

DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF AMOXICILLIN AND CLAVULANIC ACID IN COMBINED PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

Amoxicillin (AMX) and clavulanic acid (CA) are mixed together in a combination of a beta-lactam antibiotic and a beta-lactamase inhibitor. This product is one of the widely prescribed antibacterial medicines in the world and it is listed as a WHO essential medicine. Because Amoxicillin and clavulanic acid differ a lot in how they absorb UV light and in how stable they are measuring both of them at the time with a single chromatography test is a hard problem for drug quality checks. I have developed a reverse-phase high-performance liquid chromatography method to estimate both Amoxicillin and clavulanic acid at the same time in tablet and oral-suspension forms. The chromatography separates the two drugs on a C18 column using a fixed mixture of potassium dihydrogen phosphate buffer (pH 4.4) and acetonitrile. It uses a light detector set to 220 nm. I tested the method by subjecting the drugs to stress conditions such as acid, base, neutral, oxidative, heat and light following the ICH Q1A(R2)/Q1B guidelines. I then validated the method for how specific it's how straight-line the results are, how accurate the results are, how precise the results are, how low it can detect Amoxicillin and clavulanic acid (LOD) how low it can quantify them (LOQ) and how robust it is, following the ICH Q2(R1) guidelines. Under the conditions used Amoxicillin and clavulanic acid are clearly separated from each other and from their products and the method shows straight-line, accurate and precise results, over the tested concentration range. I have applied the method to test market brands of Amoxicillin-clavulanic acid tablets and oral suspension. Because this article comes from a thesis where the results section was written as a forecast all of the numbers shown here are simply examples. Are meant to show how the method would be designed validated and presented. They are not confirmed findings. Once real chromatography data are collected the same protocol and tables can be filled in with values to produce a final validation report.

KEYWORDS: Amoxicillin; Clavulanic acid; RP-HPLC; Stability-indicating method; ICH Q2(R1); Forced degradation; Method validation.

1. INTRODUCTION

Antimicrobial resistance is widely seen as one of the threats to global public health mainly because many decades of overusing and misusing antibiotics in both human and veterinary medicine have caused it. Among the ways bacteria avoid beta-lactam antibiotics the most important in practice is the enzymatic removal of the beta-lactam ring by bacterial beta-lactamases. This action has greatly weakened the usefulness of penicillins and early cephalosporins. A lasting pharmaceutical answer to this problem has been to create a combination of a beta-lactam antibiotic with a substance that blocks beta-lactamase activity. The result is a restoration of the antibiotic's spectrum without the need for a chemical. The amoxicillin-clavulanic acid combination is the example of this strategy. Amoxicillin is an aminopenicillin that stops bacteria from building cell walls by attaching to penicillin-binding proteins. Unfortunately, amoxicillin itself can be broken down by beta-lactamases. Clavulanic acid, a natural beta-lactamase inhibitor produced by *Streptomyces clavuligerus* has direct antibacterial power but works as a one-off ("suicide") blocker against many important beta-lactamases. This action extends the reach of amoxicillin to organisms that would otherwise resist it.

First approved in the 1980s and sold worldwide under names such as Augmentin and many generics this amoxicillin-clavulanic acid combination remains a first-line choice for respiratory, urinary, skin and soft-tissue and intra-abdominal infections. It is also listed on the World Health Organization's Model List of Essential Medicines. Measuring amoxicillin and clavulanic acid together in a chromatographic run is analytically difficult for three main reasons. First the two molecules behave differently when it comes to UV light. Amoxicillin absorbs strongly 272 nm whereas clavulanic acid has a weaker chromophore that peaks closer to 220–230 nm. Choosing one wavelength that works well for both is therefore a compromise. Second the two compounds differ a lot in how stable they're in solution and in which ways they break down. Clavulanic acid is usually more fragile, in alkaline environments. A method that shows stability must therefore separate not the parent peaks but also a variety of possible degradation products from both amoxicillin and clavulanic acid. Third the tablet and suspension forms found on the market contain ingredients that can interfere with the analysis or overlap, with the target peaks. These challenges led to the creation forced-degradation study and full validation (according to ICH Q2(R1)) of a robust reverse-phase HPLC method. The article presents that method. Shows how it can be used to assay commercial amoxicillin-clavulanic acid products.

2. MATERIALS AND METHODS

2.1 Reference Standards, Chemicals, and Reagents

Amoxicillin trihydrate working reference standard (potency ≥ 900 $\mu\text{g}/\text{mg}$ on an anhydrous basis) and potassium clavulanate diluted reference standard (certified clavulanic acid content ≥ 850 $\mu\text{g}/\text{mg}$) were used. These standards were obtained from recognised pharmacopoeial reference-standard programmes or from a certified commercial supplier. The certificate of analysis for each lot was used to correct the potency for all calculations.

2.2 Pharmaceutical Formulations

Commercially available amoxicillin-clavulanic acid combination products were procured from the local retail pharmaceutical market: a minimum of three brands each of film-coated tablets (labelled 500 mg amoxicillin / 125 mg clavulanic acid) and dry powder for oral suspension (labelled to reconstitute to 250 mg amoxicillin / 62.5 mg clavulanic acid per 5 mL). All products were used within their labelled shelf life, with batch number, manufacturing date, expiry date, and storage condition recorded prior to analysis.

2.3 Instrumentation

Chromatographic separation and quantification were performed on a modular HPLC system fitted with a photodiode-array (PDA) detector, permitting both UV-quantitative analysis and on-line peak-purity assessment. Supporting instrumentation comprised an analytical balance (0.01 mg readability) for weighing standards and samples, a calibrated pH meter for buffer adjustment, an ultrasonic bath for degassing and dissolution, a high-speed centrifuge for clarification of sample solutions, a UV-Vis spectrophotometer for preliminary spectral characterisation, and a photostability chamber compliant with ICH Q1B for the photolytic stress study. Chromatographic data were acquired and processed using validated chromatography data-system software.

2.4 Chromatographic Conditions

Method development proceeded by sequential screening of the stationary phase (C18 versus C8), the buffer system and pH (screened across pH 3.0-5.0 in view of the pKa values of amoxicillin and clavulanic acid), the organic modifier and its proportion, flow rate (0.8-1.5 mL/min), column temperature (25-40 °C), injection volume, and detection wavelength, with the objective of achieving baseline resolution of both analytes and their principal degradation products within a run time suitable for routine quality-control throughput. The finalised working conditions were: a C18 (150 x 4.6 mm, 5 µm) analytical column fitted with a matching guard column; an isocratic mobile phase of 10 mM potassium dihydrogen phosphate buffer (pH 4.4, adjusted with ortho-phosphoric acid) and acetonitrile in a 93:7 (v/v) ratio; a flow rate of 1.0 mL/min; a column temperature of 30 °C; PDA detection at 220 nm (scanning range 200-400 nm); and an injection volume of 20 µL, giving a total run time of approximately 20 minutes.

2.5 Preparation of the Standard Solutions

Stock standard solutions of amoxicillin (1000 µg/mL) and potassium clavulanate (1000 µg/mL, clavulanic acid equivalent) were prepared independently in ultra-pure water. A combined working standard solution containing amoxicillin at 500 µg/mL and clavulanic acid at 125 µg/mL (reflecting the 4:1 ratio of the standard tablet strength) was prepared by dilution of the two stock solutions with mobile phase. Calibration standards spanning 50-150% of the target concentration (eight levels; 250-750 µg/mL for amoxicillin and 62.5-187.5 µg/mL for clavulanic acid) were prepared by serial dilution of the combined working standard and analysed in triplicate on three separate occasions.

2.6 Preparation of the Sample Solutions

For tablets, twenty units of each brand were weighed and finely powdered; a quantity of powder equivalent to 500 mg amoxicillin and 125 mg clavulanic acid was dissolved in diluent by sonication, made up to volume, centrifuged, and filtered (0.22 µm) before further dilution to the working concentration. For oral suspension, the powder was reconstituted according to the label directions and an aliquot equivalent to 250 mg amoxicillin and 62.5 mg clavulanic acid was processed identically to the tablet samples. Each brand was analysed in triplicate, with six injections per replicate.

2.7 Forced Degradation / Stress Studies

Forced degradation was performed on the individual and combined reference standard solutions under six ICH-recommended stress conditions: acid hydrolysis (0.1 M and 1 M HCl, 60 °C, 30-120 min), alkaline hydrolysis (0.1 M and 1 M NaOH, 60 °C, 15-90 min, with a deliberately shortened exposure schedule in view of clavulanic acid's known lability), neutral hydrolysis (ultra-pure water, 60 °C, 4-48 h), oxidative stress (3% and 30% v/v H₂O₂, ambient temperature, 30-120 min), thermal (dry-heat) stress on the solid powders (60 °C/72 h and 105 °C/24 h), and photolytic

stress in a calibrated photostability chamber per ICH Q1B (≥ 1.2 million lux-h visible light and ≥ 200 Wh/m² near-UV light), with light-protected dark controls run in parallel. Exposure conditions were chosen to target 10-30% degradation, a range considered optimal for reliable specificity assessment while retaining adequate residual parent-analyte signal. Degraded samples were analysed by the optimised RP-HPLC method, and peak purity was confirmed with the PDA detector.

2.8 Validation methods

The method was validated in accordance with ICH Q2(R1). Specificity was assessed from blank and placebo injections and from the forced-degradation chromatograms, requiring resolution (R_s) ≥ 2.0 between analyte and degradation-product peaks and a PDA peak-purity index ≥ 0.999 . Linearity was assessed by least-squares regression of peak area against concentration across the eight calibration levels (acceptance: $r^2 \geq 0.9990$), supported by one-way ANOVA and residual inspection. Accuracy was assessed by the standard-addition method at 80%, 100%, and 120% of target concentration (six replicates per level; acceptance: mean recovery 98.0-102.0%). Precision was assessed as repeatability (six replicates, one day, one analyst) and intermediate precision (three days, two analysts, independently prepared reagents), with acceptance of %RSD $\leq 2.0\%$ in both cases. LOD and LOQ were calculated from the standard deviation of the response and the calibration slope (LOD = $3.3\sigma/S$; LOQ = $10\sigma/S$) and confirmed experimentally ($S/N \geq 3:1$ for LOD; $S/N \geq 10:1$ and %RSD $\leq 10\%$ for LOQ). Robustness was evaluated using a Plackett-Burman fractional-factorial design in which mobile-phase pH (± 0.2), organic-modifier proportion ($\pm 2\%$), flow rate (± 0.1 mL/min), column temperature (± 5 °C), and detection wavelength (± 2 nm) were varied simultaneously across twelve experimental runs, with system-suitability and assay outcomes examined for significant change.

The validated method was subsequently applied to the assay of the procured marketed brands. Percentage assay (percentage of labelled amount) was calculated from the peak-area ratio of sample to freshly prepared standard, corrected for sample weight, average unit weight, label claim, and standard potency; pharmacopoeial compliance required each analyte to fall within 90.0-110.0% of its labelled amount.

3. RESULTS AND DISCUSSION

3.1 Chromatographic Behaviour and System Suitability

Under the finalised conditions (Section 2.4), amoxicillin and clavulanic acid are expected to elute as sharp, well-resolved peaks, with clavulanic acid's more polar, weakly UV-absorbing character reflected in an earlier retention time and lower baseline response relative to amoxicillin's stronger chromophore. Table 1 summarises the illustrative system-suitability outcome anticipated for six replicate injections of the 100% standard solution against the pre-defined acceptance criteria.

Table 1: Illustrative system suitability outcome under the optimised chromatographic conditions (simulated values; see data-status notice).

Parameter	Acceptance criterion	Illustrative result
Theoretical plates (N)	≥ 2000	AMX: 5248; CA: 4872
Tailing factor (T)	≤ 2.0	AMX: 1.12; CA: 1.18
Resolution (R_s), AMX-CA	≥ 2.0	2.86
%RSD of peak area (n = 6)	$\leq 1.0\%$	AMX: 0.42%; CA: 0.47%
%RSD of retention time (n = 6)	$\leq 1.0\%$	AMX: 0.31%; CA: 0.38% (illustrative)

If the completed study reproduces figures of this order, all four core system-suitability parameters would satisfy the pre-set acceptance criteria, supporting adequate column efficiency, peak symmetry, and separation between the two analytes.

3.2 Linearity and Range

Calibration curves constructed for each analyte across the eight-level range (50-150% of target concentration) are anticipated to be linear and to pass close to the origin, consistent with Beer-Lambert-governed UV response over this range. Because amoxicillin possesses the stronger chromophore, its calibration slope is expected to be markedly steeper than that of clavulanic acid, even though both analytes are expected to remain well within the quantifiable range throughout. Table 2 summarises the illustrative regression outcome.

Table 2: Illustrative calibration/regression outcome for AMX and CA (simulated values; see data-status notice).

Regression parameter	Amoxicillin	Clavulanic acid
Correlation coefficient (r^2)	0.9996	0.9993
Slope	18.42	7.86
Y-intercept	312.6	98.4
Range validated ($\mu\text{g/mL}$)	250-750	62.5-187.5

Both illustrative r^2 values exceed the ICH Q2(R1) threshold of 0.9990, which, if confirmed experimentally, would support the calibration model as an acceptable basis for the assay, recovery, and degradation calculations that follow.

3.3 Specificity and Forced Degradation

Specificity is expected to be demonstrated by the absence of interfering peaks in blank and placebo chromatograms at the retention times of either analyte, together with adequate resolution ($R_s \geq 2.0$) between each analyte peak and any co-generated degradation-product peaks across all six stress conditions, and a PDA peak-purity index at or above the 0.999 threshold for both analytes. Table 3 summarises the illustrative extent of degradation anticipated for amoxicillin and clavulanic acid under each stress condition.

Table 3: Illustrative forced-degradation extent by stress condition (simulated values; see data-status notice).

Stress condition	AMX degradation (illustrative, %)	CA degradation (illustrative, %)
Acid hydrolysis	12.4	18.7
Alkaline hydrolysis	22.8	31.6
Neutral hydrolysis	5.3	8.9
Oxidative stress (H_2O_2)	16.2	11.5
Thermal (dry heat)	6.8	9.7
Photolytic	10.6	17.9

This anticipated pattern is consistent with the known chemistry of the two molecules. Clavulanic acid's more strained and electrophilic beta-lactam ring is expected to render it more susceptible than amoxicillin's bicyclic penam nucleus to hydroxide-mediated nucleophilic attack, so that alkaline hydrolysis is projected to be the most aggressive stress condition overall and clavulanic acid the more labile of the two analytes under this condition, consistent with kinetic precedent for beta-lactam and clavulanate degradation reported in the pharmaceutical stability literature. Acid and photolytic stress are likewise projected to affect clavulanic acid more than amoxicillin, the latter plausibly linked to clavulanic acid's exocyclic hydroxy-ethylidene chromophore. Conversely, oxidative stress is projected to affect amoxicillin more than clavulanic acid, potentially reflecting sulfoxide formation at amoxicillin's penicillin sulfur atom, a site that clavulanic acid's oxazolidine ring lacks. Neutral hydrolysis and dry-heat stress are projected to cause the least degradation for both analytes, consistent with the generally greater stability of beta-lactams near neutral pH and in the solid state relative to aqueous acidic or alkaline conditions. If reproduced experimentally, this pattern of differential degradation would confirm the method's stability-indicating capability and would support treating clavulanic acid as the rate-limiting, more chemically vulnerable component of the combination for shelf-life and stress-testing purposes.

3.4 Accuracy, Precision, Sensitivity, and Robustness

Table 4 consolidates the illustrative outcome anticipated across the full ICH Q2(R1) validation parameter set, alongside the corresponding acceptance criterion for each parameter.

Table 4: Illustrative ICH Q2(R1) validation summary for the developed RP-HPLC method (simulated values; see data-status notice).

Parameter	Acceptance criterion	Illustrative result
Specificity	$R_s \geq 2.0$; peak purity ≥ 0.999	$R_s = 2.86$; peak purity = 0.9997
Linearity	$r^2 \geq 0.9990$	AMX $r^2 = 0.9996$; CA $r^2 = 0.9993$
Accuracy (recovery, 80-120%)	Mean recovery 98.0-102.0%	AMX: $99.42 \pm 0.58\%$; CA: $99.16 \pm 0.71\%$
Precision - repeatability	%RSD $\leq 2.0\%$	AMX: 0.74%; CA: 0.81%
Precision - intermediate	%RSD $\leq 2.0\%$	AMX: 1.02%; CA: 1.16%
LOD	$S/N \geq 3:1$	AMX: 1.8 $\mu\text{g/mL}$; CA: 0.7 $\mu\text{g/mL}$
LOQ	$S/N \geq 10:1$; %RSD $\leq 10\%$	AMX: 5.6 $\mu\text{g/mL}$; CA: 2.1 $\mu\text{g/mL}$
Robustness	No significant change on deliberate variation	Assay difference $< 2\%$ across all 12 runs

Within this projected outcome, accuracy is expected to be marginally more consistent for amoxicillin than for clavulanic acid, in keeping with the latter's greater sensitivity to small variations in handling, temperature, and timing during sample preparation. The standard-addition experiment as designed evaluates accuracy only within the laboratory-prepared synthetic-mixture matrix rather than within each marketed brand's proprietary excipient blend, a limitation that the subsequent assay of marketed formulations (Section 3.5) is intended to partially address.

3.5 Assay of Marketed Formulations

Table 5 presents the illustrative percentage-assay outcome anticipated from application of the validated method to six procured marketed brands (three tablet products and three oral-suspension products); brand identities have been coded pending substitution of the actual procured brand names.

Table 5: Illustrative assay of marketed AMX-CA formulations (simulated values, brand codes to be replaced with actual identifiers; see data-status notice).

Brand / dosage form	AMX assay (%)	CA assay (%)	Compliance (90- 110%)
Brand A (tablet)	98.74	99.12	Complies
Brand B (tablet)	99.36	98.68	Complies
Brand C (tablet)	100.18	99.54	Complies
Brand D (oral suspension)	97.92	98.46	Complies
Brand E (oral suspension)	99.08	97.84	Complies
Brand F (oral suspension)	98.56	99.27	Complies

If reproduced experimentally, all six brands would fall within the pharmacopoeial 90.0-110.0% label-claim window for both analytes, supporting the applicability of the method to routine assay of commercial products across both principal dosage forms. A projected statistical comparison of the developed method against a pharmacopoeial (BP/USP) reference method (illustrative $p = 0.284$) would, if confirmed, indicate no statistically significant difference between the two approaches ($p > 0.05$), supporting their equivalence for routine quality-control use.

4. CONCLUSION

A single isocratic RP-HPLC method, using a C18 column, a phosphate-buffer/acetonitrile mobile phase, and PDA detection at 220 nm, was developed for the simultaneous determination of amoxicillin and clavulanic acid in combined

tablet and oral-suspension dosage forms. The method's design explicitly accounts for the two analytes' contrasting UV chromophoric strength and differing susceptibility to acid-, base-, oxidation-, heat-, and light-mediated degradation, incorporating a forced-degradation protocol tailored to each analyte's known lability, with clavulanic acid treated as the more chemically vulnerable, rate-limiting component, particularly under alkaline conditions. The full ICH Q2(R1) validation protocol described here was designed to establish specificity, linearity, accuracy, precision, sensitivity, and robustness, and the method's protocol for application to marketed tablet and suspension products was designed to confirm suitability for routine quality-control assay. As emphasised throughout Section 3, the specific numerical outcomes reported in this article are illustrative projections carried over from a thesis in which the results chapter was framed prospectively, and they await confirmation by the completion of the actual instrumental runs; once genuine data are acquired, they can be substituted directly into the tables and structure presented here to yield a stability-indicating method validated and reported in full accordance with ICH Q1A(R2)/Q1B and Q2(R1).

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