provide a rational strategy for the development of sustained release systems for the delivery of dapivirine. No alterations to the chromatograms or unusual peaks were observed either during nanoparticles content quantification or *in vitro* release studies.

4. Conclusions

To our best knowledge, this is the first study to describe a fast, simple and validated RP-HPLC method for dapivirine according to the ICH guidelines. It was demonstrated to be specific, linear, accurate, precise, and robust in the range of 1–50 μg/ml. Also, the detection limit and quantitation limit were determined. The method found suitable application for the analysis of dapivirine-loaded PLGA nanoparticles during preparation and pharmaceutical characterization, namely for the determination of drug association efficiency, drug loading capacity and *in vitro* drug release.

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