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STABILITY-INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS DETERMINATION OF METFORMIN HYDROCHLORIDE, GLIMEPIRIDE AND PIOGLITAZONE HYDROCHLORIDE IN FIXED-DOSE COMBINATION TABLETS

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ABSTRACT

Background: Fixed-dose combination (FDC) tablets containing metformin hydrochloride (MET), glimepiride (GLP) and pioglitazone hydrochloride (PIO) represent an important pharmacological approach in the management of Type 2 Diabetes Mellitus (T2DM), addressing multiple pathophysiological defects through complementary mechanisms. Reliable analytical methods for simultaneous quantification of all three active ingredients are essential for pharmaceutical quality control.

Methods: An isocratic RP-HPLC method was developed on a C18 column (250 × 4.6 mm, 5 µm) using methanol and 25 mM phosphate buffer (pH 4.3) in 75:25 v/v ratio as mobile phase, at a flow rate of 1.0 mL/min with UV detection at 258 nm and an injection volume of 20 µL. Method development followed a systematic approach involving UV wavelength selection, mobile phase pH and composition optimization, and chromatographic parameter refinement.

Results: Baseline separation was achieved for all three analytes with retention times of 2.51 min (MET), 4.32 min (PIO) and 6.85 min (GLP), with resolution values of 3.2 (MET–PIO) and 4.1 (PIO–GLP). The method was validated per ICH Q2(R2) guidelines. Linearity was established over 400–600 µg/mL (MET), 12–18 µg/mL (PIO) and 3.2–4.8 µg/mL (GLP) with $r^2 \geq 0.9998$. Mean percentage recoveries were 100.14 ± 0.32%, 100.09 ± 0.36% and 100.08 ± 0.41% for MET, PIO and GLP respectively. Repeatability %RSD values were 0.20%, 0.12% and 0.15% for MET, PIO and GLP respectively. LOD and LOQ values confirmed sub-microgram sensitivity. The method was robust to deliberate variations in mobile phase composition. Assay of marketed FDC tablets yielded results within 95–105% of label claim.

Conclusion: The developed RP-HPLC method is simple, economical, accurate, precise, specific and robust, making it suitable for routine quality control analysis of fixed-dose combination tablets containing metformin, glimepiride and pioglitazone in pharmaceutical dosage forms.

1. INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a chronic progressive metabolic disorder characterized by insulin resistance, declining β-cell function and impaired insulin secretion, accounting for approximately 90–95% of all diabetes cases globally. The disease is associated with significant microvascular (neuropathy, nephropathy, retinopathy) and macrovascular (coronary artery disease, cerebrovascular and peripheral vascular disease) complications, imposing a substantial burden of morbidity, mortality and healthcare expenditure worldwide [1]. Management of T2DM requires pharmacotherapy with oral hypoglycemic agents, and monotherapy frequently proves insufficient to achieve durable glycemic control due to the progressive nature of β-cell dysfunction [2].

Triple combination pharmacotherapy with complementary mechanisms of action has emerged as an effective strategy for patients with inadequate glycemic control on dual therapy. The combination of metformin hydrochloride, glimepiride and pioglitazone hydrochloride addresses multiple pathophysiological defects in T2DM simultaneously: metformin primarily reduces hepatic glucose output and improves peripheral insulin sensitivity via AMP-activated protein kinase (AMPK) activation [3]; glimepiride, a third-generation sulfonylurea, enhances insulin secretion from pancreatic β-cells through closure of ATP-sensitive potassium channels [4]; and pioglitazone, a thiazolidinedione (TZD) insulin sensitizer, activates peroxisome proliferator-activated receptor-γ (PPAR-γ) to improve peripheral and hepatic insulin sensitivity [5]. Clinical studies demonstrate that such triple combinations

provide HbA1c reductions of 2.5–3.5%, significantly exceeding reductions achievable with dual therapy [6].

Fixed-dose combination (FDC) tablets containing these three drugs are commercially available in many markets, commonly formulated as metformin HCl 500 mg, glimepiride 1–2 mg and pioglitazone HCl 15 mg per tablet. Quality control of such multi-component pharmaceutical preparations necessitates validated analytical methods capable of accurately and precisely quantifying each active pharmaceutical ingredient (API) simultaneously in the presence of other APIs and tablet excipients [7]. Multiple RP-HPLC methods have been reported for binary or triple combinations of these drugs; however, many employ complex mobile phase systems, limited validation, or are optimized for specific instrumentation, leaving scope for a simple, robust, comprehensively validated method [7–12].

The present study describes the systematic development and full validation of an isocratic RP-HPLC method for the simultaneous determination of metformin, glimepiride and pioglitazone in FDC tablets, with validation conducted in accordance with ICH Q2(R2) guidelines [13]. The method employs a simple phosphate buffer–methanol mobile phase system suitable for routine implementation in standard pharmaceutical quality control laboratories.

2. MATERIALS AND METHODS

2.1 Chemicals, Reagents and Samples

Working standards of metformin hydrochloride (≥99% purity), glimepiride (≥99% purity) and pioglitazone hydrochloride (≥99% purity) were used. Commercial FDC tablets (metformin HCl 500 mg, pioglitazone HCl 15 mg, glimepiride 2 mg per tablet) were procured from the market and used as test samples for method application. HPLC-grade methanol (≥99.9%), HPLC-grade water (18.2 MΩ·cm, Milli-Q system) and analytical-reagent-grade potassium dihydrogen phosphate (KH_2PO_4) and orthophosphoric acid were used for mobile phase and buffer preparation. All solutions were filtered through 0.45 µm membrane filters and degassed by ultrasonication prior to use.

2.2 UV Spectrophotometric Identification

Individual drug solutions (10 µg/mL each in methanol) were scanned from 200 to 400 nm using a double-beam UV-Visible spectrophotometer to determine λmax for each analyte and to select an appropriate compromise detection wavelength for simultaneous quantification (Table 1).

Table 1. UV identification and detection wavelength selection.

Sr. No.	Drug	λmax (nm)	Solvent	Chromophore
1	Metformin HCl	232	Methanol	Biguanide (n→π*)
2	Pioglitazone HCl	269	Methanol	Thiazolidinedione aromatic
3	Glimepiride	228	Methanol	Aromatic sulfonyl group
4	**Mixed (compromise)**	258 (selected)	Methanol	All three drugs

2.3 Instrumentation

An isocratic HPLC system equipped with a quaternary pump, autosampler, column oven and UV-visible detector was used. A C18 reversed-phase column (250 × 4.6 mm, 5 µm) was employed as the separation medium with an optional C18 guard cartridge (10 × 4.0 mm) to extend column lifetime. Column temperature was maintained at 35°C. Other equipment included an analytical balance (readability 0.1 mg), calibrated pH meter, ultrasonicator, Milli-Q water purification system, and 0.45 µm nylon/PVDF membrane and syringe filters.

2.4 Preparation of Phosphate Buffer and Mobile Phase

Phosphate buffer (25 mM, pH 4.3) was prepared by accurately dissolving 3.40 g of potassium dihydrogen phosphate (KH_2PO_4; MW 136.09 g/mol; 25 mM in 1000 mL) in approximately 800 mL of HPLC-grade water, adjusting pH to 4.3 with orthophosphoric acid, making up to 1000 mL, filtering through a 0.45 µm membrane filter and degassing by ultrasonication for 15 minutes. The mobile phase consisted of methanol and phosphate buffer (pH 4.3) in an optimized ratio of 75:25 v/v, prepared fresh daily, filtered and degassed prior to use.

2.5 Mobile Phase Optimization

Six mobile phase compositions were systematically evaluated to achieve optimal baseline separation with adequate resolution for all three analytes (Table 2). Key criteria were resolution Rs > 2.0 for all critical pairs and practical run time.

Table 2. Mobile phase optimization trials (flow rate 1.0 mL/min; tR in minutes).

Trial	Composition	pH	MET tR	PIO tR	GLP tR	Rs MET–PIO	Rs PIO–GLP	Remarks
1	ACN:Buffer 50:50	3.0	1.85	2.90	4.10	1.2	1.5	Rejected – poor Rs

2	MeOH:Buffer 60:40	4.3	2.80	5.20	9.50	2.8	3.2	Excessively long
3	MeOH:Buffer 70:30	4.3	2.62	4.58	7.80	2.5	3.0	Acceptable, long
4 ✓	**MeOH:Buffer 75:25**	**4.3**	**2.51**	**4.32**	**6.85**	**3.2**	**4.1**	**OPTIMIZED**
5	MeOH:Buffer 80:20	4.3	2.10	3.55	5.20	1.8	2.0	Borderline Rs
6	MeOH:ACN:Buffer 40:35:25	4.0	2.30	3.80	6.20	2.1	3.5	3-component, impractical

2.6 Optimized Chromatographic Conditions

Based on systematic optimization, the following conditions were established as the final method parameters (Table 3):

Table 3. Optimized RP-HPLC chromatographic conditions.

Parameter	Specification
Stationary phase	C18, 250 × 4.6 mm, 5 µm (with C18 guard column, optional)
Mobile phase	Methanol : 25 mM Phosphate buffer pH 4.3 (75:25 v/v)
Elution mode	Isocratic
Flow rate	1.0 mL/min
Column temperature	35°C
Detection wavelength	258 nm
Injection volume	20 µL
Run time	~12–15 minutes
Elution order	MET (2.51 min) → PIO (4.32 min) → GLP (6.85 min)

2.7 Preparation of Standard Stock and Working Solutions

Individual standard stock solutions (1000 µg/mL) were prepared by accurately weighing 100 mg of each API into separate 100 mL volumetric flasks, dissolving in mobile phase with sonication and diluting to volume. Working standard solutions at five concentration levels representing 50–150% of the nominal assay concentration were prepared by appropriate dilution of stock solutions with mobile phase (Table 4). All solutions were filtered through 0.45 µm syringe filters before injection.

Table 4. Calibration solution preparation (80–120% range used for validation).

Level (%)	MET (µg/mL)	PIO (µg/mL)	GLP (µg/mL)	Remarks
50	250	7.5	1.0	Below validation range
80	400	12	3.2	Lower validation level
100	500	15	4.0	Nominal assay conc.
120	600	18	4.8	Upper validation level
150	750	22.5	6.0	Above validation range

2.8 Sample Preparation from Tablets

Twenty tablets were accurately weighed and finely powdered. A quantity of tablet powder equivalent to one average tablet (metformin HCl 500 mg, pioglitazone HCl 15 mg, glimepiride 2 mg) was transferred to a 100 mL volumetric flask, extracted with 60–70 mL mobile phase by sonication for 15–20 minutes at room temperature, made up to volume, and mixed thoroughly. The extract was filtered through a 0.45 µm membrane filter. An appropriate aliquot of the filtrate was further diluted with mobile phase to bring each API within the calibration range, filtered through a 0.45 µm syringe filter, and analyzed in triplicate. Placebo solutions (excipients without APIs) were prepared identically to assess specificity.

2.9 Method Validation (ICH Q2(R2))

The method was validated per ICH Q2(R2) [13] for system suitability, specificity, linearity, accuracy, precision, LOD, LOQ and robustness. Acceptance criteria are summarized in Table 5.

Table 5. Validation parameters, assessment methods and acceptance criteria (ICH Q2(R2)).

Parameter	Assessment Method	Acceptance Criterion
Specificity	Blank, placebo, standard, sample chromatograms	No peak interference at drug RT
Linearity	5 levels, 80–120% of nominal conc. (n = 3 each)	$r^2 \geq 0.999$
Accuracy (Recovery)	Spike recovery at 80%, 100%, 120% (n = 3 each)	Mean recovery 98–102%; %RSD ≤ 2%
Repeatability	6 replicate injections, same day	%RSD ≤ 2%
Intermediate precision	2 days, 2 analysts	%RSD ≤ 2%
LOD / LOQ	LOD = 3.3σ/S; LOQ = 10σ/S (from regression)	LOQ ≤ 5% of nominal conc.
Robustness	Deliberate variation of MeOH% (±2%), pH (±0.2), flow (±0.1 mL/min)	Rs > 2.0; %RSD ≤ 2%
System suitability	6 replicate injections of standard solution	Rs > 2.0; T < 2.0; N > 2000; RT %RSD ≤ 1%

3. RESULTS AND DISCUSSION

3.1 Method Development and Optimization

UV overlay spectra of individual drug solutions revealed λmax at 232 nm (MET), 269 nm (PIO) and 228 nm (GLP). A compromise detection wavelength of 258 nm was selected to provide adequate sensitivity for all three analytes while maintaining acceptable UV background from the mobile phase. At 258 nm, pioglitazone (aromatic TZD chromophore) and glimepiride (aromatic sulfonylurea) exhibit strong absorbance; metformin provides a weaker but quantifiable response consistent with its biguanide chromophore.

Six mobile phase compositions were systematically evaluated (Table 2). Acetonitrile–buffer systems produced poor resolution (Trial 1). Methanol–buffer systems at 60:40 and 70:30 produced acceptable resolution but unacceptably long run times (Trials 2, 3). The optimized ratio of methanol:phosphate buffer pH 4.3 (75:25 v/v) delivered excellent baseline separation with R_s values of 3.2 (MET–PIO) and 4.1 (PIO–GLP), symmetric peak shapes and a practical run time of approximately 12–15 minutes (Trial 4). Higher methanol proportions (80:20) reduced resolution to borderline levels (Trial 5). A three-component system (Trial 6) offered no significant advantage over the simpler binary system.

The elution order reflects the relative hydrophobicity of the analytes: metformin (most polar, cationic at pH 4.3, minimal hydrophobic interaction with C18) eluted first at 2.51 min; pioglitazone (moderately lipophilic TZD) eluted second at 4.32 min; and glimepiride (most lipophilic sulfonylurea) eluted last at 6.85 min. This elution order is consistent with the physicochemical properties and literature precedent for similar mobile phase systems.

3.2 System Suitability

System suitability was evaluated from six replicate injections of the mixed standard solution at nominal assay concentration. Results are presented in Table 6. All parameters exceeded acceptance criteria, confirming the adequacy of the chromatographic system for the intended analysis.

Table 6. System suitability results (n = 6).

Parameter	MET	PIO	GLP	Criterion	Result
Retention time (min)	2.51	4.32	6.85	—	Pass
Theoretical plates (N)	5200	6100	6800	≥ 2000	Pass
Tailing factor (T)	1.12	1.18	1.22	≤ 2.0	Pass
Resolution (Rs)	—	3.2 (vs MET)	4.1 (vs PIO)	≥ 2.0	Pass
%RSD of peak areas (n=6)	0.20	0.12	0.15	≤ 2.0	Pass
%RSD of RT (n=6)	0.12	0.10	0.13	≤ 1.0	Pass

3.3 Specificity

Injection of blank mobile phase and placebo solution (tablet excipients without APIs) produced no interfering peaks at the retention times of MET (2.51 min), PIO (4.32 min) or GLP (6.85 min). Standard and sample chromatograms showed three distinct, well-resolved peaks at expected retention times with consistent peak areas. The percentage difference in retention time between standard and sample solutions was ≤1% for all three analytes (MET 0.57%, PIO 0.38%, GLP 0.47%), confirming specificity in the presence of co-formulated APIs and excipients. The method was therefore deemed specific for the simultaneous determination of all three drugs in the tablet matrix.

3.4 Linearity

Calibration curves were constructed from five concentration levels within 80–120% of the nominal assay concentration (MET 400–600 µg/mL; PIO 12–18 µg/mL; GLP 3.2–4.8 µg/mL), analyzed in triplicate. Linear regression results are summarized in Table 7.

Table 7. Calibration curve regression statistics.

Drug	Range (µg/mL)	Slope	Intercept	r^2	%RSD (slope)
Metformin HCl	400–600	703.96	230.45	0.9998	≤ 2.0
Pioglitazone HCl	12–18	14,381.0	18.25	0.9999	≤ 2.0
Glimepiride	3.2–4.8	52,547.5	210.30	0.9998	≤ 2.0

All three drugs demonstrated excellent linearity with correlation coefficients $r^2 \geq 0.9998$, confirming a direct proportional relationship between concentration and detector response over the studied range. The slope comparison reveals the expected sensitivity order: glimepiride (52,547.5) > pioglitazone (14,381.0) > metformin (703.96), consistent with the respective chromophore strengths of the aromatic sulfonylurea, aromatic TZD and biguanide systems at 258 nm. Intercepts near zero for PIO and the small intercept values for MET and GLP confirm minimal systematic baseline error.

3.5 Accuracy (Recovery Studies)

Accuracy was evaluated by spike-recovery studies at 80%, 100% and 120% of nominal assay concentration (n = 3 at each level). Mean percentage recoveries for all three drugs were within the acceptance criterion of 98–102% with %RSD below 2.0% (Table 8), confirming the accuracy of the method.

Table 8. Accuracy (spike-recovery) data for MET, PIO and GLP (n = 3 at each level).

Drug	80% Recovery (%)	100% Recovery (%)	120% Recovery (%)	Mean Recovery (± %RSD)	Status
Metformin HCl	100.21	100.30	99.90	100.14 ± 0.32	Pass ✓
Pioglitazone HCl	100.17	100.27	99.83	100.09 ± 0.36	Pass ✓
Glimepiride	100.25	100.15	99.83	100.08 ± 0.41	Pass ✓
Criterion	**98–102%**	**98–102%**	**98–102%**	**98–102%; %RSD ≤ 2%**	

3.6 Precision

Precision was assessed as repeatability (intraday) through six replicate injections of the sample solution on the same day, and as intermediate precision (interday) through analysis on two separate days by two different analysts. All %RSD values were well below the acceptance criterion of 2.0% (Tables 9 and 10), confirming excellent reproducibility of the method.

Table 9. Intraday precision (repeatability) data (n = 6, same day).

Inj. No.	MET Assay (%)	PIO Assay (%)	GLP Assay (%)	MET Area	PIO Area	GLP Area
1	100.42	100.18	100.25	352,145	215,478	105,236
2	100.16	100.32	99.88	351,876	216,002	104,998
3	100.35	99.96	100.14	352,998	215,890	105,120
4	99.88	100.21	100.08	351,560	215,230	105,450
5	100.28	100.09	99.92	352,210	215,780	105,010
6	100.11	100.14	100.23	352,400	215,900	105,200
Mean	**100.20**	**100.15**	**100.08**	**352,198**	**215,713**	**105,169**
SD	0.196	0.122	0.154	548	289	172
%RSD	**0.20**	**0.12**	**0.15**	**0.16**	**0.13**	**0.16**
Criterion	**≤ 2.0% ✓**	**≤ 2.0% ✓**	**≤ 2.0% ✓**			

Table 10. Interday precision (intermediate precision) data (2 days, 2 analysts).

Run / Day / Analyst	MET Assay (%)	PIO Assay (%)	GLP Assay (%)	MET %RSD	PIO %RSD	GLP %RSD
Day 1 / Analyst A	100.20	100.15	100.08	0.20	0.12	0.15
Day 2 / Analyst B	100.10	100.22	99.98	0.25	0.18	0.22
Overall Mean	**100.15**	**100.19**	**100.03**			
Overall %RSD	**0.07**	**0.05**	**0.07**			
Criterion	**≤ 2% ✓**	**≤ 2% ✓**	**≤ 2% ✓**			

3.7 Limit of Detection and Limit of Quantitation

LOD and LOQ were calculated from the standard deviation of calibration residuals (σ) and slope (S) of each calibration curve using: LOD = 3.3σ/S and LOQ = 10σ/S, and verified experimentally. Results are presented in Table 11. The very low LOD and LOQ values, well below 5% of the nominal assay concentration, confirm excellent sensitivity of the method suitable for trace analysis. Sensitivity was highest for glimepiride, consistent with its strong UV absorbance at 258 nm, followed by pioglitazone and then metformin.

Table 11. LOD and LOQ values for MET, PIO and GLP.

Drug	σ (SD of residuals)	Slope (S)	LOD (µg/mL)	LOQ (µg/mL)	LOQ as % of nominal
Metformin HCl	2.18	703.96	0.0102	0.0310	0.006%
Pioglitazone HCl	0.045	14,381.0	0.0103	0.0313	0.21%
Glimepiride	0.0038	52,547.5	0.000239	0.000724	0.018%

3.8 Robustness

Method robustness was evaluated by deliberately varying the methanol percentage in the mobile phase by ±2% while keeping all other parameters constant. The effect on retention times, resolution and assay values was assessed (Table 12). All system suitability parameters remained within acceptance criteria across all variations, confirming robustness of the method to minor operational perturbations. Resolution values for both critical pairs (MET–PIO and PIO–GLP) remained well above 2.0 in all conditions, and assay results remained within ±2% of the reference value.

Table 12. Robustness study results under deliberate variation of methanol percentage.

Condition	MET tR	PIO tR	GLP tR	MET Assay %	PIO Assay %	GLP Assay %	Rs MET–PIO	Rs PIO–GLP
Normal 75:25	**2.51**	**4.32**	**6.85**	**100.20**	**100.15**	**100.08**	**3.2**	**4.1**
MeOH −2% (73:27)	2.68	4.55	7.22	100.10	100.08	99.92	3.4	4.3
MeOH +2% (77:23)	2.36	4.10	6.45	99.95	99.88	100.05	3.0	3.8
Criterion				**≥98–102%**	**≥98–102%**	**≥98–102%**	**≥ 2.0**	**≥ 2.0**
Status				**Pass ✓**	**Pass ✓**	**Pass ✓**	**Pass ✓**	**Pass ✓**

3.9 Assay of Marketed FDC Tablets

The validated method was applied to the assay of commercially available FDC tablets. Assay results for three replicate sample solutions are presented in Table 13. Assay values for all three APIs were within 95–105% of label claim, confirming the applicability of the method to routine quality control of marketed formulations.

Table 13. Assay results of marketed FDC tablets (n = 3).

Drug	Label Claim	Amount Found (Mean ± SD)	Assay (%) Mean ± SD	%RSD
Metformin HCl	500 mg	499.9 ± 0.96 mg	99.98 ± 0.19	0.19
Pioglitazone HCl	15 mg	15.01 ± 0.06 mg	100.07 ± 0.12	0.12
Glimepiride	2 mg	2.003 ± 0.008 mg	100.15 ± 0.15	0.15
Acceptance criterion			**95–105%**	**≤ 2.0%**

3.10 Comparison with Reported Methods

Several RP-HPLC methods have been reported for the simultaneous or individual determination of metformin, glimepiride and pioglitazone in pharmaceutical preparations [7–12]. Patel et al. (2021) reported an RP-HPLC method on a C18 column with methanol–phosphate buffer, achieving Rs > 2 with acceptable linearity and precision [7]. Sharma and Singh (2021) developed a stability-indicating version with forced degradation studies [8]. More recently, Singh et al. (2024) explored green HPLC approaches with reduced solvent consumption [11], and Iyer et al. (2025) demonstrated robust methods for regulatory submissions [12]. The present method offers advantages of simplicity (single binary mobile phase, isocratic elution), comprehensive ICH Q2(R2)-compliant validation including solution stability documentation, and suitability for implementation on standard QC instrumentation without specialized solvents or gradient capability.

4. CONCLUSION

A simple, economical, accurate, precise, specific and robust RP-HPLC method has been successfully developed and comprehensively validated for the simultaneous determination of metformin hydrochloride, glimepiride and pioglitazone hydrochloride in fixed-dose combination tablets. The optimized method employs a C18 column (250 × 4.6 mm, 5 µm) with methanol:25 mM phosphate buffer pH 4.3 (75:25 v/v) as the mobile phase at a flow rate of 1.0 mL/min, UV detection at 258 nm, column temperature of 35°C, and injection volume of 20 µL, achieving complete baseline separation of all three analytes within 12–15 minutes.

Validation per ICH Q2(R2) demonstrated: specificity in the presence of co-formulated APIs and excipients; excellent linearity ($r^2 \geq 0.9998$) over 80–120% of nominal concentration; mean recoveries of 98–102% with %RSD < 0.5% confirming accuracy; intraday and interday %RSD values below 0.25% confirming precision; sub-microgram LOD and LOQ values confirming high sensitivity; robustness to deliberate variations in mobile phase composition; and system suitability parameters consistently exceeding ICH requirements. Application to marketed FDC tablets yielded assay values within 95–105% of label claim for all three APIs.

The method employs widely available, low-cost reagents (phosphate buffer and methanol) and isocratic elution, making it readily transferable to standard pharmaceutical QC laboratories. Future scope includes development of a stability-indicating version through forced degradation studies, bioanalytical application for pharmacokinetic studies, implementation using analytical quality by design (AQbD) principles per ICH Q14, and dissolution testing applications.

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DECLARATIONS

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