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Development and Validation of an RP-HPLC Method for Estimation of Related Substances of Tetracycline Hydrochloride 1% w/w Ophthalmic Ointment: A Review

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Abstract: Tetracycline hydrochloride 1% w/w eye ointment is a WHO essential medicine for superficial bacterial eye infections and neonatal conjunctivitis prophylaxis. Tetracycline degrades readily by C4-epimerisation and C6-dehydration to 4-epitetracycline (ETC), anhydrotetracycline (ATC) and the nephrotoxic 4-epianhydrotetracycline (EATC), and it carries the fermentation impurities 2-acetyl-2-decarboxamidotetracycline (ADTC) and chlortetracycline (CTC). Control of these related substances in the finished ointment is therefore a critical quality attribute.

This review surveys the pharmacopoeial requirements and the published chromatographic methods for tetracycline and its related substances, from early silica-based RP-HPLC and poly(styrene-divinylbenzene) methods to modern C18 gradient, porous graphitic carbon, silica-hydride, fluorescence and HPTLC approaches. For each method the chromatographic conditions, validation findings and limitations are summarised.

Most published methods were developed for the drug substance, capsules or oral suspensions; few address the ointment matrix, and none reports a fully validated, stability-indicating related-substances method specific to the 1% ophthalmic ointment. Gaps include incomplete impurity coverage (most dosage-form methods control only EATC), lack of validated extraction from the paraffin base, solution instability during analysis, reliance on high-temperature polymeric columns and ion-pairing agents, and absence of ICH Q2(R2)/Q14-aligned validation and forced degradation in the ointment matrix. A development strategy is proposed to close these gaps.

Keywords: Tetracycline hydrochloride; ophthalmic ointment; related substances; 4-epianhydrotetracycline; RP-HPLC; stability-indicating method; ICH Q2(R2)

1. Introduction

Tetracycline is a broad-spectrum polyketide antibiotic that inhibits bacterial protein synthesis by binding the 30S ribosomal subunit [3]. Despite rising resistance, it remains widely used. Tetracycline hydrochloride 1% w/w eye ointment is listed in the WHO Model List of Essential Medicines as an ophthalmic anti-infective [1,2]. It is used for superficial bacterial infections of the eye, trachoma and prophylaxis of neonatal conjunctivitis [2,4].

The ointment is a sterile, anhydrous preparation in which tetracycline hydrochloride is dispersed in a hydrocarbon base, typically white soft paraffin with liquid paraffin. The anhydrous base protects the

drug from hydrolysis, but tetracycline remains chemically labile. It epimerises at C4 in weakly acidic solution and dehydrates at C6 under strongly acidic conditions or heat, giving 4-epitetracycline (ETC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC) [7].

These degradants matter clinically. ETC has much lower antibacterial activity, so its formation reduces potency. EATC, and to a lesser extent ATC, are toxic: degraded, outdated tetracycline was linked to a reversible Fanconi-type renal tubular syndrome in the 1960s [6]. Pharmacopoeias therefore set specific limits for these impurities in the drug substance and, for EATC, in dosage forms [8–10].

ICH Q3B(R2) requires degradation products in a finished product to be reported, identified and qualified above defined thresholds [5]. Meeting this requirement for the eye ointment needs an analytical procedure that can extract tetracycline and its related substances quantitatively from the paraffin base, separate all specified impurities from the drug and from base-derived interferences, and remain stability-indicating. Ophthalmic products also carry stricter expectations for quality because they are applied to a sensitive, poorly buffered tissue.

This review collates the published work relevant to that task. It summarises the degradation chemistry, the pharmacopoeial methods and limits, and the chromatographic methods reported for tetracycline and its related substances, with their findings and limitations. It then identifies the gaps in current knowledge and sets out the scope for developing and validating an RP-HPLC method for related substances in tetracycline hydrochloride 1% w/w ophthalmic ointment.

2. Drug profile and degradation chemistry

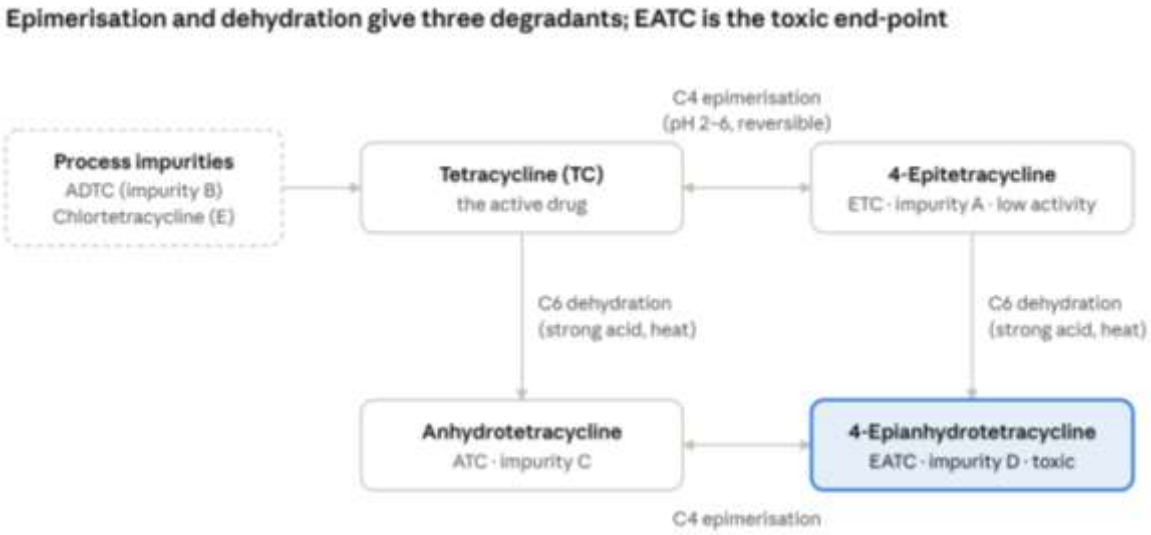
2.1 Drug profile

Table 1. Physicochemical profile of tetracycline hydrochloride

Property	Description
Chemical name	(4S,4aS,5aS,6S,12aS)-4-(Dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide hydrochloride
Molecular formula	$C_{22}H_{24}N_2O_8 \cdot HCl$
Molecular weight	480.9 g/mol
Description	Yellow, hygroscopic crystalline powder
Solubility	Soluble in water, slightly soluble in ethanol, practically insoluble in acetone
Ionisation	Amphoteric; three pKa values near 3.3, 7.7 and 9.7
UV absorption	Maxima near 270 and 360 nm in acidic media
Key reactivity	C4 epimerisation, C6 dehydration, chelation of Ca^{2+}, Mg^{2+}, Fe^{3+} and other metal ions
Dosage form reviewed	Sterile eye ointment, 1% w/w, in a white soft paraffin and liquid paraffin base

2.2 Degradation pathways and impurities

Tetracycline degrades by two main routes. In weakly acidic solution (about pH 2–6) the C4 dimethylamino group epimerises reversibly to give ETC, which reaches an equilibrium with the parent drug [7]. Under strongly acidic conditions or heat, the C6 hydroxyl and C5a hydrogen are eliminated to give the aromatised anhydro compounds ATC and, from ETC, EATC [7]. In alkaline conditions the C ring cleaves to form isotetracycline. Fermentation-derived impurities include 2-acetyl-2-decarboxamidotetracycline (ADTC) and chlortetracycline (CTC) [10].



Degradation and process impurities of tetracycline impurity letters as in the Ph. Int. draft / Ph. Eur. [9,10]

Because the anhydro compounds are more lipophilic and more conjugated, they elute after tetracycline in reversed-phase systems and absorb strongly at longer wavelengths. ETC is close in polarity to tetracycline, and the ETC/TC and EATC/ATC pairs are the critical separations in every published method [10,15].

Table 2. Specified related substances of tetracycline hydrochloride

Impurity (Ph. Eur./Ph. Int.)	Chemical name	Origin	RRT*	Limit in drug substance
A	4-Epitetracycline (ETC)	Degradation (epimerisation)	~0.9	≤ 3.0%
B	2-Acetyl-2-decarbamoyltetracycline (ADTC)	Fermentation	~1.1	≤ 1.5%
C	Anhydrotetracycline (ATC)	Degradation (dehydration)	~1.7	≤ 0.5%
D	4-Epianhydrotetracycline (EATC)	Degradation (both routes)	~1.5	≤ 0.5%
E	Chlortetracycline (CTC)	Fermentation	—	Not limited in the LC test

*RRT relative to tetracycline in the C18 phosphoric acid–acetonitrile gradient of the WHO draft monograph [10]. Limits from the same draft; check against the current Ph. Eur., USP and IP editions.

3. Pharmacopoeial and regulatory requirements

Pharmacopoeial control of related substances is thorough for the drug substance but thin for the eye ointment. The USP monograph for Tetracycline Hydrochloride Ophthalmic Ointment requires 90.0–125.0% of label claim, sterility and metal-particle testing, and refers its assay to the Tetracycline Hydrochloride Ointment monograph [8]. That assay disperses the ointment in cyclohexane, extracts the drug with methanol under sonication, and analyses it by the drug-substance LC system [8]. The edition reviewed sets no individual limits for ETC, ATC or ADTC in the ophthalmic ointment.

The Ph. Eur. related-substances test was historically built on poly(styrene-divinylbenzene) (PS-DVB) columns at 60 °C with a mobile phase of 2-methyl-2-propanol, phosphate buffer and sodium edetate, following the work of Hoogmartens and co-workers [15,19]. Sister monographs for doxycycline and oxytetracycline still use this format [9]. The 2019 WHO draft monograph for the International Pharmacopoeia replaced it with a C18 gradient of 0.1% phosphoric acid and acetonitrile at 50 °C, with detection at 280 nm and an autosampler held at 10 °C [10]. That draft requires a resolution of at least 2.5 for both critical pairs (ETC/TC and EATC/ATC) and specifies limits for impurities A–D [10].

Table 3. Pharmacopoeial methods relevant to tetracycline hydrochloride and its eye ointment

Source	Scope	Column and conditions	Detection	Related-substances control
USP, Tetracycline Hydrochloride [8]	Drug substance	L7 (C8); ammonium oxalate–dimethylformamide–ammonium phosphate buffer, pH about 7.6, isocratic **[verify current edition]**	UV 280 nm	Limit of EATC; assay
USP, Tetracycline Hydrochloride Ointment and Ophthalmic Ointment [8]	Ointments	As drug substance; sample dispersed in cyclohexane, extracted into methanol	UV 280 nm	Assay only (90.0–125.0%); no individual impurity limits
Ph. Eur., Tetracycline hydrochloride [9]	Drug substance	Classically PS-DVB, 60 °C, 2-methyl-2-propanol–phosphate–edetate **[verify current edition]**	UV 254 nm	Impurities A–D specified
Ph. Int. draft QAS/17.740 [10]	Drug substance	C18 base-deactivated, 150 × 4.6 mm, 3 µm; 50 °C; 0.1% H_3PO_4–acetonitrile gradient, 10 min	UV 280 nm	A ≤ 3.0%, B ≤ 1.5%, C ≤ 0.5%, D ≤ 0.5%
IP / BP, Tetracycline Eye Ointment [11]	Eye ointment	**[insert from current IP/BP]**	—	**[insert]**

The ICH framework adds requirements that the pharmacopoeial texts do not address for this product. ICH Q3B(R2) sets reporting, identification and qualification thresholds for degradation products in finished products [5]. ICH Q1A(R2) and Q1B call for stress testing to show that the analytical procedure is stability-indicating [28,29]. ICH Q2(R2) and Q14, both adopted in 2023, require validation of specificity, range, accuracy, precision and quantitation limits for each impurity, supported by a documented development rationale [26,27].

4. Review of published methods

More than four decades of chromatographic work on tetracycline fall into five groups: silica-based RP-HPLC with acidic or ion-pair mobile phases (1976–1982), PS-DVB methods underpinning the Ph. Eur. (from 1986), alternative selectivity and detection (1998–2013), modern C18 gradient methods aimed at monograph modernisation (2014–2019), and dosage-form methods for ointments (2019–2025). Table 4 summarises each method in chronological order.

Table 4. Published methods for tetracycline and its related substances

#	Author, year [ref]	Matrix	Column and mobile phase	Detection	Key findings	Observations and limitations
1	Tsuji and Robertson, 1976 [12]	Drug substance, pharmaceutical products	C18, 10 µm, 300 mm; gradient 10–60% acetonitrile in 0.02 M phosphate pH 2.5 over 11 min; 1.0 mL/min	UV	ETC/TC resolution improved 150% and EATC/ATC 250% over the earlier method; 16 min run; RSD 0.66%; EATC recovery linear over 0.3–100%; product recovery > 99.6%; agreed with microbiological	First practical RP-HPLC for products. Large particles; ADTC and CTC not addressed; validation predates ICH

					assay	
2	Muhammad and Bodnar, 1980 [13]	Tetracycline raw material	RP-HPLC (silica-based)	UV	Separated and quantified CTC, ETC, EATC and ATC	Poor precision for impurities: 2σ RSD 3.7% (CTC), 4.5% (ETC), 7.6% (EATC), 2.8% (ATC)
3	Hermansson and Andersson, 1982 [14]	Tetracycline, analogues, potential impurities	Reversed-phase ion-pair chromatography	UV	Ion-pairing controlled tailing of the basic dimethylamino group and improved selectivity	Ion-pair reagents give slow equilibration and column memory; not suited to MS
4	Wolfs et al., 1986 [15]	Tetracycline and related substances	PS-DVB copolymer, elevated temperature; 2-methyl-2-propanol, phosphate buffer, edetate	UV	Complete separation of TC, ETC, ATC, EATC, ADTC and CTC on a metal-free polymeric phase	Basis of the Ph. Eur. method. Needs 60 °C, alkaline pH and long runs; polymeric columns have low efficiency and limited suppliers
5	J.W. Muritu et al., 1994 [16]	Raw materials, 12 batches of skin ointment, 4 eye ointments, capsules (Kenyan market)	Compendial-type LC **-18**	UV	Quantified TC, ETC, EATC, ATC and ADTC; all samples met compendial limits	The only survey found that reports related substances in tetracycline eye ointments. Method not re-validated for the ointment matrix
6	Pena et al., 1998 [17]	Tetracycline and degradants (residue-level)	C8, 100 × 3 mm; 0.01 M oxalic acid pH 2.0 with 20–40%	Fluorescence after post-column Mg^{2+}/bora	TC, ETC, EATC and ATC separated within 15 min; LOQ 0.25 ng (TC,	Oxalic acid suppresses metal chelation. Post-column

			acetonitrile	te reagent	ETC), 25 ng (EATC), 50 ng (ATC) injected	derivatisation adds complexity; anhydro compounds 100–200 times less sensitive than TC
7	Monser and Darghouth, 2000 [18]	Five tetracyclines and 6-epidoxycycline in products	Porous graphitic carbon; 0.05 M KH_2PO_4 pH 2.0–acetonitrile (40:60), isocratic	UV 268 nm	Rapid, selective isocratic separation; PGC is stable across the full pH range	Designed to separate different tetracyclines, not the TC degradation profile; PGC retention can drift
8	[Authors], Malaysian J Pharm Sci [19]	Tetracycline raw material	PLRP-S (PS-DVB); 60 °C, pH 9.0; 2-methyl-2-propanol, phosphate buffer, edetate; isocratic	UV	Complete separation of TC, ETC, ADTC, ATC and EATC; LOD 0.01% (ETC), 0.05% (EATC), 0.1% (ATC)	High temperature and alkaline pH can generate degradants on-column; raw material only
9	Joshua E Young et al., c. 2013 [22]	Tetracycline, doxycycline, minocycline and degradants	Silica-hydride Bidentate C8, Phenyl Hydride and Cholesterol columns, 75 × 4.6 mm, 4 µm; water–acetonitrile with 0.1% TFA	UV	Silica hydride reduced tailing of the dimethylamino compounds without ion-pairing; selectivity differed between the three phases	Column comparison only; no validation or dosage-form application
10	Hussien, 2014 [20]	Drug substance, oral suspension	C18 (L1), 150 × 4.6 mm, 3 µm; 0.1% H_3PO_4–acetonitrile gradient; 1.0 mL/min	UV 280 nm	All related compounds eluted in < 8 min; TC spectrally pure under stress; solution degradation slowed by 4 °C autosampler and 0.1% H_3PO_4 diluent; EATC LOQ 0.1 µg/mL, 20 times below the limit	Basis of the WHO draft. Validated only for assay and the EATC limit; ETC, ATC and ADTC not quantified; not applied to ointments
11	Hussien,	Capsules	As method 10	UV 280	Linear 80–160%	Extends

	2014 [21]			nm	of assay level (r > 0.9998) and 50–150% of EATC limit (r > 0.997); recovery 99–101%; RSD < 0.6%	method 10 to capsules; still EATC only
12	MicroSolv application note [23]	Heat- and acid-stressed capsules	Bidentate C18, 75 × 4.6 mm, 4 µm; 0.1% formic acid in water–acetonitrile, 10–80% in 7 min	UV 360 nm then 430 nm	ETC, TC, EATC and ATC resolved in 7 min without ion-pair; retention-time RSD < 0.1%	Vendor note, not validated; MS-compatible mobile phase is a useful lead
13	Varaganti et al., 2019 [24]	Hydrocortisone–tetracycline ointment	RP-HPLC (C18), isocratic	UV	Simultaneous assay; linear (r^2 = 0.999); TC recovery 99.39–99.61%; forced degradation performed	Assay only; related substances of TC neither identified nor quantified; ointment extraction scheme not optimised for impurities
14	Gashaw et al., 2024 [2]	API, 1% eye ointments (3 brands), 3% skin ointments	HPTLC, silica plates	Densitometry	Stability-indicating assay; LOD 31.9 ng/band, LOQ 96.7 ng/band; results interchangeable with the USP HPLC assay	Assay, not impurity quantitation; resolution of ETC from TC limited on HPTLC
15	Chen and Meng, 2025 [25]	Chlortetracycline HCl for ophthalmic ointment	HPLC with 2D-LC-HRMS/MS heart-cutting	UV, HRMS/MS	Identified a degradation impurity not described in Ph. Eur., distinct from the listed impurity E	Shows that unknown peaks in tetracycline-class eye ointments need structural confirmation; analogous drug only

5. Critical observations across the literature

5.1 Low-pH silica-based C18 gradients are now the method of choice. Early silica methods suffered from tailing caused by silanol interactions and metal chelation [12,13]. The response was either ion-pairing [14] or a move to metal-free PS-DVB phases at high temperature [15,19]. Modern base-deactivated, high-purity C18 columns with 0.1% phosphoric acid achieve the same critical-pair resolution in under 10 min at

moderate temperature [10,20]. This shift reflects better column chemistry rather than a change in the analytes.

5.2 Metal chelation must be controlled. Tetracyclines bind trace metals in the HPLC system and on silica, which broadens peaks and lowers recovery. Published methods suppress this with edetate [15,19], oxalic acid or oxalate buffers [8,17], or strongly acidic phosphoric acid mobile phases [20]. The choice affects selectivity and MS compatibility.

5.3 The analytes degrade during analysis. Tetracycline epimerises in solution, and EATC itself degrades. Hussien showed that degradation in the autosampler is slowed by an acidic diluent (0.1% H_3PO_4) and a 4 °C sample tray [20], and the WHO draft fixes the autosampler at 10 °C [10]. Methods that ignore solution stability risk reporting ETC formed in the vial as a product impurity.

5.4 Detection wavelength is a compromise. Tetracycline and ETC absorb strongly near 270–280 nm and 355 nm, while ATC and EATC absorb further into the visible range (about 430–440 nm) [23]. Most impurity methods use 280 nm as a single compromise [10,20]. Wavelength switching or PDA extraction at two wavelengths can improve sensitivity for the anhydro compounds [23]. Fluorescence offers very low limits for TC and ETC but is less sensitive for the anhydro compounds [17].

5.5 Dosage-form methods focus on assay. Of the 15 methods reviewed, only four were applied to ointments [2,16,24,25], and only the 1994 market survey quantified the full impurity profile in eye ointments [16]. Recent ointment methods report assay figures and recoveries for tetracycline but do not identify or quantify its degradants [2,24].

5.6 Validation reporting is uneven. Older methods report precision and recovery but not specificity, quantitation limits or robustness [12,13]. Modern methods follow ICH Q2(R1) for the drug and EATC but not for ETC, ATC and ADTC [20,21]. None was validated against ICH Q2(R2) and Q14, which took effect in 2023 [26,27].

5.7 Sample preparation for the paraffin base is borrowed. The USP extraction (cyclohexane dispersion, methanol extraction, sonication) was designed for assay at about 300 mg of drug per test [8]. At 1% w/w, the impurity test needs larger ointment weights, and methanol can promote epimerisation. The extraction efficiency of each impurity from the base, and the effect of extraction on ETC formation, have not been reported.

6. Gaps identified

No published method provides a validated, stability-indicating RP-HPLC procedure for the full related-substances profile of tetracycline hydrochloride 1% w/w eye ointment. Table 5 sets out the specific gaps and how a new method can address each.

Table 5. Gaps in the literature and the improvement each calls for

#	Gap	Evidence	Improvement needed
1	No dedicated related-substances method for the 1% eye ointment	Pharmacopoeial ointment monographs control assay only [8]; ointment papers report assay [2,24]	A method validated in the eye-ointment matrix for impurities A–E and unknowns
2	Incomplete impurity coverage in dosage-form methods	Modern methods quantify only EATC [20,21]	Individual quantitation of ETC, ADTC, ATC, EATC and CTC with relative response factors
3	Extraction from the paraffin base not validated for impurities	USP extraction designed for assay [8]; no impurity recovery data	Optimised and validated extraction (solvent, ratio, sonication time, temperature) with impurity recovery and placebo checks
4	Sample-solution instability	Degradation of TC and EATC in solution [20]	Acidic diluent, cooled autosampler, protection from light, and a defined solution-stability window
5	Reliance on PS-DVB columns, 60 °C and alkaline pH, or ion-pair	Ph. Eur.-type and ion-pair methods [14,15,19]	A robust low-pH method on a widely available base-deactivated C18 column at ≤ 40 °C, without

	reagents		ion-pair reagents
6	Forced degradation not performed in the ointment matrix	Stress studies reported for drug substance and capsules only [20,21]	Acid, base, oxidative, thermal, humidity and photolytic stress on drug, placebo and ointment, with peak purity and mass balance
7	Quantitation limits not tied to ICH Q3B(R2) thresholds	Limits reported for EATC only [20]	LOQ at or below the reporting threshold (0.1% for a dose ≤ 1 g) for every specified impurity [5]
8	Validation predates ICH Q2(R2) and Q14	Methods validated to Q2(R1) or earlier [12,20,21]	Q14-based development (ATP, risk assessment, design of experiments) and Q2(R2) validation
9	Unknown degradants not characterised	Unlisted impurities found in a related tetracycline eye ointment [25]	MS-compatible mobile phase or orthogonal LC-MS to identify unknown peaks above the identification threshold
10	Little data on marketed eye ointments	One market survey, from 1994 [16]	Application of the validated method to current marketed batches and stability samples

7. Scope and proposed strategy for the new RP-HPLC method

The gaps above define clear scope for a new method. The most promising starting point is the low-pH C18 gradient of Hussien [20] and the WHO draft [10], extended to every specified impurity, transferred to the ointment matrix with a validated extraction, and developed and validated under ICH Q14 and Q2(R2) [26,27].

7.1 Analytical target profile

The procedure should separate and quantify impurities A–E and any unknown degradant in tetracycline hydrochloride 1% w/w eye ointment, with resolution of at least 2.5 for the ETC/TC and EATC/ATC pairs, a quantitation limit at or below 0.05% of the tetracycline concentration, and accuracy of 80–120% at the LOQ and 90–110% at the specification level, across the product shelf life.

7.2 Proposed starting conditions

Table 6. Suggested initial chromatographic conditions, to be optimised

Parameter	Proposed starting point	Rationale
Column	Base-deactivated C18, 150 × 4.6 mm, 3 µm (or 2.7 µm core-shell)	Achieves critical-pair resolution at low pH [10,20]
Mobile phase A	0.1% v/v orthophosphoric acid in water (0.1% formic acid if MS identification is needed)	Suppresses silanol and metal interactions; formic acid allows LC-MS [20,23]
Mobile phase B	Acetonitrile	Sharper peaks and lower back-pressure than methanol
Elution	Linear gradient from about 12% to 60% B over 15 min, then re-equilibration	Elutes polar ETC early and lipophilic ATC/EATC within the run
Flow rate	1.0 mL/min	Standard for a 4.6 mm column
Column temperature	30–40 °C	Avoids on-column epimerisation at 50–60 °C
Detection	PDA; quantitation at 280 nm, spectra 200–500 nm for peak purity, extraction at 430 nm for ATC/EATC	Covers both chromophores [20,23]
Diluent	0.1% orthophosphoric acid (or 0.01 M HCl)	Slows epimerisation in solution [20]
Autosampler	4–10 °C, protected from light	Solution stability [10,20]

Test concentration	About 0.5 mg/mL tetracycline hydrochloride	Places 0.05% (0.25 µg/mL) above the expected LOQ [20]

7.3 Sample preparation to be developed

1. Weigh ointment equivalent to about 25 mg of tetracycline hydrochloride (about 2.5 g of 1% ointment) into a centrifuge tube or separator.
2. Disperse the paraffin base in a non-polar solvent such as n-hexane or cyclohexane [8].
3. Extract the drug into acidic aqueous diluent in two or three portions with shaking or brief sonication at controlled temperature.
4. Separate phases by centrifugation, combine the aqueous extracts, dilute to volume and filter through a validated 0.45 µm membrane.
5. Compare candidate schemes by impurity recovery from spiked placebo, ETC formed during preparation, and placebo interference.

7.4 Forced degradation in the ointment matrix

Stress the drug substance, placebo base and finished ointment under acid (0.1 M HCl), base (0.1 M NaOH), oxidation (3% H_2O_2), dry heat, humidity and ICH Q1B light [28,29]. Target 5–20% degradation. Confirm peak purity of tetracycline and each impurity by PDA, assess mass balance, and characterise unknowns above the identification threshold by LC-MS [25].

7.5 Validation plan

Table 7. Proposed ICH Q2(R2) validation of the related-substances method

Parameter	Design	Typical acceptance criterion
System suitability	Resolution solution of TC with ETC, ADTC, ATC and EATC	Rs ≥ 2.5 for ETC/TC and EATC/ATC; tailing ≤ 2.0; %RSD of replicate standards ≤ 5.0%
Specificity	Blank, placebo, individual impurities, spiked sample, stressed samples	No interference at impurity retention times; peak purity passes
LOD and LOQ	Signal-to-noise or calibration slope for each impurity	S/N ≈ 3 (LOD), ≈ 10 (LOQ); LOQ ≤ reporting threshold
Linearity and range	LOQ to 150% of each specification limit, ≥ 5 levels	r ≥ 0.99; residuals random
Relative response factor	Slope of impurity vs slope of TC	Applied where RRF lies outside 0.8–1.2
Accuracy	Placebo spiked at LOQ, 50%, 100% and 150% of limit, triplicate	Recovery 80–120% at LOQ, 90–110% at other levels
Precision	Six spiked preparations at the limit; second analyst, day and column	%RSD ≤ 10% (≤ 15% near LOQ)
Robustness	Design of experiments: pH, temperature ±5 °C, flow ±10%, initial %B ±2%	System suitability met across the design space
Solution stability	Standard and sample at 4–10 °C and room temperature over 24–48 h	Change in each impurity within agreed limits

7.6 Expected outcome

A validated method of this design would give manufacturers and regulators a single procedure for release and stability testing of the eye ointment. It would quantify every specified impurity, avoid ion-pair reagents and high-temperature polymeric columns, and provide a basis for revising pharmacopoeial ointment monographs to include a related-substances test.

8. Conclusion

Tetracycline hydrochloride degrades by epimerisation and dehydration to ETC, ATC and the toxic EATC, and carries the fermentation impurities ADTC and CTC. Chromatography of these compounds has moved from silica, ion-pair and high-temperature PS-DVB systems to fast, low-pH C18 gradients that resolve both critical pairs in under 10 min. However, published and pharmacopoeial methods for the ophthalmic ointment control assay, and at most EATC. None provides a validated, stability-indicating related-substances method in the paraffin matrix.

The main gaps are incomplete impurity coverage, an unvalidated extraction from the ointment base, sample-solution instability, missing forced degradation in the finished product, and the absence of ICH Q14/Q2(R2)-aligned development and validation. A low-pH, base-deactivated C18 gradient with PDA detection, an acidic cooled diluent, and a validated hydrocarbon-dispersion extraction offers a practical route to close these gaps. Such a method would support release and stability testing of tetracycline hydrochloride 1% w/w eye ointment, a WHO essential medicine.

Conflict of interest

The authors declare no conflict of interest.

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