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Mass balance in rapamycin autoxidation

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ABSTRACT

The immunosuppressant drug rapamycin is a complex polyene-containing natural product which undergoes autoxidation. The resulting product mixtures contained numerous monomeric and oligomeric compounds, which represented challenges for addressing mass balance in forced degradation studies and in analysis of aged developmental drug-eluting stents. A combination of SEC with ultraviolet and refractive index detection and RP-HPLC was used to account for drug loss and product formation. The mass balance methodology was subsequently validated for the determination of rapamycin and composite rapamycin autoxidation product material in developmental stent samples. This mass balance approach may find general applicability in other situations where drugs degrade to a plethora of products, which cannot be determined individually and summed.

1. Introduction

Understanding the degradation chemistry of a drug is vital for the development of stable and safe pharmaceutical dosage forms and is a regulatory requirement [1]. Furthermore, this knowledge is a prerequisite for the systematic development of validated stability-indicating methods [2]. Forced degradation reactions are used for these drug chemistry studies in conjunction with the analysis of stressed dosage forms [3–5]. One goal of such studies is to account for product material in addition to determining the amount of drug lost [6,7]. Typically, mass balance is achieved by adding the drug assay value to the amounts of individual degradation products and comparing the resulting sum to the initial drug amount. Some possible reasons and corresponding solutions for the lack of mass balance have been presented [6,8]. Alternative HPLC detectors, such as the chemiluminescent nitrogen-specific detector, have been used to address the variability of UV response factors in some mass balance determinations [9–10]. Radiotracers have been used in a few difficult cases [11].

Achieving mass balance may become especially problematic when dealing with individual drug product units (*e.g.*, a single tablet or a single medical device) because of uncertainty in the initial drug amounts. Thus, the amount of drug actually incorporated into each unit during the manufacturing process may be somewhat different from the label claim because variations in the drug addition step or loss of drug during processing (*e.g.,* physical loss, drug–excipient interactions, or drug degradation). Therefore determining mass balance in any given product unit or composite, at the time of manufacture and later, will require the ability to measure the total degradation product amounts directly. A similar need to directly determine impurities has also been expressed for drug substances [7].

Unfortunately, some drugs may degrade to especially complex product mixtures under certain circumstances and present major analytical challenges for the determination of the total degradation product amounts and mass balance. For example, polyene compounds, such as vitamin D analogs [12], simvastatin [13], carotenes [14], retinoids [15], and various polyene-containing lipids [16–21] can readily react with atmospheric oxygen (autoxidation) to give such mixtures. These reactions are typically radical chain processes involving peroxyl radicals as key intermediates [16–23], but additional pathways may be involved in some cases [24]. Among other reactions, a peroxyl radical (that is derived from a polyene molecule) can abstract a hydrogen atom from another molecule or add to a double bond of a second polyene molecule

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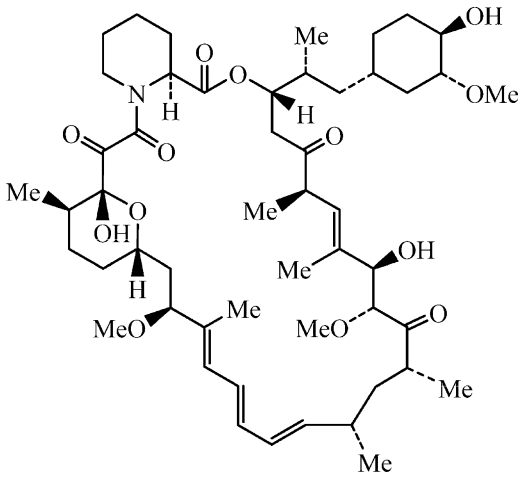

Fig. 1. Rapamycin structural formula.

[23,25]. Ultimately these types of reactions can yield numerous monomeric products (*e.g.,* hydroperoxides, epoxides, aldehydes, ketones, alcohols) as well as numerous dimeric and oligomeric products, which contain monomeric units joined by peroxide linkages and containing some terminal hydroperoxide groups [12–21]. Some dimeric polyene autoxidation products have been demonstrated to be the major source of autocatalytic radical generation [16,17]. Small amounts of such material in drug substance or drug products could lead to future rapid drug degradation. Therefore these product types should be considered in any effort directed toward mass balance determinations and the development of suitable stability-indicating methods.

The immunosuppressant drug rapamycin (Fig. 1) is used in drug-eluting stents [26–29]. This polyene drug, like the polyenes mentioned above, was found to undergo autoxidation [30] and, in the present study, to produce a complicated mixture of monomeric and oligomeric products during forced degradation studies and in aging developmental stents. As described below, mass balance was addressed by using a novel combination of size-exclusion chromatography (SEC) with refractive index (RI) detection and HPLC to determine the amounts of composite monomeric and oligomeric degradation product material.

SEC has not typically been used in such pharmaceutical mass balance studies [6] although SEC (sometimes in conjunction with NP- and RP-HPLC) has been used to analyze autoxidation product mixtures [16–18,31–33] and related materials [34–37]. For example, SEC was used to measure the composite monomeric and oligomeric autoxidation products of some polyene-containing lipids as well as unreacted starting material [18,31]. Resolution between monomeric products and starting compounds (molecular weight, MW = ~300 g/mol) was not totally complete. In the case of rapamycin (MW = 914 g/mol), the drug and monomeric products were not resolved by SEC (see below). Therefore, the combination SEC/HPLC approach was developed.

2. Experimental

2.1. Materials and equipment

Rapamycin was supplied by Wyeth Pharmaceutical (Pearl River, NY). All solvents were HPLC grade (EMD, Gibbstown, NJ). Azobisisobutyronitrile (AIBN) was obtained from Aldrich (Milwaukee, WI) and formic acid from EM Science (Gibbstown, NJ). Water was purified with a Millipore Milli-Q® system (Billerica, MA). Polystyrene standards with narrow molecular weight distributions were provided by Phenomenex (Torrance, CA; PN AL0-2761). Agilent (Santa Clara, CA) 1100 series HPLC systems which consisted of quaternary pumps, autosamplers diode array UV, and RI detectors were used. HPLC-MS data were acquired with a Thermo-Finnigan (Madison, WI) LCQ Deca® XP Plus instrument equipped with ESI and APCI probes. Columns were supplied by Phenomenex, Agilent, and Waters (Milford, MA).

Table 1
Method parameters.

Method	RPa	RPb	SECa	SECb
Column	Eclipse® XDB-C18	”	Three Phenogel® columns (50, 100, and 500 Å)	Styragel® HR 1 THF
Particle size (μm)	3.5	”	5	”
Dimensions (mm)	150 × 4.6	”	300 × 7.8	”
Supplier	Agilent	”	Phenomenex	Waters
Column temperature (°C)	35	”	35	”
Mobile phase A	Formic acid–water–THF (0.02:95:5, v/v/v)	Formic acid (0.02% v/v in water)–THF (95:5, v/v)	THF	”
Mobile phase B	Formic acid–water–THF (0.02:5:95, v/v/v)	Formic acid (0.02% v/v in water)–THF (5:95, v/v)	–	”
Flow rate (mL/min)	1.0	”	0.9	”
Linear gradient (step #1)	A:B (65:35, v/v) to A:B (50:50) over 35 min	A:B (65:35, v/v) to A:B (52:48) over 30 min	100% A for 35 min	100% A for 15 min
Linear gradient (step #2)	A:B (50:50, v/v) to 100% B over 30 min	–	–	”
Equilibration time (min)	12	”	1	”
Injection volume (μL)	5	30	10	30
Detector #1	278, 230 nm	”	230 nm	”
Range (mg/mL)	0.02–2	0.05–0.25	0.02–5	0.00015–0.25
Slope	$1.440 \pm 0.006 \times 10^4$ mAU s mL/mg	$9.32 \pm 0.04 \times 10^4$ μV s mL/μg	$3.92 \pm 0.05 \times 10^3$ mAU s mL/mg	$1.22 \pm 0.004 \times 10^4$ μV s mL/μg
Intercept	58 ± 47 mAU s	$3 \pm 6 \times 10^4$ μV s	$-6 \pm 9 \times 10^1$ mAU s	$9 \pm 5 \times 10^3$ μV s
R^2	0.99969	0.99986	0.99817	0.99980
Detector #2	–	–	RI	–
Range (mg/mL)	–	–	0.02–20	–
Slope	–	–	$8.98 \pm 0.02 \times 10^4$ nRIU s mL/mg	–
Intercept	–	–	$-3 \pm 11 \times 10^2$ nRIU s	–
R^2	–	–	0.99995	–

(–) not used. (”) same as cell to left. Detector response linearity data (slope, intercept, and R^2) are for drug.

Table 2
SEC-RI data (Method SECa) for a typical rapamycin autoxidation product mixture.

Rapamycin and products	Hypothetical structure	Corres-ponding MW (g/mol)	Retention time (min)	M_p, predicted from observed retention time (g/mol)
Rapamycin	Drug	914	22.4	951
Monomers	Drug + 1O	930	22.4	951
Dimers	2 drug + 4O	1892	20.4	2175
Trimers	3 drug + 6O	2839	19.7	3120
Tetramers	4 drug + 8O	3785	19.1	4090

Reaction: 20 mg/mL with AIBN; 1.5 h; 81% rapamycin remaining. Drug: $C_{51}H_{79}NO_{13}$; MW = 914.17.

2.2. Autoxidation of rapamycin

In a typical study, a reaction solution containing 0.0215 M (19.7 mg/mL) rapamycin and 0.108 M AIBN was prepared in acetonitrile. Aliquots of this solution were placed into vials and the vials were placed into each of seven 450 mL stainless steel reactors (Parr Instruments, Moline IL). Additional vials of acetonitrile were included to saturate the atmospheres with solvent. The reactors were pressurized to 300 psi with oxygen and maintained at 50 °C in an oven. Periodically, a reactor was removed and the reaction solutions were analyzed by RP-HPLC-UV and by SEC-UV-RI at 20 mg/mL (no dilution) and 1 mg/mL (after appropriate dilutions with acetonitrile).

The autoxidation product mixture used for SEC method validation contained rapamycin (0.1 mg/mL; 1%), monomeric products (12.5 mg/mL; 73%), and dimeric plus oligomeric degradation products (4.5 mg/mL; 26%) according to RP-HPLC and SEC.

2.3. RP-HPLC-UV-MS

RP-HPLC-UV was used to determine an assay value for rapamycin (and to separate rapamycin from degradation product material). HPLC-MS data were collected for selected samples in both the (+)-ESI and (−)-APCI modes. The initial research RP-HPLC-UV method (Method RPa) was slightly modified to give Method RPb, which was validated for the assay of rapamycin in developmental stent samples (Table 1). The modifications included reducing the formic acid content of the mobile phases, increasing the injection volume, and eliminating the last segment of the solvent program. Reference standard solutions were typically prepared at concentrations of 150 μg/mL in acetonitrile. Dosage form samples were prepared at a concentration of approximately 150 μg/mL by placing an appropriate number of stents into an 8-mL vial, adding acetonitrile, and shaking for 30 min.

2.4. SEC-UV-RI

SEC-UV-RI was used to determine the composite amounts of monomeric and oligomeric degradation products of rapamycin. The initial research method (Method SECa) utilized 50 Å, 100 Å, and 500 Å columns in series to provide selectivity over the molecular weight range of approximately 160–10,000 g/mol (Table 1). Solutions of polystyrene molecular weight standards (peak-maximum MW, M_p = 10,400, 5440, 2240, 1220, 685, 381, 266, and 162 g/mol) were prepared in THF (0.5–1.3 mg/mL). The polystyrene calibration data were fitted to Log (M_p mol/g) = $(A \times T^2) + (B \times T) + C$ where M_p is peak maximum-molecular weight and T is elution time in minutes; $A = 0.0073 \pm 0.002\ \text{min}^{-2}$; $B = -0.495 \pm 0.09\ \text{min}^{-1}$; $C = 10.4 \pm 1.0$; $R^2 = 0.9942$. This research method (Method SECa) was slightly modified to give Method SECb, which was validated for the determination of autoxidation product material in developmental stent samples (Table 1). The original column train was replaced with a single column covering the MW range of 1000–5000 g/mol, the injection volume was increased, and UV (230 nm) detection was used. Solutions were prepared as described for Method RPb. The elution times for rapamycin + monomeric products (monomer peak), dimeric products (dimer peak), and oligomeric products (oligomer peak; combined trimeric and tetrameric products) with Method SECb were approximately 7.6, 7.0, and 6.6 min, respectively. The corresponding values for the research method (Method SECa) are given in Table 2.

3. Results and discussion

3.1. Forced degradation studies

Forced degradation studies of rapamycin to address autoxidation were conducted in acetonitrile under oxygen with AIBN as an initiator [38–40]. The resulting product mixtures were analyzed by RP-HPLC-UV (Fig. 2) and SEC-UV-RI (Fig. 3; Tables 1 and 2). A key requirement for these studies was a universal chromatographic detector and in this situation a RI detector was found to be suitable [41–43]. Nearly identical chromatographic data (not shown) were collected for aged developmental stents which indicated that the forced degradation studies were relevant to drug products.

3.2. Mass balance

The gradient RP-HPLC-UV chromatograms (Fig. 2) showed a few distinct peaks on top of very broad bands covering much of the elution time range. These bands represented numerous low-level products with varying response factors (different UV spectra; see below). The individual or small collections of individual products that were represented by distinct chromatographic peaks did not

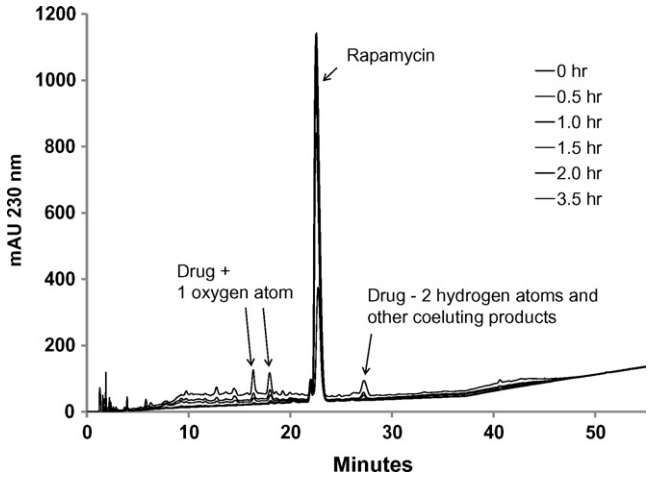

Fig. 2. RP-HPLC chromatograms (230 nm; Method RPa) for a reaction with rapamycin at 20 mg/mL in acetonitrile under oxygen at 50 °C using AIBN (5 M equivalents; see Table 3). The distinct peaks eluting between 15 and 20 min and at ~28 min gave *m/z* values of 952.5 ([drug + 1O + Na]$^+$) and 934.5 ([drug − 2H + Na]$^+$), respectively. The peak eluting just before the drug was a drug isomer (not a degradation product).

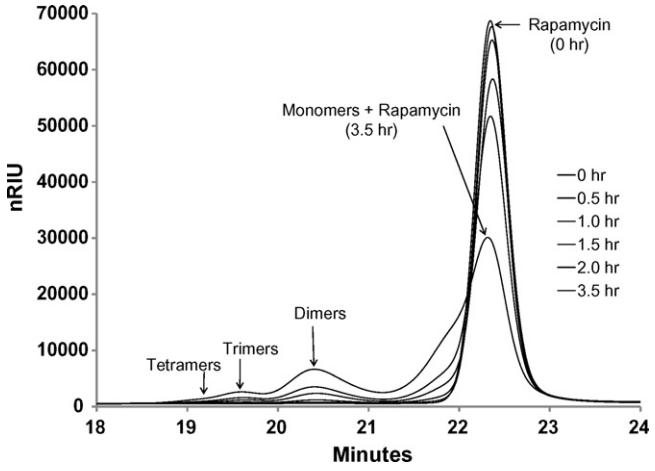

Fig. 3. SEC-RI chromatograms (Method SECa) for a reaction with rapamycin at 20 mg/mL in acetonitrile under oxygen at 50 °C using AIBN (5 M equivalents; see Table 2).

account for a major portion of drug loss. Most importantly, integration of the very broad bands, even with other detector types, was impractical. Thus, mass balance could not be readily achieved with the RP-HPLC-UV alone since the total amount of drug-related degradation product material in any given stent (or collection of stents) could not be easily deduced by this technique.

To address this broad-band problem with RP-HPLC, SEC was pursued as a means to compact the elution of various product types into narrow bands for quantitation as composite mixtures. The SEC-RI chromatograms showed a large peak at 22.4 min which corresponded to unreacted rapamycin and monomeric degradation products (Fig. 3 and Table 2). This chromatographic peak (monomer peak) broadened as the degradation proceeded but unfortunately SEC was not able to resolve the monomeric products from the drug, as in the case of smaller substrate molecules [18,31]. In fact, monomer/drug separation was not expected here since the addition of only one or two oxygen atoms to the large rapamycin molecule should not alter the molecular size significantly. A second distinct SEC peak appeared at 20.5 min and, according to polystyrene calibration, represented dimeric products (Table 2). Likewise, a third distinct peak appeared at 19.7 min which corresponded to trimeric products. There was also an indistinct band at ~19.1 min (Table 2) which corresponded to tetrameric products according to the polystyrene calibration. For quantitation purposes the areas for these fused trimeric and tetrameric peaks were combined (oligomer peak). Fig. 4 presents UV spectra recorded at different retention times in the course of SEC analysis. The UV spectra (Fig. 4) corresponding to the dimer and oligomer peaks showed that the triene UV bands of rapamycin (~260–300 nm) had mostly disappeared and new UV bands appeared at lower wavelengths (~210–260 nm). Thus, the triene functional group region of the drug was a major site of degradation in dimers and oligomers. The UV spectrum recorded at the apex of the monomer peak still shows strong triene absorption, even for the sample with ~25% drug remaining.

Clearly, the RP-HPLC and SEC-UV-RI data together showed that a complicated mixture of monomeric, dimeric, and oligomeric products were being formed from the beginning of the reaction. However, neither method could be used by itself for mass balance determinations. Therefore RP-HPLC, which gave excessively broad bands, was used only to determine the drug amount and this value was used to deal with the problem of the non-resolved drug and monomeric product bands with SEC as described below.

A refractive index detector was used for SEC since the autoxidation products had varying UV spectra and therefore varying UV response factors. However, the RI response factors may also vary. For oligomers, the RI detector signal may have a dependence upon the molecular weight of the individual oligomer as described elsewhere [44]. To address this potential issue, the total RI response was monitored over the course of two autoxidation reactions (Table 3) and found to be reasonably constant. For example, after 76% of the rapamycin had oxidized (in a 20 mg/mL AIBN reaction), the combined SEC-RI area for all peaks was still 94% of the initial area for rapamycin. Therefore, equal RI response factors for the drug and each product were assumed.

Conclusions about the absolute amounts of monomeric, dimeric, and oligomerc products were deduced from the SEC-RI data. While the monomeric products and unreacted rapamycin nearly coeluted, the concentration of the drug (C_{Drug}) in the same sample was determined independently by RP-HPLC-UV. By knowing the drug concentration and the SEC-RI response factor (area/concentration) for the drug by itself (RF_{Drug}), the area contributed by the drug ($C_{Drug} \times RF_{Drug}$) to the area of the composite (monomer) SEC peak ($A_{Total\ Monomers}$) was calculated. Hence (by difference) the area for the total monomeric products (A_M) could be calculated (Eq. (1)). Furthermore, the RI peak areas for the dimeric products and the oligomeric products could be directly determined since the corresponding peaks were separated from the monomer peak. Thus, with this area information and RF_{Drug}, the concentration (C) of each of composite product type was calculated (Eq. (2)–(4); Table 3). Typically, the monomeric products represented the majority of the product material.

$$A_M = A_{Total\ Monomers} - (C_{Drug} \times RF_{Drug}) \quad (1)$$

$$C_M = A_M \times \left(\frac{1}{RF_{Drug}}\right) \quad (2)$$

$$C_D = A_D \times \left(\frac{1}{RF_{Drug}}\right) \quad (3)$$

$$C_O = A_O \times \left(\frac{1}{RF_{Drug}}\right) \quad (4)$$

where subscripts M, D, O indicate monomeric, dimeric, and oligomeric products, respectively. $A_{Total\ Monomers}$ refers to the area of the composite chromatographic peak that represented non-resolved drug and monomeric degradation products.

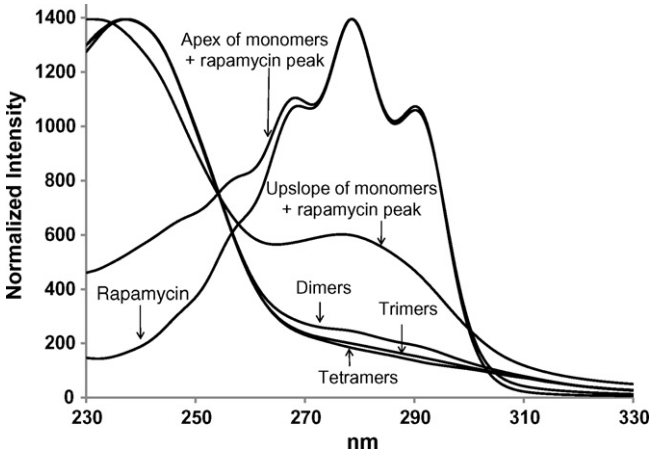

Fig. 4. SEC-UV spectra for components of a typical rapamycin autoxidation product mixture: rapamycin at 20 mg/mL in acetonitrile under oxygen at 50 °C using AIBN (5 M equivalents; 3.5 h; see Table 2).

Table 3
SEC-UV-RI data for typical initiated rapamycin autoxidation reactions.

Elapsed time (h)	Drug (% of initial drug mg/mL)	Monomeric products (% of initial drug mg/mL)	Dimeric products (% of initial drug mg/mL)	Oligomeric products (% of initial drug mg/mL)	Total drug + products (% of initial drug mg/mL)	% of initial SEC-RI area observed	$RRF230_M$ (monomeric products)	$RRF230_D$ (dimeric products)	$RRF230_O$ (oligomeric products)
Method	RP-HPLC	SEC-RI	SEC-RI	SEC-RI	RP-HPLC SEC-RI	SEC-RI	SEC-UV (230 nm)	SEC-UV (230 nm)	SEC-UV (230 nm)
Reaction: rapamycin = 20 mg/mL; AIBN = 5 M equivalents; solvent = acetonitrile; temp. = 50 °C									
0.00	101	2	0.1	0	103	100	Initial sample	Initial sample	Initial sample
0.25	100	3	0.3	0.1	104	101	1.3	2.1	2.6
0.50	99	4	0.4	0.1	103	101	2.5	2.7	2.3
0.75	99	4	1	0.3	104	101	1.6	2.3	2.1
1.00	94	8	2	1	104	101	1.5	2.3	1.9
1.50	81	14	4	2	102	99	2.1	2.6	2.3
2.00	66	24	8	2	100	97	2.0	2.6	2.8
3.50	24	49	18	5	97	94	2.3	2.4	2.3
Reaction: rapamycin = 80 mg/mL; AIBN = 5 M equivalents; solvent = acetonitrile; temp. = 50 °C									
0.00	98	5	0.2	0.0	103	100	Initial sample	Initial sample	Initial sample
0.25	98	1	0.7	0.4	100	97	2.2	2.2	2.7
0.50	92	6	1	1	101	97	1.3	2.7	2.6
0.75	87	9	3	2	102	98	1.8	2.5	2.4
1.00	79	12	4	3	99	96	2.0	2.7	2.6
1.50	61	19	9	6	95	92	2.6	2.5	2.4
2.00	45	28	13	9	95	92	2.5	2.4	2.3
3.50	5	49	23	12	89	86	2.4	2.1	2.3
						Average	2.0	2.4	2.4
						S.D.	0.4	0.2	0.3

In order to perform similar analyses on HPLC instruments without RI detectors, the composite SEC-UV (230 nm) relative response factors (RRF230; with respect to the drug) were estimated for each of the three types of autoxidation products (Table 3). The 230 nm data channel was chosen for this exercise because the parent drug exhibited relatively little absorbance at this wavelength while the products had significant absorption (Fig. 4). By analogy to SEC, the UV 230 nm area contribution of the drug to the composite monomer peak ($C_{Drug} \times RF230_{Drug}$) was calculated from the drug concentration (C_{Drug}) obtained from RP-HPLC data and from the measured drug response factor for SEC-UV (230 nm; $RF230_{Drug}$). The area contribution by the monomeric products ($A230_M$) was simply the total area ($A230_{Total\ Monomers}$) for the monomer peak minus the area contributed solely by the drug (Eq. (5)). The SEC-UV (230 nm) areas for the dimeric and oligomeric products could be determined by direct peak integration. These three composite product areas and their corresponding concentrations from SEC-RI (Eqs. (2)–(4)), as well as the SEC-UV response factor for the drug, were then used together to calculate the relative response factors (RRF230; Eqs. (7) and (8); Table 3).

$$A230_M = A230_{Total\ Monomers} - (C_{Drug} \times RF230_{Drug}) \quad (5)$$

$$RRF230_M = \frac{(A230_M/C_M)}{RF230_{Drug}} \quad (6)$$

$$RRF230_D = \frac{(A230_D/C_D)}{RF230_{Drug}} \quad (7)$$

$$RRF230_O = \frac{(A230_O/C_O)}{RF230_{Drug}} \quad (8)$$

For dimeric and oligomeric products, there did not seem to be any trends for the RRF230 values with respect to the level of degradation or with respect to the initial concentration of drug. $RRF230_M$ seemed to increase with the level of rapamycin degradation. However, the uncertainty in the average $RRF230_M$ value was greater than the uncertainties in the average $RRF230_D$ and $RRF230_O$ values and this prevented more detailed evaluation of the data at low levels of conversion. The higher uncertainty in the former value was probably due to the need to incorporate the separate RP-HPLC drug assay value in the individual $RRF230_M$ determinations.

Finally, these mass balance studies showed the formation of dimeric and oligomeric products which were presumably related structurally to the dimeric autoxidation products from polyene-containing lipids, which were shown to be a major source of autocatalytic activity [16–17]. Thus, the presence of such compounds in any given drug product could portend a future propensity for autoxidative drug degradation. However, the methodology described herein detected and quantitated the total amounts of these potentially problematic dimeric/oligomeric degradation products.

3.3. Validation of RP-HPLC-UV method

The Method RPb (Table 1) was formally validated for the analysis of developmental stents. *Specificity* was addressed by noting the absence of significant changes in the UV and MS spectra across the rapamycin peak for a typical autoxidation product mixture (20 mg/mL reaction; 1.5 h; Fig. 2). Moreover, there were no interferences from components in extracts of placebo developmental stents. *Linearity* was addressed with rapamycin solutions that were prepared in duplicate over the range of 50 μg/mL to approximately 249 μg/mL (Table 1). *Accuracy* was addressed by determining the recovery of rapamycin from placebo stents spiked with rapamycin at 33%, 50%, 67%, 83%, 100%, 133%, and 167% of the target assay

concentration. Six replicate solutions were prepared at the 100% level and triplicate solutions were prepared at the other levels. The recovery values ranged from 100.1% to 101.5% (R.S.D. = 0.2–1.0%). *Precision* (analysis repeatability) was addressed by analyzing six developmental stents at each of two target assay concentrations of rapamycin. The R.S.D. values were 1.0% and 2.0%, respectively. *Intermediate precision* (accuracy) was addressed with six placebo stents spiked with rapamycin at 100% of the target assay concentration. These samples were analyzed by two different analysts in different laboratories. The recovery values were 100.9% (R.S.D. = 0.4%) and 97.1% (R.S.D. = 0.3%), respectively. *Intermediate precision* (repeatability) was addressed by the analysis of two sets of six stents by two different analysts in two laboratories. The R.S.D. values were 1.0% and 2.0% for the first analyst and 0.8% and 1% for the second analyst. The *limit of detection* (LOD) was studied with a solution of rapamycin at a nominal concentration of 0.03% of the target assay concentration (150 μg/mL). Six replicate injections or this solution gave signal-to-noise-ratios of 10–12. The *limit of quantitation* (LOQ) was studied with a solution of rapamycin at a concentration of 0.15 μg/mL (0.1% of the target rapamycin assay concentration of 150 μg/mL). Six replicate injections of this solution gave signal-to-noise ratios of 82–105. *Robustness* of the method was evaluated by modifying various method parameters and comparing results to those obtained with the prescribed method. Parameter (% difference): 20 min extraction time (0.7%), 40 min extraction time (0%), 1.1 mL/min flow rate (1.3%), 0.9 mL/min flow rate (1%), 33 °C column (1%), 37 °C column (0%), 27 min gradient (1%), 33 min gradient (2%). Standard and sample *solution stability* was evaluated by analyzing solutions stored at ambient conditions and at refrigerated conditions (0–8 °C) protected from light over a 5-day period. The values for % difference (versus initial assay) were: standard/ambient (0.1%), standard/refrigerated (2.2%), sample/ambient (0.3%), and sample/refrigerated (0.3%).

3.4. Validation of SEC-UV method

The Method SECb was also validated for developmental stent samples. *Specificity* was addressed by noting the absence of interference from components in extracts of placebo stents. However, this method was intended to be non-specific for monomeric autoxidation products (*i.e.*, they eluted together along with rapamycin). *Linearity* was addressed with solutions of rapamycin and with solutions of autoxidation product material that resulted from an AIBN-initiated reaction in which nearly all of the rapamycin had been consumed. Linearity data for rapamycin are presented in Table 1. The range of the method for the monomeric and dimeric/oligomeric products was 0.15–80 μg/mL. Based upon accuracy results (see below), the accuracy range for monomeric and dimeric/oligomeric products was established at 1.5–37.5 μg/mL concentration. Linearity was observed for the monomeric products with 230 nm data: Area (in μV s) = $M \times$ concentration (in μg/mL) + B, where $M = 2.52 \pm 0.01 \times 10^4$ μV s mL/μg, $B = 4 \pm 3 \times 10^3$ μV s, and $R^2 = 0.99981$. Linearity was observed for the dimeric/oligomeric products with 230 nm data: Area (in μV s) = $M \times$ concentration (in μg/mL) + B, where $M = 2.53 \pm 0.01 \times 10^4$ μV s mL/μg, $B = -1.1 \pm 0.3 \times 10^4$ area counts, and $R^2 = 0.99988$. The *accuracy* of the method was evaluated by determining the recovery of monomeric and dimeric/oligomeric products in the presence of placebo stents. The levels evaluated were 1%, 5%, and 25% of the target assay value (150 μg/mL). Six solutions were prepared at the 5% level and triplicate solutions were prepared at the 1% and 25% levels. The corresponding recovery values were 96, 98, and 100% (R.S.D. = 2%, 3%, and 3%) for the monomers and 88%, 101%, and 106% (R.S.D. = 11%, 3%, and 3%) for the oligomers. *Precision* (analysis repeatability) was addressed with six aged developmental stents. The R.S.D. values for the monomers and dimers/oligomers were 9% and 7%, respectively. *Intermediate precision* (accuracy) was evaluated with six placebo stents spiked with monomeric and dimeric/oligomeric products at 5% of the target assay concentration (150 μg/mL). These samples were analyzed by two different analysts in two different laboratories. The average recovery values were 98% (R.S.D. = 3%) for monomers and 101% (R.S.D. = 4%) for dimers/oligomers for the first analyst. The corresponding values for the second analyst were 97% (R.S.D. = 0.4%) and 92% (R.S.D. = 4%). *Intermediate precision* (analysis repeatability) was likewise evaluated with six aged developmental stents and two analysts. R.S.D. values for both monomers and dimers/oligomers were 9% for the first analyst and 5% for the second analyst. The LOD was evaluated with spiked solutions prepared with monomeric and dimeric/oligomeric products present at 0.03% of the target rapamycin assay concentration of 150 μg/mL. Triplicate injections of each solution were made. The signal-to-noise-ratios for the monomeric and dimeric/oligomeric products were 8–10 and 3–4, respectively. The LOQ was evaluated with solutions containing monomeric and dimeric/oligomeric products at 0.1% of the target rapamycin assay concentration of 150 μg/mL. Six replicate injections of each solution were made. The signal-to-noise-ratios for the monomeric and dimeric/oligomeric products were 10–15 and 10–13, respectively. *Robustness* of the method was evaluated by modifying various method parameters and comparing results to those obtained with the prescribed method. Parameter (% difference): 20 min extraction time (0%), 40 min extraction time (4%), flow rate 1 mL/min (9%), flow rate 0.8 mL/min (9.7%), column temperature 32 °C (4%), and column temperature 38 °C (8%). A dosage form extract solution was stable when refrigerated (0–8 °C) for up to 3 days (3% difference from initial value).

4. Conclusions

A combination of SEC-UV-RI and HPLC-UV analytical data was used to address mass balance in a situation where the summation of individually determined drug degradation products was not feasible. Furthermore, these combined methods were subsequently validated for the determination of rapamycin and rapamycin autoxidation product material in drug-eluting stents. This mass balance approach may have general applicability to other drugs which degrade to a plethora of products in pathways such as autoxidation and photooxidation.

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