A Sensitive High-Throughput HPLC Assay for Simultaneous Determination of Everolimus and Clobetasol Propionate

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Abstract

A novel sensitive high-throughput high-performance liquid chromatography assay is developed and validated for the simultaneous determination of everolimus and clobetasol propionate in pharmaceutical formulations. The chromatographic separation is achieved on a Zorbax Eclipse XDB-C18 reversed-phase column using a gradient elution, with solvent A: ammonium acetate (pH 6.8; 0.01 M) and solvent B: acetonitrile. The mean recovery ranges from 95.1% to 100.0% for clobetasol propionate and from 97.9% to 103.7% for everolimus. The limit of quantitation for each analyte is 0.02 µg/mL. The percent relative standard deviations are less than 3% for intra- and inter-day analyses. The proposed method can be used for the routine quality control of everolimus and clobetasol propionate in complex pharmaceutical formulations, especially the drug-delivery systems with a low total drug-load.

Introduction

Everolimus (Figure 1) is a potent immunosuppressive agent with anti-proliferative activity (1–3). Clobetasol propionate (Figure 1) is very potent as a vasoconstrictor as well as an anti-inflammatory agent (4–6). The combination of anti-proliferative and anti-inflammatory agents can potentially be used to treat or prevent a wide variety of disorders (7–12). The mode of delivery can be local or systemic. The local delivery of a therapeutic substance is a preferred method of treatment because the substance is concentrated at a specific site and thus smaller total levels of medication can be administered in comparison to systemic dosages that often produce adverse or even toxic side effects for the patient.

Drug-delivery systems such as drug-eluting stents loaded with everolimus and cobetasol propionate can be used for the treatment of restenosis and vulnerable plaque (13). Due to the low total levels of everolimus (100 µg) and especially clobetasol propionate (< 3 µg) loaded on the drug-eluting stents to minimize the potential toxicity, sensitive analytical methods would be invaluable in developing formulations and quality control tools that can determine the capability of a formulation to release the drug into solution in a predictable manner necessary for base scale manufacturing.

Several high-performance liquid chromatography (HPLC) and gas chromatography (GC)–mass spectrometric (MS) methods for the quantitation of everolimus or clobetasol propionate in biological samples have been reported so far (14–21). Although these methods are well-suited for application in biological samples, the analysis involves extensive sample pretreatment before determining the everolimus or clobetasol propionate. Moreover, the use of the MS technique to guide the formulation design and control the product quality has not been recognized due to the following limitations. First, the mass detector is still relatively expensive for routine quality control and it is not always available in analytical laboratories. Second, few well-defined analytical procedures fit current Good Manufacturing Practice requirements; for instance, due to the suppression of ionization from the matrix (e.g., excipient, in-vitro release rate medium, and sample diluent), quantitation is oftentimes limited to the use of internal standards. Third, the method ruggedness and robustness issues associated with MS are the result of an unstable ionization source and/or significant interference from the formulation excipients and in vitro release-rate media (22).

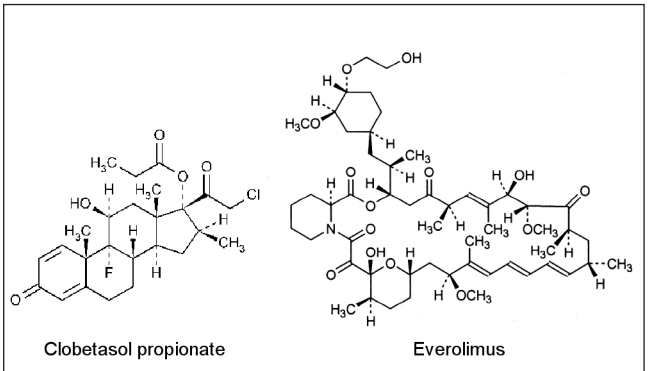

Figure 1. Chemical structures of everolimus and clobetasol propionate.

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Also, several HPLC methods with UV detection for the quantitation of everolimus in biological samples (23–26) or clobetasol propionate in ointment (27,28) have been previously described. To increase the sensitivity and make them suitable to adequately measure everolimus in biological samples or in ointment, these assays utilize large sample volumes, complex extraction procedures, preconcentration, and large injection volumes, and they might not be suitable for the direct, routine analyses of everolimus or clobetasol propionate in complex pharmaceutical formulations which call for consistent results, high accuracy, simplicity, and rapidity. In addition, due to the difference in the log *P*/hydrophobicity values between clobetasol propionate and everolimus (3.6 vs. 5.8), the designed chromatographic conditions of the published HPLC methods do not allow the simultaneous determination of both analytes. As the formulation development trends toward more complex drug delivery systems, the pharmaceutical scientists continue to search for higher resolution, faster, and more economically viable HPLC methods compatible with complex pharmaceutical matrices.

The purpose of this work was to develop and validate a convenient, sensitive, specific, and reproducible high-throughput method for the direct, simultaneous determination of everolimus and clobetasol propionate in complex pharmaceutical samples. This could potentially improve the efficiency of the analysis and reduce laboratory supply costs associated with revalidation and testing of methods for individual drugs. Strategies to increase the sensitivity, reduce the run time, and lower the cost of assay were considered. The scope of the assay application was targeted to address in-vitro release rate samples of eluting stents, which could have lower drug concentrations, especially clobetasol propionate, in the presence of antioxidants and surfactant media.

Experimental

Instrumentation and chromatography

Chromatography was performed with a Waters series HPLC system (Waters Technologies, Inc., Palo Alto, CA) provided with a binary pump, a thermostatted autosampler, a thermostatted column compartment, and a multiple wavelength photo diode array (PDA) detector. Data were collected and analyzed using Empower Software, (Waters, Inc.). The separation of analytes was accomplished using an Eclipse XDB-C18 reversed-phase column (4.6 mm i.d. × 50 mm, 1.8 µm) (Agilent Technologies, Inc., Palo Alto, CA) maintained at 60°C. Final chromatographic conditions involved a gradient elution, with solvent A: ammonium acetate (pH 6.8; 0.01 M) and solvent B: acetonitrile (ACN). The pump flow rate was 1.2 mL/min. The mobile phase program is described in Table I. The injection volume was 40 µL. The optimum wavelength of 239 nm was selected for clobetsol propionate and 277 nm for everolimus.

Table I. Mobile Phase Program for the Gradient Elution

Time (min)	Flow (mL/min)	%A*	%B*	Curve
0	1.2	48	52	Linear
2.5	1.2	35	65	Linear
2.6	1.2	32	68	Isocratic
4.9	1.2	32	68	Isocratic
5.0	1.2	48	52	Linear
6.5	1.2	48	52	Isocratic

* A = Amonium acetate (pH 6.8; 0.01 M) and B = Acetonitrile.

Chemicals

All chemicals were at least analytical grade. HPLC grade ACN was obtained from Fisher Scientific (Waltham, MA). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade water was prepared by purifying demineralized water in a Milli-Q filtration system (Millipore, Bedford, MA). The reference standard for clobetasol propionate was purchased from Sigma-Aldrich (St. Louis, MO) and for everolimus from Novartis (Basel, Switzerland). Butylated hydroxytoluene (BHT) was obtained from Sigma-Aldrich (St. Louis, MO).

Standard preparation

The standard stock solutions of clobetasol propionate and everolimus were prepared in 50-mL volumetric flasks with 10.0 mg of clobetasol propionate and 30.0 mg of everolimus weighed exactly and dissolved with BHT (0.02% w/v) in ACN. The solutions were sonicated for 10 min and adjusted to the volume with BHT (0.02% w/v) in ACN. The working standards for clobetasol propionate (0.02–4.0 µg/mL) and everolimus (0.2–40 µg/mL) were freshly prepared from the standard stock solutions by serial dilution with in vitro release rate medium (0.7% Triton X-405 and 0.01 M potassium phosphate buffer at pH 6.0).

Preparation of spiked recovery sample

To pick up the possible recovery problems during both the chromatographic procedures and the sample preparation, the spiked recovery samples were prepared by spiking known levels of clobetasol propionate and everolimus into the product matrix

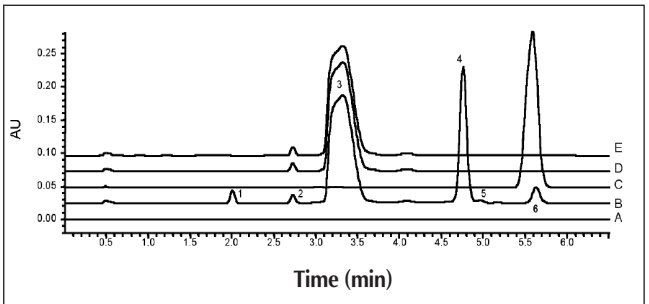

Figure 2. Representative chromatograms of placebo stent extract (A), working standard of clobetasol propionate (1.0 µg/mL) and everolimus (10.0 µg/mL) in the in vitro release medium (B), 0.02% BHT (w/v) in ACN (C), aged in vitro release rate medium at 37°C for 24 h (D), fresh in vitro release medium (E). Peaks: 1, clobetasol propionate; 2 and 3, release medium (Triton X-405); 4, everolimus; 5, everolimus isomer; 6, BHT. The broad band of Triton X-405 is related with the restricted diffusion due to the hydrodynamic diameter of the molecule. Also, due to its flexible linear chain, Triton X-405 can exist as two or more distinct or inter-converting species that migrate through the column with different retention time (peaks 2 and 3).

(placebo). The following amounts of clobetasol propionate: 1, 2, and 4 µg and of everolimus: 10, 50, and 100 µg were spiked into the product matrix and diluted to the volume of 10 mL with the release medium. Three replicate samples of each level along with the respective controls (clobetasol propionate and everolimus spiked in the release medium only) were prepared. All samples were incubated at 37°C in the release rate bath for 24 h prior to analyses.

Sample preparation

The in-vitro release rate profile of eluting stents loaded with clobetasol propionate and everolimus was studied using USP apparatus 7. Stents were loaded on the stent holders and dipped in 10 mL release rate medium (37°C ± 0.5°C) at a rate of 40 dips per min for 10 min, 1, 4, 7, and 24 h. At the end of each dipping time interval, the solutions were transferred into HPLC vials and injected into HPLC column for the quantitation of everolimus and clobetasol propionate. After the last time point, the stents were analyzed for the residual of everolimus and clobetasol propionate. They were separately transferred into 1-mL volumetric flasks containing 1 mL of 0.02% BHT–ACN solution, and the flasks were sonicated for 30 min. The extracted solutions were transferred into HPLC vials and analyzed for everolimus and clobetasol propionate.

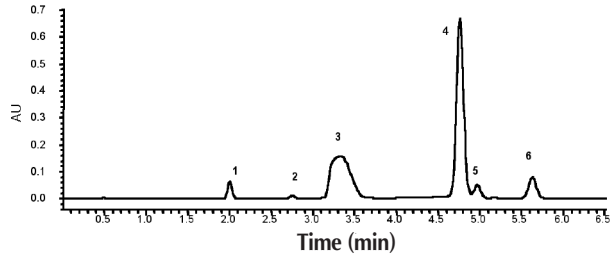

Figure 3. Representative chromatogram of an unknown in vitro release rate sample. Peaks: 1, clobetasol propinate; 2 and 3, release medium (Triton X-405); 4, everolimus; 5, everolimus isomer; 6, BHT.

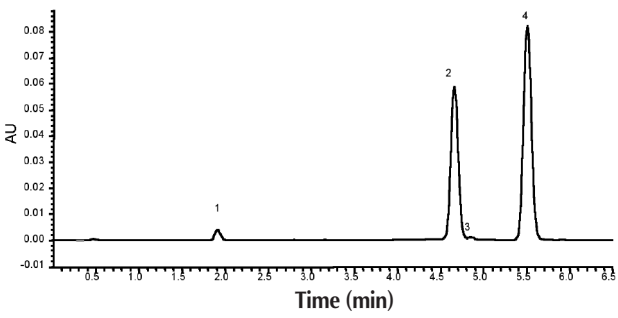

Figure 4. Representative chromatogram of an extracted clobetasol propionate and everolimus residual from the stent. Peaks: 1, clobetasol propionate; 2 everolimus; 3, everolimus isomer; 4, BHT.

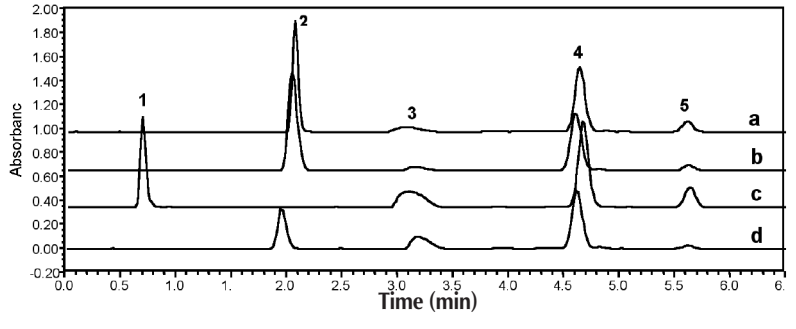

Figure 5. Representative chromatogram momethasone and everolimus (a), momethasone and sirolimus (b), dexamethasone and everolimus (c), clobetasol propionate, and sirolimus (d). Peaks: 1, dexamethasone; 2a,b, momethasone; 2d, clobetasol propionate; 3a–d, release medium (Triton X-405); 4a,c, everolimus; 4b,d, sirolimus; 5a–d, BHT.

Results and Discussion

Selection of HPLC conditions

The initial stage of this work consisted of a number of qualitative experiments to determine which column, mobile phase, column temperature, and flow rate should be employed. These investigations used typical production batches of drug eluting-stents to judge the degree of resolution of clobetasol propionate and everolimus from the sample matrices. On the bases of these preliminary studies, a Zorbax Eclipse (XDB-C18, 4.6 mm × 50 mm, 1.8 µm) high-speed column was selected as especially suited for our application. The small particle (1.8 µm) provided the high resolving power (high column efficiency due to the decrease of peak volume), while the short column length (50 mm) enabled high-speed analyses (Figures 2–5). On the other hand, the extra-dense coating of bonded phase and exhaustive endcapping simultaneously deactivate the silica surface from deleterious interactions with analytes and also protect the silica support from dissolution in intermediate and higher pH environments (29). These characteristics are particularly important for use in methods that need long-term stability and reproducibility.

The selection of the mobile phase was the second most important step in the development of this method. Because the bonded stationary phase of the selected column is nonpolar in nature, an ACN–aqueous mixture was used as a mobile phase. The mobile phase pH was controlled by adding ammonium acetate (pH 6.8; 0.01M). Buffer capacity and UV absorbance were taken into consideration in selecting this particular buffer. A buffer concentration of 0.01M was found adequate to give a reproducible separation and without distorted peaks. ACN was selected as the organic modifier because it provided adequate retention of all sample compounds within a very short run time (6.5 min). In addition, ACN has a low viscosity (minimizing the column back-pressure), little adsorption at low wavelengths, and a long history of proven reliability in RP-HPLC.

The gradient elution provided more efficiency because of the compression effects and was selected over the isocratic elution. Indeed, due to the gradient elution, the eluted peaks have essen-

Table II. System Suitability*

Injection	Clobetasol t_R	Clobetasol Area	Clobetasol N	Clobetasol R_s	Clobetasol A_s	Everolimus t_R	Everolimus Area	Everolimus N	Everolimus R_s	Everolimus A_s
1	2.0	69176	6750	6.9	1.0	4.8	1183779	14793	1.5	1.2
2	2.0	68925	6961	6.9	1.0	4.8	1184418	15027	1.5	1.2
3	2.0	69236	6790	7.0	1.0	4.8	1183940	14835	1.5	1.2
4	2.0	69035	6913	6.9	1.0	4.8	1183340	15128	1.5	1.2
5	2.0	69308	6963	7.0	1.0	4.8	1181864	14928	1.5	1.2
Mean	2.0	69136	6875	6.9	1.0	4.8	1183468	14942	1.5	1.2
%RSD	0.01	0.2	1.4	1.2	0.0	0.3	0.1	0.9	0.0	0.9

* t_R: retention time; N: theoretical plate number; R_s: resolution between adjacent peaks (clobetasol propionate and Triton-X 405; everolimus and everolimus isomer); A_s: peak asymmetry factor. The theoretical plate count numbers (N) were calculated using the equation: $N = 5.54(t_R/W_{1/2})^2$ where, t_R: retention time; $W_{1/2}$: the width at half the peak height measured in the same units as t_R.

Table III. Accuracy and Intra-Day Precision*

Drug	Spiked amount (µg/mL)	Mean recovery (%)* (%RSD)	Grand mean recovery (%)† (grand %RSD)
Clobetasol	0.10	95.3 (0.6)	98.1 (2.6)
	0.20	99.2 (1.5)	
	0.40	98.8 (0.8)	
Everolimus	0.95	99.1 (0.6)	99.4 (1.6)
	4.76	101.2 (0.8)	
	9.52	97.9 (0.6)	

* Mean values represent three spiked samples for each concentration.
† Grand mean values represent nine spiked samples (three samples at three concentrations) analyzed on the same day.

tially the same peak width, so sensitivity is similar throughout the chromatogram. On the other hand, the gradient elution exposes the column continuously to solvents of increasing strength, minimizing the sample and impurity buildup.

Because the high temperature HPLC is a practical means of achieving significant improvements in the HPLC throughput, the chromatographic separation was performed at high temperatures. The benefits of conducting LC at elevated temperatures (> 40°C) have been discussed extensively in the scientific literature (30,31). For example, the decrease in viscosity of the mobile phase with increasing temperature allows the use of higher flow rates for higher throughput. The lower viscosity factors also allow for the use of smaller particle sizes (< 3 µm). Furthermore, there is a significant reduction in analyte diffusion as it goes through the column at an elevated temperature, minimizing the band broadening and therefore maximizing the efficiency. A temperature of 60°C was found to be the most efficient, without lowering the column performance. Maintaining the column at 60°C did not only ensure reproducible separations and improve the resolution between the everolimus and its isomer, but also lowered the required concentrations of organic solvent. Increasing the temperature of the system will change the dielectric constant of water, giving more organic character to the aqueous mobile phase, resulting in a significant reduction in the use of organic solvent and reducing the cost. In addition, the high temperature (60°C) was also the only approach that allowed a significant fraction of the column plate count to be retained as the column linear velocity was increased from 0.5 to 1.2 mL/min.

Under the given conditions, clobetasol propionate and everolimus eluted at retention times of 2.0 and 4.8 min, respectively, and the total analysis time required was only 6.5 min per sample. The retention time as an index of compound quality is the most important parameter for the length of analyses. The short run time reduces the time consumption for routine series of analyses and moreover, reduces the solvent consumption. Representative chromatograms obtained by the described method are shown in Figures 2–4.

Validation

The parameters essential to ensure the acceptability of the performance of the analytical method were determined: such as the system suitability, specificity, linearity and range, precision, accuracy, solutions stability, and the limit of quantitation.

System suitability parameters

The system precision was assessed by analyzing five injections from the same sample (concentration, 1.0 µg/mL for clobetasol propionate and 10.0 µg/mL for everolimus). The results of the system suitability testing are given in Table II. For the definition of the efficiency, various parameters can be used (29). In our study, the theoretical plate number was used. Apparently, the chosen chromatographic conditions provided a large enough N value for the separation (Table II), indicating that the selected column was reliable and had the ability to produce sharp, narrow peaks achieving a good resolution of band pairs. The peak resolution describes the rate of analytes' separation. As shown in Table II, the resolutions between clobetasol propionate and the adjacent peak (Triton X-405) at the retention time of 2.7 min, and between the everolimus and the adjacent peak (evrolimus isomer) at the retention time of 5.0 min were 6.9 and 1.5, respectively, indicating that the assay achieves the necessary level of discrimination. The peak asymmetry is important for precise peak integration and thus for quantitative information. All of the values meet the ICH requirements for validation, which recommends the value range of 0.5–1.5.

Table IV. Inter-Day Precision*

Drug	Spiked amount (µg/mL)	Mean recovery (%) (%RSD)/Day 1	2	3	Grand mean recovery (%) (Grand %RSD)
Clobetasol	0.10	95.3 (0.6)	95.4 (0.6)	95.1 (2.3)	97.0 (2.1)
	0.20	99.2 (1.5)	97.8 (0.6)	95.7 (1.0)	
	0.40	98.8 (0.8)	100.0 (1.3)	97.9 (0.4)	
Everolimus	0.95	99.1 (0.6)	101.6 (0.3)	100.1 (0.3)	100.9 (1.7)
	4.76	101.2 (0.8)	103.7 (0.1)	101.7 (0.1)	
	9.52	97.9 (0.6)	101.7 (0.8)	101.5 (0.2)	

* Mean values represent 3 spiked samples for each concentration. Grand mean values represent 27 spiked samples. Nine spiked samples of both analytes were prepared and run by three analysts, using three instruments, on three different days, in one laboratory.

Table V. Stability of Clobetasol Propionate and Everolimus Standard Solutions

	% Difference from T_0					
	Clobetasol propionate (µg/mL)			Everolimus (µg/mL)		
Standard solution/conditions	0.02	1.2	4.0	0.2	10.0	40.0
Long term stock solution (28 days; –20°C)	2.0	1.2	1.7	0.8	0.1	–0.3
Freeze-thaw (three cycles) of stock solution	–1.3	–0.3	0.1	–0.9	1.0	–0.6
Working standard (7 days; 2–8°C)	–0.1	–0.4	0.1	1.2	0.7	–1.3
Working standard (7 days; room temperature)	1.9	–1.1	0.1	1.3	–0.1	0.8

Specificity

The specificity was evaluated by an injection of a mixture of the working standard solution, BHT (0.02% w/v) in ACN, fresh and aged in-vitro release media, and the placebo stent extract. There was evidence that the substances being quantitated were the intended analytes. No interference was observed at the same or within ±5% of the retention time of the analytes of interest (Figure 2).

Linearity and range

Standard curves were constructed for each analyte's concentration range by fitting a regression line to the test results (peak area vs. analyte concentration) using the method of least squares. Seven standards were used to define adequately the relationship between the concentration and response. The sum of squares for residuals was equal to zero, indicating that the calibration lines for each analyte were described by a linear relationship. The following equations were derived from the calibration curves: $y = 58431x - 1370$ and $y = 92962x + 2761$ for clobetasol propionate and everolimus, respectively. The correlation coefficients between the concentration and the peak area for each analyte were > 0.999. A calibration curve was generated for each analytical run and was used to calculate the concentrations of each analyte in the unknown samples assayed with that analytical run. The range over which each analyte can be determined (1–4 µg for clobetasol propionate and 10–100 µg for everolimus) was defined based on the approximately 50% of the lowest to 150% of the highest amount eluted from the stent when immersed in the in-vitro release medium, as well as on the evaluation of actual samples over the range, including their statistical variation.

Accuracy and precision

The accuracy and precision with which known concentrations of each analyte in the release rate samples can be determined were evaluated. The recovery assessment was performed by analyses of samples spiked with known amounts of clobetasol propionate and everolimus at three concentrations representing the entire range of expected concentrations in the unknown samples. The mean recovery ranged from 95.1% to 100.0% for clobetasol propionate and from 97.9% to 103.7% for everolimus (Table III). Intra-assay precision (repeatability) was assessed using nine determinations from the accuracy study covering the specified range for the procedure (three concentrations/three replicates each for both analytes). The grand %RSD was 2.6% for clobetasol propionate and 1.6% for everolimus (Table III). The inter-day (intermediate) precision was assessed by running nine spiked samples of both analytes by three analysts, using three instruments, on three different days, in one laboratory. Different sources of reagents and multiple lots of columns were included in this study. The samples were prepared freshly at each day. The grand %RSD was < 3% for both analytes (Table IV).

Stability studies

The studies were performed to gain information on the stability of standard and sample solutions under defined storage conditions and to assure that the solutions are stable enough to allow for delays such as instrument breakdowns or overnight analyses using auto-samplers. The stability of the standard stock solutions was evaluated over a 28-day period at –20°C; working standard solutions were tested at 2–8°C and room temperature over a 7-day period, and sample solutions were tested at 2–8°C, room temperature, and 37°C over a 3-day period. In addition, the stability of the standard stock solutions was determined after three freeze-thaw cycles. The standard stock solutions were stored at –20°C for 24 h, thawed unassisted for 8 h at room temperature and refrozen for 24 h under the same conditions. The freeze-thaw cycle was repeated two more times and the samples were analyzed on the third cycle. The stability of clobetasol propionate and everolimus was tested using six independent sets of sample preparations. The acceptable stability was defined as ≤ 5% change in the standard or sample response, relative to freshly prepared solutions (T_0). The quantitation of analytes was determined using freshly prepared standards. There was no significant loss in everolimus and clobetasol propionate as shown by % difference concentration values obtained during stability tests (Tables V and VI).

Table VI. Stability of Clobetasol Propionate and Everolimus Sample Solutions

	% Difference from T_0					
	Clobetasol propionate (µg/mL)			Everolimus (µg/mL)		
Standard solution/conditions	0.1	0.2	0.4	1.0	4.8	9.5
3 days at 2–8°C	–0.2	–0.7	–0.5	0.1	–0.2	–0.1
3 days at room temperature	–0.2	–0.1	–0.1	–0.4	–0.6	–0.7
3 days at 37°C	–0.1	–0.1	–0.2	–0.4	–0.1	–1.1

Limit of quantitation

The limits of quantitation (LOQ) for clobetasol propionate and everolimus were determined based on signal-to-noise (S/N) ratio approach. Determination of the S/N ratio was performed by comparing measured signals from samples with known low concentrations of analytes (0.01, 0.02, 0.03, and 0.04 µg/mL for each analyte) with those of blank samples. The lowest concentration that gave the S/N = 10 was 0.02 µg/mL for both analytes. The LOQ was subsequently validated by the analyses of five independent samples spiked with known amounts (0.02 µg/mL) of clobetasol propionate and everolimus into the product matrix. The mean recovery was 104.5% for clobetasol propionate and 96.1% for everolimus. The %RSD was 1.5% for clobetasol propionate and 5.4% for everolimus. Therefore, the LOQs for both clobetasol propionate and everolimus were defined at 0.02 µg/mL. Due to the low LOQ, the assay allows the quantitaion of clobetasol propionate and everolimus even at ranges that are more typically addressed by liquid chromatography (LC)–mass spectrometry (MS).

Advantages of the present assay

Compared with previously published assays for the determination of clobetasol propionate (27–28) or everolimus (23–26), the present assay has several advantages.

It is the first assay reported for the simultaneous determination of both compounds. This could potentially improve the efficiency of the analysis and reduce laboratory supply costs associated with revalidation and testing of methods for individual drugs.

The total run time of 6.5 min is considerably shorter than the other methods (23–28). The direct injection of the sample and the short chromatographic analysis time allow the analyzing of ~75 samples within an 8-h shift. This makes it a rapid mean of analytical pharmaceutical sample screening and quality control sample analysis. The column may be changed every 6 months, which in our daily routine corresponds to 1000 injections.

The present assay allows analyses of everolimus and clobetasol propionate without prior sample clean-up and pre-concentration, and the analytes of interest can be separated and quantified directly in the presence of excipient (BHT used as an antioxidant) and surfactant in-vitro release medium, which could create significant bias if a spectrometric method were to be used (22). Direct injection eliminates multiple sample pre-treatment and pre-concentration steps involved in the previous assays (23–26), which require a large quantity of sample volume (0.5–1.0 mL) to achieve sufficient sensitivity and a significant amount of time by the analyst to complete this task. It avoids analyte degradation or loss associated with the use of cartridges and sample concentration and assures more consistent, accurate, and quicker results. In addition, the direct injection limits the creation of unnecessary biohazardous waste (i.e., used cartridges), reduces potentially dangerous sample handling, eliminates the use of large amounts of toxic and expensive organic solvents, and offers the lowest cost solution.

Only a small volume (40 µL) of sample solution is needed for the analysis, which is of particular interest when small volumes of the release medium are employed for the evaluation of the in vitro release rate of eluting stents loaded with very low drug amount, especially clobetasol propionate. This could also minimize chromatographic peak distortion problems with the volume overload.

Although we have not done so, it is likely that the present assay could be also adapted for the simultaneous analysis of other combinations, including dexamethasone and everolimus/sirolimus, momethasone and everolimus/sirolimus, and clobetasol and sirolumus, because the dexamethasone, sirolimus, and momethasone do not appear to interfere with clobetasol propionate or everolimus (Figure 5). Additional work is needed, though, to confirm that this assay could be employed for the simultaneous analyses of these combinations.

Because the purpose of this work was to develop and validate a convenient, sensitive, specific, and reproducible high-throughput method for the direct simultaneous determination of everolimus and clobetasol propionate in complex pharmaceutical samples, the method has not been evaluated for biological samples. We think that after employing the sample purification and concentration steps and a higher injection volume (25), it is likely that the present method could be adapted and be an interesting alternative for the simultaneous analyses of clobetasol propionate and everolimus in biological samples. Taking into consideration that the rapid resolution high throughput 1.8-µm column employed in our assay provides 40% more resolving power with 2× the efficiency of the 3.5-µm column (32), the present assay could provide a LOQ even lower than the previously reported one (1 ng) for the routine therapeutic monitoring of everolimus and clobetasol propionate (25). Further investigations may be started in the future to find out if the assay can be adapted for the simultaneous analyses of clobetasol propionate and everolimus in biological samples.

Conclusion

In conclusion, the HPLC method described here is sensitive, selective, linear, and reproducible. It can be used for the simultaneous determination of clobetasol propionate, and everolimus in complex pharmaceutical preparations, and especially for the in-vitro release testing of complex drug delivery systems containing very low drug doses. In addition, the method described is simple, easy to perform, and is distinguished by its speed, which significantly improves the efficiency of the analyses, reduces the con-

sumption of the mobile phase solvent per analysis, and decreases the cost and the environmental impact.

Acknowledgment

We thank Dr. Mikael Trollsas for his critical reading of the manuscript and invaluable comments.

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Manuscript received February 6, 2007;
Revision received April 29, 2007.