Forced degradation of tacrolimus and the development of a UHPLC method for impurities determination

TANJA ROZMAN PETERKA*
TINA TRDAN LUŠIN
JURE BERGLES
ZORAN HAM
ROK GRAHEK
UROŠ URLEB

*Lek Pharmaceticals d.d.
SI-1526 Ljubljana, Slovenia*

An ultra-high performance liquid chromatography method for simultaneous determination of tacrolimus impurities in pharmaceutical dosage forms has been developed. Appropriate chromatographic separation was achieved on a BEH C_{18} column using gradient elution with a total run time of 14 min. The method was applied to analyses of commercial samples and was validated in terms of linearity, precision, accuracy, sensitivity and specificity. It was found to be linear, precise and accurate in the range of 0.05 to 0.6 % of the impurities level in pharmaceutical dosage forms. Stability indicating power of the method was demonstrated by the results of forced degradation studies. The forced degradation study in solution revealed tacrolimus instability under stress alkaline, thermal, light and photolytic conditions and in the presence of a radical initiator or metal ions. The drug was stable at pH 3–5. Solid-state degradation studies conducted on amorphous tacrolimus demonstrated its sensitivity to light, elevated temperature, humidity and oxidation.

Tacrolimus (TAC), also known as FK-506, is a natural product originally isolated from the bacterium *Streptomyces tsukubaensis* (1–3). It is an immunosuppressive drug used in preventing organ and tissue rejection in patients after transplantation and in the treatment of other autoimmune diseases (4–7).

TAC is a complex 23-membered macrolide lactone with incorporated masked tricarbonyl and ʟ-pipecolic acid moieties (Fig. 1a) (8). Due to its structural characteristics, TAC can undergo different kinds of transformations including dehydration, epimerization, rearrangement and isomerization of double bonds (8). Several potential degradation products are reported in the literature in addition to some closely related process impurities, which may be present in the final drug due to the manufacturing process of the drug substance (8–15). Moreover, two kinds of conformational heterogeneity of TAC are known (16, 17): *cis-trans* conformational isomerization due to restricted rotation of the amide bond

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* Correspondence, e-mail: tanja.rozman_peterka@novartis.com

in the pipecolic acid moiety (16), and epimerization observed in polar solvents, where TAC undergoes a hydration reaction to a diol, which is then converted into the C-10 epimer, reaching equilibrium with all the three co-existing forms (16, 17) (Fig. 1a). Both TAC equilibrium compounds, herein named TAC compound I and II, are not considered as impurities since both demonstrate equivalent biological activity as TAC (11, 18).

Several methods have been reported for the analysis of TAC-related substances and degradation products (14, 15, 19, 20). In the *United States Pharmacopeia* (USP) official monograph of TAC and TAC-capsules, two high performance liquid chromatography (HPLC) methods are described for the analysis of TAC organic impurities, since not all potential impurities could be measured by one method alone (14). The first USP method is based on normal phase chromatography and demonstrates use of two consecutively connected columns for separation of impurities with a total separation time of more than 45 min. The second USP method is a reverse-phase (RP) HPLC method with a total run time of 60 and 85 min for the TAC drug substance and TAC-capsules, resp. In the *European Pharmacopoeia* (EP) monograph, a gradient method with a run time of 53 min is described and is applied for the TAC drug substance only (15). The most promising pre-existing method applied for pharmaceutical formulations seemed to be a UHPLC method with a total run time of 25 min developed by Subasranjan *et al.* (20), where efficient separation of four known impurities from TAC and its equilibrium compounds was demonstrated. In our previous work, we used a fast UHPLC method for evaluation of TAC incompatibility with excipients (13). However, the reported method was not finally optimized with regard to its sensitivity and selectivity with respect to all the relevant TAC impurities that might be present in the final drug product.

Previously described methods are time consuming and have limited ability to detect all important impurities. In this context, the aims of the present study were to develop and validate a fast and sensitive UHPLC method that would make it possible to simultaneously detect all the relevant TAC impurities and to evaluate the intrinsic stability of TAC through forced degradation studies.

EXPERIMENTAL

Samples, reagents and apparatus

TAC monohydrate drug substance (purity by HPLC 97.1 %) and TAC working standard (purity by HPLC 97.7 %) were available from Lek Pharmaceuticals, Slovenia. Impurities TAC-H1, TAC-RI, TAC-EPI, TAC-diene, TAC-dehydro and ascomycin were available from Lek Pharmaceuticals, Slovenia. Tacrolimus 8-propyl analog was obtained as a USP reference standard (USP, USA). Identity and purity of the used impurities was as follows: TAC-H1 (identity: ^{1}H NMR, ^{13}C NMR, MS, IR; purity by ^{1}H NMR 94 %), TAC-RI (identity: ^{1}H NMR, ^{13}C NMR, MS, IR; purity by ^{1}H NMR 88 %), TAC-EPI (identity: ^{1}H NMR, ^{13}C NMR, MS, IR; purity by ^{1}H NMR 101 %), TAC-diene (identity: ^{1}H NMR, ^{13}C NMR, MS, IR; purity by ^{1}H NMR 94 %), TAC-dehydro (identity: ^{1}H NMR, ^{13}C NMR, MS, IR; purity by ^{1}H NMR 88 %) and ascomycin (identity: ^{1}H NMR; purity by ^{1}H NMR 98 %), Tacrolimus 8-propyl analog (USP RS, 100 % purity). Structures of impurities were originally elucidated and disclosed by different groups in earlier works (8–11, 13). Their structural formulae are shown in Figs. 1b,c.

Samples of Advagraf® 0.5 mg and 5 mg capsules (Astellas Pharma, batch No. 0M6041A and batch No. 5M4297A, Canada) were obtained from the Canadian market.

a)

Tacrolimus (TAC)

Tacrolimus diol
(TAC compound I)

Tacrolimus 10-epimer
(TAC compound II)

b)

Ascomycin

Tacrolimus propyl analog
(TAC propyl analog)

c)

Tacrolimus alpha-hydroxy acid
(TAC-H1)

Tacrolimus epimer
(TAC-EPI)

Tacrolimus regioisomer
(TAC-RI)

Tacrolimus diene
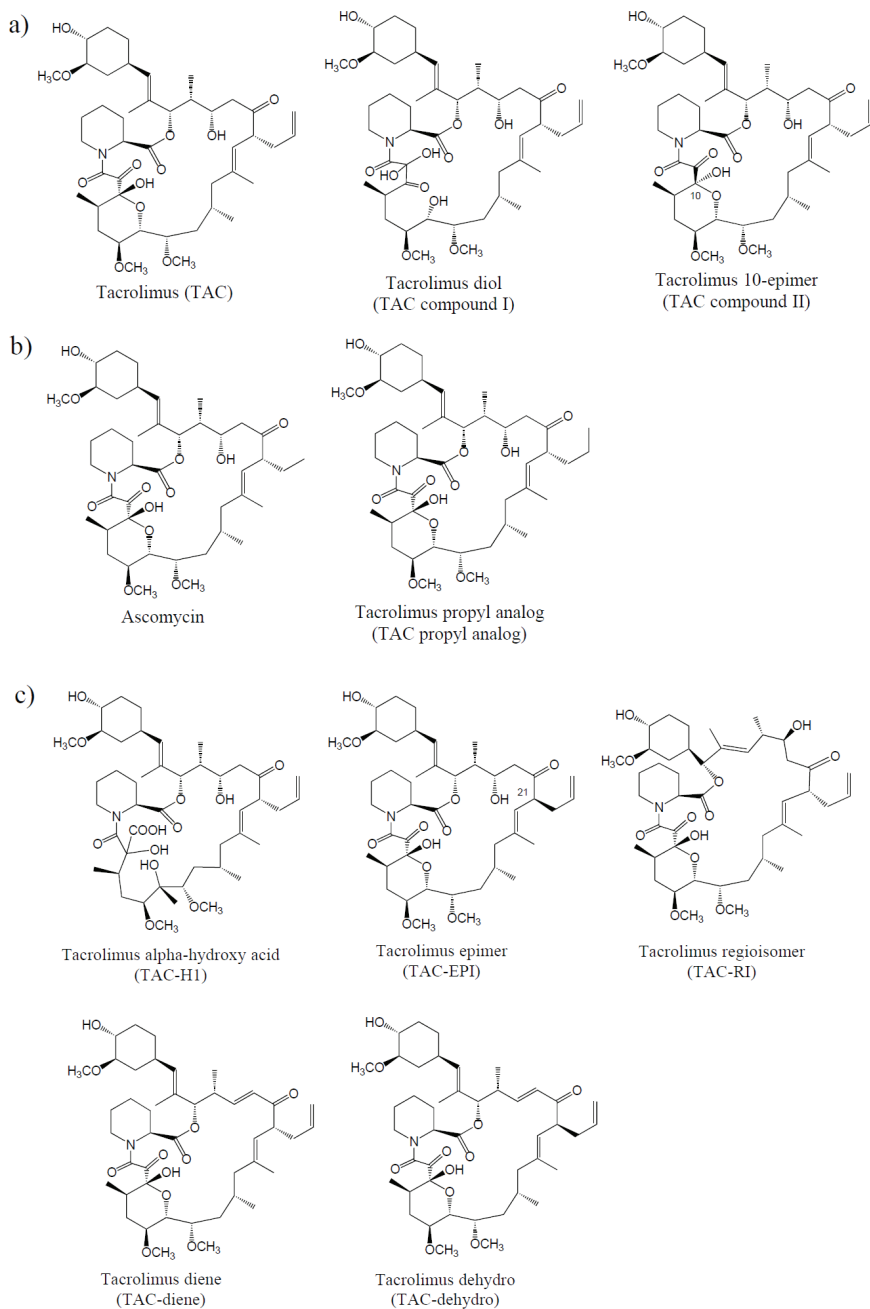(TAC-diene)

Tacrolimus dehydro
(TAC-dehydro)

Fig. 1. Structural formulae of: a) TAC and its equilibrium compounds I and II in solution, b) TAC-related substances and by-products, c) TAC degradation products.

Phosphoric acid (85 %), iron(III) chloride hexahydrate, hydrogen peroxide (30 %), sodium hydroxide solution Titrisol®, hydrochloric acid solution Titrisol® and buffer solutions Centipur® were supplied by Merck (Germany). 4,4′-Azobis(4-cyanovaleric acid) (ACVA) was obtained from Sigma-Aldrich (USA). Solvents for chromatography, acetonitrile (ACN) and methyl *tert*-butyl ether (MTBE) were purchased from J. T. Baker (USA) and Merck (Germany), resp. Deionized water was prepared using a Millipore Milli-Q water purification system (Millipore, USA). The following stability chambers were used for stability studies: Suntest XLS+ from Atlas (USA), thermostatic chamber BF 720 from Binder (Germany) and climate chamber VP 1300 from Weiss Technik (Germany).

Preparation of standard and reference solutions

TAC standard solutions were prepared at a concentration of 15.0 µg mL^{-1} using ACN-water in a ratio of 70:30 (*V/V*) as a solvent for forced degradation studies and a mixture of ACN and 5 mmol L^{-1} ammonium acetate buffer (pH 5) in a ratio of 80:20 (*V/V*) for the analysis of capsules. Reference solution for impurity identification was prepared by dissolving TAC and its impurities (ascomycin, TAC-propyl analog, TAC-H1, TAC-RI, TAC-EPI, TAC-diene, TAC-dehydro) in a mixture of ACN and 5 mmol L^{-1} ammonium acetate buffer (pH 5) in a ratio of 80:20 (*V/V*) at a concentration of 3000 µg mL^{-1} and 15.0 µg mL^{-1} for TAC and the aforementioned impurities, resp.

UHPLC instrumentation and conditions

Chromatographic separations were performed with a Waters ACQUITY UPLC system controlled by the Empower 3 software, using an ACQUITY UPLC^{TM} BEH C$_{18}$ column, 100 mm × 2.1 mm, 1.7 mm (Waters, USA). Column temperature was 70 °C and the mobile phase was preheated before reaching the column. Mobile phase A was a phosphoric acid solution (0.1 %, *V/V*). Mobile phase B was prepared by mixing 850 mL of ACN and 80 mL of MTBE. It was filtered through a 0.1-µm PTFE filter (Merck Millipore, USA) prior to use. The following gradient program was employed: mobile phase A:B (*V/V*)/time (min); 63:37/0 min (initial conditions), 63:37/1.0 min, 52:48/9.0 min, 30:70/11.0 min, 30:70/13.5 min, 63:37/14.0 min; a linear gradient was applied. The flow rate was 0.75 mL min^{-1}. The system returned to the initial conditions after 14.0 min and continued under the same conditions for further 2.0 min. UV detection was carried out at 220 nm using a photodiode array detector (PDA) equipped with a high sensitivity (25 mm) flow cell. Injection volume was 3 mL.

X-ray powder diffraction measurement

X-ray powder diffraction measurement was made with an Xpert PRO MPD instrument (PANalytical, The Netherlands) in Bragg-Brentano geometry using CuKα radiation (45 kV, 40 mA) in the range of 2–40 ° 2θ, step size of 0.033 ° 2θ and step time of 50 s. Automatic divergence slits were used to irradiate 10 mm of sample length.

Forced degradation in solid state

Amorphous TAC was obtained by dissolving 5 g of crystalline TAC drug substance in 50 mL of methanol. The solvent was removed under reduced pressure (35 kPa) at 80 °C

Table I. Forced degradation study design and storage conditions

Degradation type	Experimental conditions	Storage conditions	Storage time
Forced degradation in solid state			
Initial	n.a.	n.a.	n.a.
Thermal/humidity	Open dish, stability chamber	50 °C/30 % RH	1 month
	Open dish, stability chamber	50 °C/50 % RH	1 month
	Open dish, stability chamber	50 °C/75 % RH	1 month
Oxidation	Sealed vial, nitrogen headspace	50 °C	1 month
	Sealed vial, air in headspace	50 °C	1 month
	Sealed vial, oxygen headspace	50 °C	1 month
Photolytic	Artificial sunlight	SUNTEST XLS+, 300–800 nm, 250 W m^{-2}	16 hours
	Dark control (in aluminum foil)	SUNTEST XLS+, 300–800 nm, 250 W m^{-2}	16 hours
	Artificial light	Cool white fluorescent lamp, 2000 lux	22 hours
	Dark control (in aluminum foil)	Cool white fluorescent lamp, 2000 lux	22 hours
Forced degradation in solution			
Initial	n.a.	n.a.	n.a.
Hydrolysis	0.01 mol L^{-1} HCl	Room temperature	24 hours
	0.1 mol L^{-1} HCl	Room temperature	24 hours
	0.01 mol L^{-1} NaOH	Room temperature	24 hours
	0.1 mol L^{-1} NaOH	Room temperature	24 hours
	pH: 3, 5, 7, 9, 11	Room temperature	24 hours
Oxidation	3 % H$_2$O$_2$	Room temperature	24 hours
	ACVA (1 mg mL^{-1})	60 °C	24 hours
Metal catalysis	FeCl$_3$ (1 mg mL^{-1})	60 °C	24 hours
Photolytic	Artificial sunlight	SUNTEST XLS+, 300–800 nm, 250 W m^{-2}	2 hours
	Dark control (in aluminum foil)	SUNTEST XLS+, 300–800 nm, 250 W m^{-2}	2 hours
	Artificial light	Cool white fluorescent lamp, 2000 lux	22 hours
	Dark control (in aluminum foil)	Cool white fluorescent lamp, 2000 lux	22 hours
Thermal	Thermostatic chamber	60 °C	24 hours
	Heat control	Room temperature	24 hours

ACVA – 4,4-azobis(4-cyanovaleric acid), n.a. – not applicable, RH – relative humidity

until it reached a very viscous consistence. After evaporation under reduced pressure, the residue was lyophilized overnight to obtain dry amorphous TAC. Samples were exposed to elevated temperature, dry/wet environment and different headspace atmosphere (nitrogen/air/oxygen), as summarized in Table I. Samples were analyzed before and after storage under the described conditions. In addition, photo stability testing was conducted by exposing the material to artificial sunlight or cool white fluorescent light (Table I). Prior to UHPLC analysis, samples were dissolved in a solvent (ACN-water, 70:30, *V/V*) to give a solution containing approximately 3000 µg mL^{-1} of TAC.

Forced degradation in solution

TAC monohydrate (750 mg) was dissolved in ACN (25 mL). After transferring 2-mL aliquots of this initial solution into 20-mL flasks, 1 mL of the respective stress medium was added (acid, base, buffer, water, FeCl$_3$ solution, peroxide, ACVA, *etc.*). Photo-degradation studies were performed with 1 mL of water added to 2 mL of initial solution, followed by exposure to artificial sunlight using SUNTEST XLS+ or cool white fluorescent light. Suitable controls were kept under the same conditions protected with aluminum foil (dark control). For thermal stress, TAC dissolved in 2 mL of ACN with 1 mL of water was heated at 60 °C. Samples were withdrawn at appropriate time and subjected to UHPLC analysis. Before analysis, samples were further diluted with a solvent consisting of ACN-water (70:30, *V/V*) to get a concentration of approximately 3000 µg mL^{-1} of TAC. Details on storage conditions and times are given in Table I.

Analysis of capsules

Applicability of the proposed UHPLC method was evaluated by analyses of commercially available capsules (Advagraf®) of two doses: 0.5 mg and 5 mg. Samples in the original packaging configuration were analyzed before being placed under accelerated stability conditions (40 °C/75 % relative humidity) and also after 1, 3 and 6 months of storage under accelerated stability conditions. For the purpose of sample preparation, a mixture of ACN and 5 mmol L^{-1} ammonium acetate buffer (pH 5) in the ratio of 80:20 (*V/V*) was used as a solvent.

For UHPLC analysis, capsules were opened and the amount of capsule content corresponding to 15 mg of TAC was weighed and dissolved in 5 mL of solvent. Samples were then sonicated for 20 min with occasional manual shaking. After centrifugation of samples at 5000 rpm for 10 min, the supernatant was filtered through a 0.2-µm PTFE filter (Merck Millipore) and analyzed *via* UHPLC.

Method validation

Specificity. – Specificity of the method was investigated for all available impurities, sample solution, placebo solution and blank. Specificity was demonstrated by spiking the drug product (3000 µg mL^{-1}) with an appropriate level of impurities (15.0 µg mL^{-1}).

Linearity. – Linearity of the method was investigated in the range 1.5–18 µg mL^{-1} using nine points (1.5, 3, 4.5, 6, 7.5, 9, 12, 15, 18 µg mL^{-1} TAC). TAC concentration ranged from 0.05 to 0.6 % of TAC concentration in the sample solution (3000 µg mL^{-1}).

Accuracy (recovery). – For accuracy experiments, samples were spiked with degradation products TAC-H1, TAC-RI, TAC-EPI and TAC-diene at 0.05, 0.5 or 0.6 % of TAC concentration in the sample solution (3000 μg mL^{-1}). As small quantities of impurities may already be present in sample solutions, their content was estimated by injecting a sample solution without addition of any impurity stock solution. These results were then subtracted from the content of impurities measured after spiking to obtain the determined amount of added impurities for recovery calculations.

Precision. – For precision experiments, an in-house drug product (composed of TAC and excipients hypromellose, ethylcellulose, lactose monohydrate and magnesium stearate) was stressed at 60 °C/50 % relative humidity for 7 days or aged under accelerated stability conditions of 40 °C/75 % relative humidity for 3 months, to obtain a sufficient level of degradation products for evaluation. Six replicates of sample solutions were prepared and analyzed.

*Limit of detection (*LOD*) and limit of quantitation (*LOQ*).* – *LOD* and *LOQ* were evaluated on the basis of the signal-to-noise (*S/N*) ratio ≥ 3:1 and ≥ 10:1, resp., for six replicate determinations containing 0.02 or 0.05 % of the compound of interest, relative to the concentration of TAC in sample solution (3000 μg mL^{-1}).

<div align="center">RESULTS AND DISCUSSION</div>

Optimization of the UHPLC method

A reversed phase UHPLC method was developed to quantitate TAC impurities. Effects of various factors such as mobile phase composition, stationary phase, gradient and temperature were evaluated, and parameters that assure the most robust and fast separation of analytes were selected.

pH does not significantly affect method selectivity and repeatability, since TAC and all known impurities except TAC-H1 are not ionized under conditions normally used for reversed phase liquid chromatography. The only known impurity that could be affected by the selection of the mobile phase pH is TAC-H1. The TAC-H1 impurity is acid; therefore, the polarity of the molecule depends on the pH of the mobile phase. At pH two units below pK_a (estimated cca. 4.6, typical pK_a for carboxylic acids) the molecule is not ionized and thus less polar; it consequently elutes later in the reverse phase mode. In the case of pH above pK_a, TAC-H1 would be poorly retained and difficult to quantify by the liquid chromatography (LC) method. We therefore decided to use 0.1 % phosphoric acid for the mobile phase A preparation to ensure that TAC-H1 will be un-ionized, which improves its peak shape and increases its retention time. TAC-H1 is much more polar than the other impurities and TAC itself; therefore, selectivity of a method for TAC-H1 determination is not critical. Separation of other impurities was more challenging, and it was found that method selectivity was largely dependent on MTBE percentage in the mobile phase and on temperature.

Stationary phase selection was less critical than expected as several tested columns (BEH phenyl, CSH C$_{18}$, BEH C$_{18}$, CSH fluoro-phenyl, CSH phenyl-hexyl) could be used for method optimization. However, the elution strength of mobile phases needs to be adjusted according to the selected stationary phase to achieve suitable retention of analytes and the best possible method efficiency. Waters BEH C$_{18}$ column proved to be most suitable because

of its performance at elevated temperature and adequate retention of all analytes. A 100-mm column with 1.7 µm particles was sufficient for fast separation of all structurally similar TAC impurities from the main peak and from each other.

Optimized mobile phase B is a mixture of two organic modifiers, which are crucial for method selectivity. ACN is suitable because of its low viscosity and adequate elution strength and in combination with MTBE analysis time and peak width were markedly reduced. In general, use of MTBE has to be carefully considered as MTBE is not miscible with water but has a low UV cut-off and high elution strength, which is favorable for this analysis. Selection of suitable parameters for separation of TAC and its impurities is challenging due to molecular similarity and high hydrophobicity, and the use of MTBE considerably improves method selectivity and shortens analysis time. However, if the percentage of MTBE is too high, co-elution of TAC-RI and ascomycin is observed, as well as co-elution of TAC-EPI with the TAC-propyl analogue. Other organic modifiers, including methanol, 2-propanol and tetrahydrofuran, were also tested, but resulted in broad peaks, increased back pressure and reduced sensitivity due to the baseline at low wavelengths.

In the case of TAC, selection of suitable temperature is also significant for improving resolution between the known impurities as well as for reducing analysis time. At 30 °C, several co-elutions can be seen under the same chromatographic conditions. At 65 °C, all impurities are separated, but to increase method robustness, 70 °C was selected as the most suitable temperature. Increase in temperature also facilitates rotation of the amide bond, which has a considerable effect on the narrowing of peaks. This is essential for separation of the known impurities TAC-RI and ascomycin from TAC and TAC compound II. Further increase of temperature impairs resolution between TAC-RI and TAC compound II and, in addition, it is not recommended because it could shorten the column lifetime. In general, higher temperature allows optimization of methods with a higher flow rate due to reduced mobile phase viscosity.

A slow gradient was required to achieve separation of the most structurally similar analytes. After the initial 1 min hold, the percentage was increased by only 9 % in 8 min in the mixture of organic modifiers. Subsequent gradient slope was increased markedly to
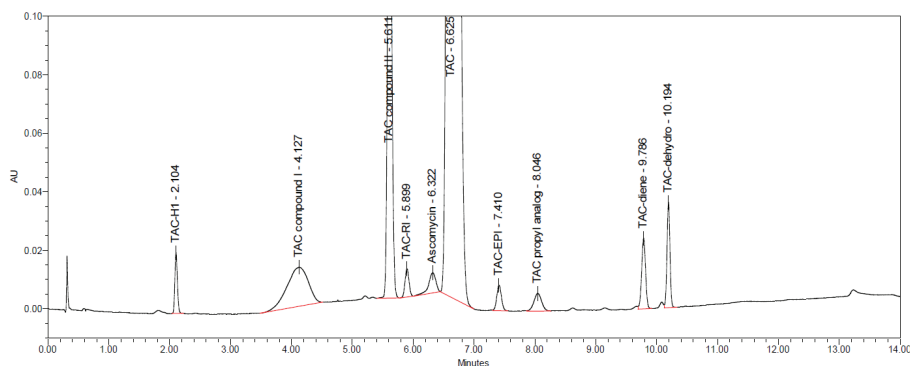

Fig. 2. Typical UHPLC chromatogram of TAC spiked with its isolated impurities: related substances (ascomycin and TAC-propyl analog) and degradation products (TAC-H1, TAC-EPI, TAC-RI, TAC-diene and TAC-dehydro).

speed up elution of the remaining impurities. A representative chromatogram of TAC spiked with its impurities is presented in Fig. 2.

Detection wavelength was selected on the basis of the recorded UV spectra of analytes and in compliance with the USP monograph for TAC (14). At 220 nm, all impurities can be precisely quantified. Lower wavelengths are less appropriate due to increased baseline noise and a high slope in UV spectra, which can impair repeatability when using different detectors. To improve sensitivity, a 25-mm detector flow cell was used instead of the standard 10-mm flow cell, since the *LOQ* of TAC was expected to be 1.5 µg mL^{-1} when injecting 3 µL. According to the International Conference on Harmonisation (ICH) guidelines, the *S/N* ratio for TAC at *LOQ* concentration (which corresponds to 0.05 % of the reporting limit for TAC impurities) needs to be checked in every analysis, and sufficient method sensitivity must be ensured, so that *S/N* ratio ≥ 10 can be repeatedly achieved on all tested UHPLC systems (21).

Other reported LC methods for TAC impurities determination are not capable of detecting and quantitating all relevant TAC impurities in a single run with sufficient accuracy and *LOQ*. The existing compendial HPLC methods involve a long analysis time and lack of sensitivity as the method *LOQ* needs to be the same as the reporting limit for all impurities. In addition, none of the previously published UHPLC methods was demonstrated to be appropriate for determination of various TAC impurities that may be present in the final dosage form. The newly developed method represents a significant advancement in the determination of TAC impurities. It has improved selectivity for several identified degradation products (TAC-H1, TAC-RI, TAC-EPI, TAC-diene and TAC-dehydro) and TAC related substances (ascomycin and TAC-propyl analogue) also with respect to unknown impurities found during the degradation study. Moreover, the overall analysis time is only 14 min. Improved *S/N* ratio, increased selectivity and reduced analysis time are very important factors for the methods used in the pharmaceutical industry.

Appropriate method specificity, accuracy, precision, linearity, *LOQ, LOD* and range were confirmed according to ICH requirements (21, November 1996). Chromatographic peaks were also found to be spectrally pure in various degradation studies.

Forced degradation in solid state

Forced degradation studies in solid state were performed on amorphous TAC. Complete transformation of crystalline TAC to amorphous TAC during the amorphization process was confirmed by X-ray powder diffraction. X-ray diffraction pattern of amorphous TAC exhibits no characteristic diffraction peaks of crystalline TAC (Fig. 3). An approach using amorphization was used to mimic the stability behavior of TAC in commercially available formulations, where TAC exists in amorphous form obtained by solid dispersion preparation techniques (22). Amorphous material has a higher thermodynamic activity and tends to be chemically and/or physically less stable than the corresponding crystalline material. For stress studies, only degradation products (unknown and known) were taken into consideration, and no production process related impurities were reported as they are not expected to increase during TAC exposure to different stress conditions and are therefore not relevant for stability evaluation. Results are gathered in Table II. For each tested stress condition, the results of all identified degradation products (TAC-H1, TAC-RI, TAC-EPI, TAC-diene and TAC-dehydro), maximal unknown impurity designated by its relative
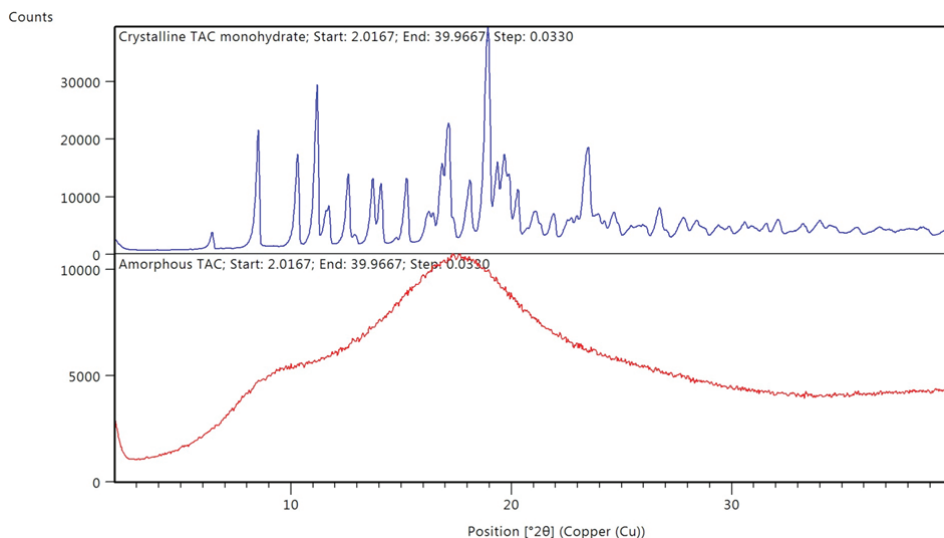
Fig. 3. XRPD patterns of crystalline TAC monohydrate (used as starting material for the preparation of amorphous material) and the prepared amorphous TAC.

retention time (RRT) and total impurities are reported. Results suggested the following degradation behavior of TAC in the solid state.

Elevated temperature under different humidity conditions. – Amorphous TAC was prone to degradation at elevated temperature (50 °C). Proportion of impurities in amorphous material increased with higher moisture content. This can be explained by the fact that amorphous material is more hygroscopic than crystalline material, and water absorbed on the surface increases the chemical degradation of amorphous material by providing reaction media or acting as a reactant. Thermal degradation was associated with a rise in two known degradation products, TAC-RI and TAC-EPI, along with several minor degradation products, which primarily eluted before the TAC main peak (see Fig. 4b). It was assessed that most of these unknown small peaks were oxidative degradation products (see also *Oxygen induced degradation*). On the other hand, increase in TAC-RI is mostly associated with high temperature and humidity.

Oxygen induced degradation. – Solid state studies showed that amorphous TAC is susceptible to oxidation. Studies were performed at elevated temperature (50 °C) under air (~21 % oxygen), oxygen, and nitrogen as reference conditions. Degradation under oxygen resulted in a significant increase in total impurities (17.93 %) compared to that under an air atmosphere (3.47 %). A large number of different unidentified degradation products were formed, indicating complex oxidation reactions (see Fig. 4c). When amorphous TAC was exposed to elevated temperature under an inert (nitrogen) atmosphere, almost no decomposition was observed.

Photolytic conditions. – After exposure to artificial sunlight, substantial degradation of TAC resulted in numerous unknown degradation products and an increase in the known

Table II. Results of forced degradation study

	TAC-H1 (%)	TAC-RI (%)	TAC-EPI (%)	TAC-diene (%)	TAC-dehydro (%)	Max. unknown impurity (%) (RRT)	Sum (%)
Forced degradation in solid state							
Initial	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.13 (RRT 0.93)	0.25
50 °C/30 % RH, 1 m	< 0.05	0.19	0.28	< 0.05	< 0.05	0.20 (RRT 0.72)	1.37
50 °C/50 % RH, 1 m	< 0.05	0.29	0.33	< 0.05	< 0.05	0.22 (RRT 0.72)	2.01
50 °C/75 % RH, 1 m	< 0.05	1.07	0.38	0.06	< 0.05	0.65 (RRT 0.93)	5.81
50 °C, nitrogen, 1 m	< 0.05	0.12	0.05	< 0.05	< 0.05	0.13 (RRT 0.93)	0.30
50 °C, air, 1 m	< 0.05	0.28	0.49	< 0.05	< 0.05	0.33 (RRT 0.27)	3.47
50 °C, oxygen, 1 m	< 0.05	0.74	1.18	0.09	< 0.05	1.49 (RRT 0.27)	17.93
SUNTEST, 16 h	< 0.05	< 0.05	0.23	< 0.05	< 0.05	3.99 (RRT 0.56)	14.28
SUNTEST, 16 h, dc	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.13 (RRT 0.93)	0.24
Fluor. light, 22 h	< 0.05	< 0.05	0.09	< 0.05	< 0.05	0.13 (RRT 0.93)	0.56
Fluor. light, 22 h, dc	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.13 (RRT 0.93)	0.24
Forced degradation in solution							
Initial	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.10 (RRT 0.93)	0.26
0.1 mol L⁻¹ HCl, 24 h	< 0.05	< 0.05	< 0.05	0.08	< 0.05	0.10 (RRT 0.93)	0.31
0.01 mol L⁻¹ HCl, 24 h	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.10 (RRT 0.93)	0.24
0.01 mol L⁻¹ NaOH, 24 h	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	11.17 (RRT 0.49)	16.88
0.1 mol L⁻¹ NaOH, 24 h	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	34.26 (RRT 0.49)	118.52[a]
pH 3, 24 h	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.11 (RRT 0.93)	0.24
pH 5, 24 h	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.11 (RRT 0.93)	0.24
pH 7, 24 h	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.09 (RRT 0.93)	0.36
pH 9, 24 h	< 0.05	< 0.05	0.09	< 0.05	< 0.05	20.24 (RRT 0.49)	31.43
pH 11, 24 h	< 0.05	< 0.05	0.21	< 0.05	< 0.05	57.30 (RRT 0.49)	91.94
Water, RT, 24 h	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.08 (RRT 0.93)	0.25
Water, 60 °C, 24 h	< 0.05	0.48	< 0.05	< 0.05	< 0.05	0.21 (RRT 0.38)	0.93
3 % H₂O₂, RT, 24 h	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.49 (RRT 0.39)	0.69
ACVA, 60 °C, 24 h	< 0.05	0.46	0.36	< 0.05	< 0.05	0.72 (RRT 0.10)	4.12
FeCl₃, 60 °C, 24 h	1.66	0.47	0.25	0.88	< 0.05	0.91 (RRT 1.06)	10.19
SUNTEST, 2 h	< 0.05	< 0.05	0.30	< 0.05	< 0.05	2.40 (RRT 1.21)	6.79
SUNTEST, 2 h, dc	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.09 (RRT 0.93)	0.09
Fluor. light, 22 h	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.09 (RRT 0.93)	0.09
Fluor. light, 22 h, dc	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.09 (RRT 0.93)	0.09

ACVA – 4,4′-azobis(4-cyanovaleric acid), dc – dark control, m – month, RT - room temperature

[a] Total degradation in 0.1 mol L⁻¹ NaOH and formation of several secondary/tertiary degradation products resulting in the sum of impurities > 100 % due to different response factors (UV spectra) of formed degradation products compared to parent TAC.
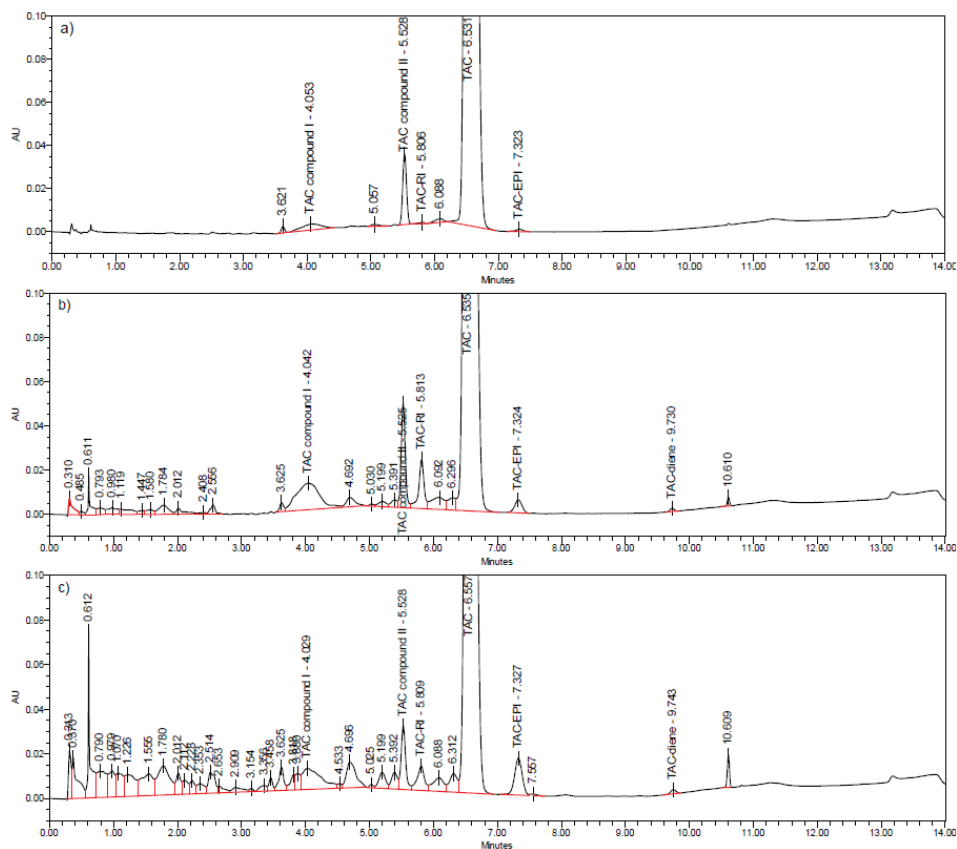
Fig. 4. Chromatograms of amorphous TAC samples: a) initial sample, b) thermal/humidity degradation (50 °C/75 % RH, open dish), and c) oxygen induced degradation.

degradation product TAC-EPI. The largest unidentified impurity peaks were observed at RRT 0.29 (1.08 %), RRT 0.43 (2.45 %), RRT 0.56 (3.99 %) and RRT 1.05 (1.20 %). After exposure to cool white fluorescent light, a slight increase in total impurities and TAC-EPI was detected. No degradation of dark control was observed under the conditions applied.

Forced degradation in solution

For stress testing of TAC in solution, ACN was used as a co-solvent because TAC is poorly soluble in water. Results of forced degradation studies in solution are gathered in Table II and revealed the following.

Hydrolytic conditions. – TAC was found to be highly labile and prone to degradation under alkaline conditions and relatively stable under neutral or mildly acidic conditions. TAC degradation under basic conditions at pH ≥ 9 and in 0.01 mol L^{-1} NaOH was associated with a substantial rise in the major degradation product at RRT 0.49 (see Fig. 5a).

Moreover, some other degradation products at RRT 0.36, RRT 0.61 and RRT 1.08 were also increased. Complete degradation of TAC was observed when the drug was exposed to more severe basic conditions in 0.1 mol L^{-1} NaOH for 24 hours. The amount of total degradation products in 0.1 mol L^{-1} NaOH was calculated to be > 100 %, indicating the formation of several secondary/tertiary degradation products with response factors different from the parent drug (TAC). On the other hand, TAC is stable under acidic conditions at pH 3–5 and in 0.01 mol L^{-1} HCl, where practically no increase in degradation products was detected. Some minor increase in unknown inpurities under neutral conditions at pH 7 was observed.

Thermal degradation. – Upon heating the TAC aqueous solution at 60 °C for 24 hours, an increase of up to 0.48 % for the impurity TAC-RI was observed, with some minor increase in the degradation product at RRT 0.38 (0.21 %) (see Fig. 5b). In contrast, no degradation of TAC was observed in aqueous solution at room temperature (used as reference for evaluation of the influence of elevated temperature).

Oxidation conditions. – Susceptibility of TAC to oxidation in solution was studied using hydrogen peroxide, radical initiator 4,4′-azobis(4-cyanovaleric acid) (ACVA) and metal ion (Fe^{3+}). TAC was not found to be very susceptible to oxidation by hydrogen peroxide in aqueous medium. After treatment with 3 % H_2O_2 at room temperature, increase in only one degradation product at RRT 0.39 (0.49 %) was detected. In the presence of the radical initiator ACVA (at 60 °C), TAC was degraded to a main degradation product at RRT 0.10 (0.72 %) and TAC-EPI (0.36 %), along with several other minor unidentified degradation products. Decomposition of TAC catalyzed by Fe^{3+} ions in the solution led to an increase in the known degradation products TAC-H1, TAC-diene and TAC-EPI, together with some other unidentified degradation products at RRT 0.38, RRT 0.41 and RRT 1.06 (see Fig. 5c). Increase in TAC-RI was associated with the elevated temperature of 60 °C and was not due to the impact of radical initiator or Fe^{3+} ions (TAC-RI levels in water bath at 60 °C and in the presence of ACVA or Fe^{3+} ions at 60 °C were comparable).

Photolytic conditions. – The drug was found to be labile when exposed to sunlight according to ICH conditions in solution (data not presented). Subsequently, studies were performed for a shorter period of 2 hours (300–800 nm, 250 W m^{-2}) to obtain appropriate degradation levels of total impurities (6.79 %). Degradation was associated with a rise in TAC-EPI and several unknown impurities, the most significant eluting at RRT 0.97 (1.60 %) and RRT 1.21 (2.40 %). Under a cool white fluorescent lamp, no degradation was observed and the level of total impurities was comparable to the dark control.

Application of the method

Applicability of the proposed UHPLC method was also evaluated by examining commercially available capsules (Advagraf®). Results are gathered in Table III.

In samples prepared from capsules in the solvent used for stress stability testing of TAC (ACN-water, 70:30, *V/V*), an unidentified impurity (t_R cca. 3 min) was found to increase over time in some cases. Given TAC sensitivity to alkaline conditions, a potential cause for degradation was thought to be alkaline leachables from the surface of glass vials. Since our primary solvent did not have any buffer capacity, we decided to implement ammonium acetate buffer to avoid variations in pH. Therefore, the final solvent selected for the preparation of samples from capsules was a mixture of ACN and 5 mmol L^{-1} ammonium acetate buffer (pH 5) in a ratio of 80:20 (*V/V*).
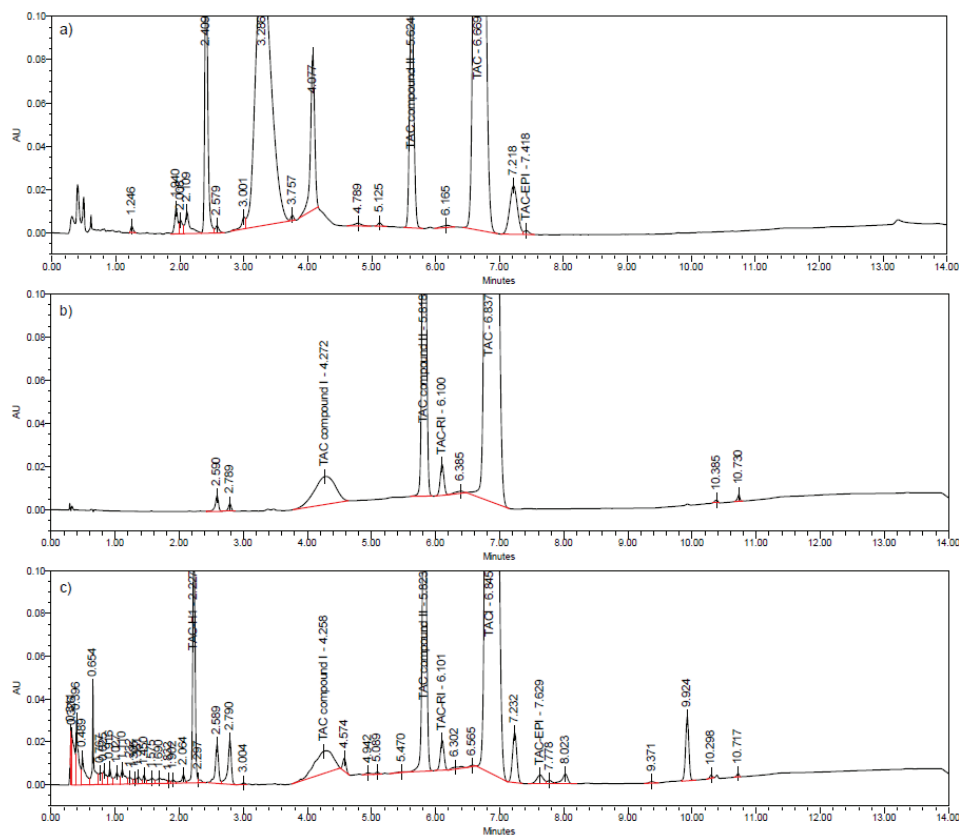
Fig. 5. Chromatograms of TAC forced degradation in solution: a) alkali degradation (pH 9), b) thermal degradation, c) metal ion catalyzed degradation (Fe^{3+}).

Summary of validation studies

The developed method was validated in accordance with ICH guidelines (21, November 1996). In line with ICH guidelines, accuracy was evaluated only for the relevant known degradation products formed in forced degradation studies or during accelerated stability studies of capsules (TAC-H1, TAC-RI, TAC-EPI, TAC-diene). Nevertheless, specificity was also checked for other known impurities, including process related impurities originating from the TAC drug substance.

Specificity. – Specificity was demonstrated for all available known impurities. Specificity was also examined relative to the blank and placebo solution (placebo was composed of hypromellose, ethylcellulose, lactose monohydrate and magnesium stearate). Solvent and placebo did not interfere with the determination of TAC impurities in pharmaceutical dosage forms.

Table III. Analyses of commercial pharmaceutical dosage forms (%)

	Advagraf® 0.5 mg				Advagraf® 5 mg			
	Initial	1 m	3 m	6 m	Initial	1 m	3 m	6 m
	40 °C/75 % RH				40 °C/75 % RH			
TAC-H1 (%)	< 0.05	0.07	0.09	0.08	0.05	0.09	< 0.05	< 0.05
TAC-RI (%)	0.06	0.11	0.20	0.31	0.06	0.12	0.19	0.27
TAC-EPI (%)	0.05	0.07	0.09	0.14	< 0.05	0.06	0.06	0.10
TAC-diene (%)	< 0.05	< 0.05	< 0.05	0.06	< 0.05	< 0.05	< 0.05	0.05
TAC-dehydro (%)	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Max. unknown impurity (%)	0.08	0.18	0.09	0.09	0.08	0.09	0.09	< 0.05
RRT of max. unknown impurity	RRT 1.23	RRT 1.63	RRT 1.23	RRT 1.23	RRT 1.23	RRT 1.23	RRT 1.24	RRT n.a.
Sum	0.30 %	0.78 %	0.68 %	0.83 %	0.24%	0.41 %	0.39 %	0.42 %

m – month, n.a. – not applicable

Linearity. – Calibration curve showed good linearity (R^2 = 0.9999) over the range of 1.5–18 µg mL^{-1} for TAC (from *LOQ* level of 0.05 to 0.6 % of nominal concentration). Regression equation for plotted calibration curve was y = 4039.3047x + 219.5273.

Accuracy. – Recovery results for spiked impurities were obtained within acceptance limits of 70–130 % for impurities at levels of ≤ 0.2 %, 80–120 % for impurities at levels of 0.3–0.5 % and 90–110 % for impurities at levels of 0.6–5 %. Results are presented in Table IV.

Table IV. Results of accuracy studies

Impurity spiked	Impurity target (%)	Recovery (%)	Accuracy range (mg mL^{-1})
	0.05	99.98	
TAC-H1	0.50	98.45	1.4–17.1
	0.60	97.79	
	0.05	85.25	
TAC-RI	0.50	93.68	1.3–15.8
	0.60	93.37	
	0.05	84.19	
TAC-EPI	0.50	94.95	1.6–18.7
	0.60	95.66	
	0.05	123.52	
TAC-diene	0.50	99.47	1.4–17.3
	0.60	99.93	

The method was proven to be accurate within the range of 0.05 % (*LOQ*) to 0.6 % of the individual impurity level in pharmaceutical formulations.

Precision. – RSD of replicate determinations was found to be < 5 % for the measured content of individual impurities and sum of impurities, which indicates that the method is precise.

*Limit of detection (*LOD*) and limit of quantitation (*LOQ*).* – *LOD* and *LOQ* were confirmed to be 0.6 µg mL^{-1} (0.02 % of the nominal sample concentration) and 1.5 µg mL^{-1} (0.05 % of the nominal sample concentration), resp.

Range. – Method range was derived from linearity, accuracy and precision experiments. The method was confirmed to be linear, accurate and precise in the concentration range from 0.05 to 0.6 % of the nominal value for impurity level.

CONCLUSIONS

Forced degradation study in solution showed that TAC is a labile compound, prone to degradation under basic, thermal, photolytic conditions and in the presence of a radical initiator or metal ions. It is stable under slightly acidic conditions at pH 3–5. Forced degradation study in solid-state, conducted on amorphous TAC, revealed that this material is sensitive to light, elevated temperature, humidity and is susceptible to oxidation. This study demonstrated the importance of appropriate design of forced degradation studies, taking into consideration both physiochemical characteristics of the drug substance and the aspects of the developed formulation. The acquired knowledge could be very helpful during development or when solving stability-related problems of commercial formulations. In addition, an advanced UHPLC method was developed for simultaneous separation of several TAC impurities. In comparison with the pre-existing TAC methods, the developed method demonstrated excellent specificity and a substantially shorter analysis run time of 14 minutes. The method was successfully applied to the analysis of commercial samples and validated in terms of linearity, precision, accuracy, sensitivity and specificity. The method was found to be suitable for UHPLC determination of degradation products of TAC in the range of 0.05 to 0.6 % of the impurities level in pharmaceutical dosage forms. Furthermore, the stability indicating power of the method was demonstrated by the results of forced degradation studies.

The developed method is fast, simple and cost-effective, and is therefore suitable for the routine analysis of TAC degradation products in pharmaceutical dosage forms.

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