



Rp-Hplc Method Development and Validation for the Simultaneous Estimation of Citicoline and Piracetam in Bulk Drug and Tablet Dosage Form

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Abstract

Introduction: Simultaneous estimation of citicoline and piracetam, a fixed-dose nootropic combination widely used for cerebrovascular and cognitive disorders, is essential to ensure the quality of combined pharmaceutical formulations. This study aimed to develop and validate a simple, rapid, accurate, precise, robust and economical reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the simultaneous estimation of citicoline and piracetam in bulk drug and tablet dosage form in accordance with International Council for Harmonisation (ICH) guidelines.

Materials and Methods: Chromatographic separation was carried out on a Hypersil BDS C18 column (250 × 4.6 mm, 5 µm) using ammonium dihydrogen orthophosphate buffer (pH 3.5, adjusted with ortho-phosphoric acid) as the mobile phase in isocratic mode, at a flow rate of 1.0 mL/min with ultraviolet detection at 210 nm. The method was validated for system suitability, specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), limit of quantitation (LOQ) and assay as per ICH Q2 guidelines.

Results: The optimized method produced well-resolved, symmetrical peaks for citicoline (retention time 3.197 min) and piracetam (retention time 10.723 min) (resolution, $R_s = 9.42$; tailing factor ≤ 1.4 ; theoretical plates > 6700) within a 20 min isocratic run. Linearity was established over 25-75 $\mu\text{g/mL}$ for citicoline and 40-120 $\mu\text{g/mL}$ for piracetam, with correlation coefficients (R^2) of 0.9997 and 0.9996, respectively ($n = 5$ concentrations, six replicate injections each). Mean percentage recoveries were $99.52 \pm 0.13\%$ for citicoline and $98.98 \pm 0.23\%$ for piracetam ($n = 9$ per drug), with %RSD values below 2.0% for all precision parameters (repeatability, intermediate precision and reproducibility). The LOD and LOQ, calculated from the residual standard deviation of the regression line and its slope, were 0.06 and 0.18 $\mu\text{g/mL}$ for citicoline, and 0.09 and 0.26 $\mu\text{g/mL}$ for piracetam, respectively. Peak area %RSD remained $\leq 0.36\%$ under small, deliberate variation of flow rate, detection wavelength and mobile-phase pH, confirming robustness.

Discussion: All validation parameters satisfied the acceptance criteria of the ICH Q2(R2) guideline, and no co-eluting peak from tablet excipients or from acid-, base-, peroxide-, thermal- or photolytically generated degradation products was observed at the retention times of either analyte, confirming both the specificity and the stability-indicating capacity of the method.

Conclusion: The developed RP-HPLC method is simple, rapid, accurate, precise, robust and cost-effective, and is suitable for the routine simultaneous quality-control estimation of citicoline and piracetam in bulk drug and pharmaceutical dosage forms.

Keywords: Citicoline; Piracetam; RP-HPLC; Method Development; Method Validation; Simultaneous Estimation; ICH Guidelines

Introduction

Pharmaceutical analysis plays a crucial role in ensuring the quality, safety, efficacy and regulatory compliance of pharmaceutical products throughout their lifecycle, encompassing the identification, characterization and quantitative determination of active pharmaceutical ingredients, impurities, degradation products and excipients in bulk drugs and finished dosage forms [1,2]. Among the analytical techniques available, reversed-phase high-performance liquid chromatography (RP-HPLC) remains one of the most versatile and widely employed techniques in pharmaceutical analysis, offering excellent resolution, high sensitivity, reproducibility and rapid analysis, and its capability to separate and quantify multiple analytes within a single run substantially reduces analysis time, solvent consumption and operational cost [3].

Disorders of the nervous system affect a substantial proportion of the global population and represent a major public-health burden, with an estimated one billion people worldwide living with a neurological disorder [4]. The combination of citicoline and piracetam is widely used as a nootropic or cognitive enhancer because of its reported synergistic neuroprotective and memory-enhancing effects, and finds application in the management of head trauma, cerebrovascular disease, Parkinson's disease and attention-deficit hyperactivity disorder [5]. Since both drugs

are frequently formulated together, a reliable analytical method for their simultaneous estimation is essential to ensure product quality, dosage uniformity and regulatory compliance.

Several methods, including HPLC [6-12], ultra-performance liquid chromatography [13] and spectrophotometry [14-16], have previously been reported for the estimation of citicoline and/or piracetam. However, many of these methods are limited by low sensitivity, long run times, failure to meet compendial peak-purity thresholds, or a lack of a documented stability-indicating capability against the principal ICH-recommended stress conditions (acid, base, oxidative, thermal and photolytic degradation). Building on recent advances in HPLC-based estimation of this combination [17,18], and given that citicoline sodium ($\text{pK}_a \approx 4.4$, ionizable) and piracetam (a neutral, non-ionizable lactam) differ substantially in their acid-base behaviour, chromatographic selectivity for this pair is expected to be particularly sensitive to mobile-phase pH, a variable that was therefore prioritized during method optimization. The present study was undertaken with the specific objectives of (i) developing a simple, rapid, selective RP-HPLC method for the simultaneous estimation of citicoline and piracetam in bulk drug and tablet dosage form, and (ii) validating the method for system suitability, specificity, linearity, accuracy, precision, robustness, LOD, LOQ and assay in accordance with the revised ICH Q2(R2) guideline on validation of analytical procedures [19-21].

Materials and Methods

Chemicals and reagents

Citicoline sodium (CTS) and piracetam (PIC) reference standards were obtained from Micro Labs Limited, Bangalore, India. Acetonitrile and methanol of HPLC grade were purchased from Spectrochem, Mumbai. Analytical-reagent-grade ammonium dihydrogen orthophosphate was procured from Merck (India), and other chemicals were of analytical grade from SD Fine Chemicals, India. Deionized, ultra-pure water used throughout the study was obtained from a Milli-Q water purification system (Millipore, USA), and solutions were filtered through 0.45 µm nylon membrane filters (Advanced Microdevices, Mumbai, India). A marketed tablet formulation (label claim: 500 mg citicoline and 800 mg piracetam per tablet) was procured from the local market.

Instrumentation

Chromatographic analysis was performed on a Waters HPLC system equipped with an LC-20AD solvent delivery module, a photodiode array detector and an autosampler, with data acquisition using Empower™ 2 chromatography software. An analytical balance (Mettler Toledo XS205), a UV-visible spectrophotometer (Lab India UV-3000) and a digital pH meter (Metrohm 827 pH lab) were also used.

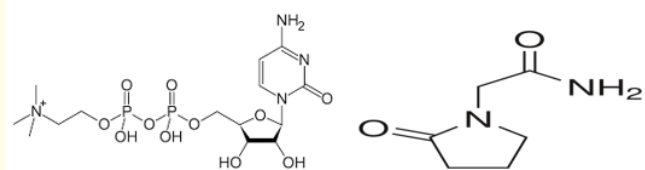


Figure 1: Chemical structures of citicoline (left) and piracetam (right).

Chromatographic conditions

Chromatographic optimization was carried out systematically by varying the column, buffer pH, flow rate and column temperature over six trials. Separation was finally achieved on a Hypersil BDS C18 column (250 × 4.6 mm, 5 µm) using an isocratic mobile phase of ammonium dihydrogen orthophosphate buffer (pH 3.5, adjusted with ortho-phosphoric acid), at a flow rate of 1.0 mL/min, with detection at 210 nm using a photodiode array detector, an injection volume of 20 µL and a column temperature

of 25 °C. This combination gave symmetrical, well-resolved peaks that passed the peak-purity threshold for both analytes, with no interfering hump in the baseline (Table 1).

Preparation of mobile phase and diluent

Ammonium dihydrogen orthophosphate (5.75 g) was dissolved in 500 mL of water in a 1000 mL beaker, diluted to volume and mixed. The pH was adjusted to 3.5 with ortho-phosphoric acid, and the buffer was sonicated to degas and filtered through a 0.45 µm nylon membrane filter. Water was used as the diluent.

Preparation of standard solution

Citicoline sodium working standard (54 mg) and piracetam working standard (80 mg) were accurately weighed into a 100 mL volumetric flask, dissolved in about 70 mL of diluent by sonication and made up to volume. An aliquot (5 mL) of this solution was further diluted to 50 mL with diluent to obtain final concentrations of approximately 50 µg/mL CTS and 80 µg/mL PIC, and the solution was filtered through a 0.45 µm nylon membrane filter before injection.

Preparation of sample solution

Twenty tablets were weighed and finely powdered. Tablet powder equivalent to 50 mg citicoline and 80 mg piracetam was accurately weighed into a 100 mL volumetric flask, dissolved in about 70 mL of diluent by sonication for 10 min and made up to volume. An aliquot (5 mL) was diluted to 50 mL with diluent, filtered through a 0.45 µm nylon membrane filter, and 20 µL of the resulting solution was injected into the chromatographic system.

Statistical analysis

All chromatographic determinations were performed with a minimum of three to six replicate injections per condition, as specified for each parameter below, and results are expressed as mean ± standard deviation (SD), with precision reported as percentage relative standard deviation (%RSD = SD/mean × 100). Calibration data were fitted by unweighted least-squares linear regression of peak area (y) against nominal analyte concentration (x, µg/mL) using Empower™ 2 chromatography software, and the correlation coefficient (R²), slope and y-intercept were recorded for each analyte. Acceptance of each validation parameter was based on the numerical criteria specified in the ICH Q2(R2) guideline (e.g., %RSD ≤ 2.0% for precision, tailing factor < 2, theoretical

plates > 2000, recovery within 98-102%), as detailed under the relevant subsection.

Method validation

The optimized method was validated as per ICH Q2 guidelines for system suitability, specificity, linearity, accuracy, precision, robustness, LOD, LOQ and assay [19-21].

- **System suitability:** System suitability was assessed from six replicate injections of the standard solution, evaluating peak area, retention time, tailing factor, theoretical plate number and resolution.
- **Linearity:** Standard solutions were prepared over the concentration range of 25-75 µg/mL for CTS and 40-120 µg/mL for PIC. Each concentration was injected in triplicate, and linearity was evaluated by linear regression analysis of peak area versus concentration.
- **Limit of detection and limit of quantitation:** LOD and LOQ were calculated from the standard deviation of the response (σ) and the slope of the calibration curve (S) using the equations $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$.
- **Accuracy:** Recovery studies were performed by the standard addition method at three concentration levels (50%, 100% and 150% of the target concentration), with three replicate preparations injected in triplicate at each level.
- **Precision:** Repeatability (intra-day) and intermediate precision (inter-day, by two analysts) were assessed at three concentration levels (37.5, 50 and 62.5 µg/mL for CTS; 60, 80 and 100 µg/mL for PIC) and expressed as percentage relative standard deviation (%RSD).
- **Robustness:** Robustness was evaluated by making small, deliberate changes to the flow rate (0.9, 1.0 and 1.1 mL/min), detection wavelength (205, 210 and 215 nm) and mobile-phase pH (2.8, 3.0 and 3.2), and observing the effect on peak area, tailing factor and theoretical plate count.
- **Specificity:** Forced degradation was carried out under acid (0.1 N HCl), base (0.1 N NaOH), oxidative (3% H₂O₂), thermal (105 °C, 8 h) and UV-light (4 h) stress conditions, and the standard, placebo and stressed samples were analyzed to confirm that the method could specifically quantify the analytes in the presence of degradation products and excipients.

- **Assay:** The content of citicoline and piracetam in the marketed tablet formulation was determined by injecting solutions prepared to yield nominal concentrations of 50 µg/mL CTS and 80 µg/mL PIC, in six replicate preparations, under the optimized chromatographic conditions.

Results

Method development

Chromatographic conditions were optimized over six exploratory trials, varying the column (Inertsil ODS 3V and Hypersil BDS), buffer pH (3.0-4.5), flow rate (0.5-1.0 mL/min) and column temperature (25-40 °C). Earlier trials with the Inertsil ODS 3V column at pH 3.0-4.5 failed the peak-purity threshold for one or both analytes and showed a baseline hump near the citicoline peak. Replacing the column with Hypersil BDS (250 × 4.6 mm, 5 µm) and using ammonium dihydrogen orthophosphate buffer (pH 3.5) at a flow rate of 1.0 mL/min and 25 °C (Trial 6) produced a clean baseline with no interfering humps, and the peak-purity threshold was passed for both citicoline and piracetam; these conditions were therefore selected as final (Table 1).

Parameter	Condition
Column	Hypersil BDS C18 (250 × 4.6 mm, 5 µm)
Mobile phase	Ammonium dihydrogen orthophosphate buffer, 100%
pH	3.5 (adjusted with ortho-phosphoric acid)
Elution mode	Isocratic
Flow rate	1.0 mL/min
Detection wavelength	210 nm
Injection volume	20 µL
Column temperature	25 °C
Run time	20 min

Table 1: Optimized chromatographic conditions.

System suitability

System suitability parameters obtained from six replicate injections ($n = 6$) of the standard solution were within ICH/ USP-recommended limits for both analytes — tailing factor < 2, theoretical plates > 2000, and peak-area and retention-time %RSD ≤ 2.0% — confirming that the chromatographic system performed

reproducibly and was suitable for the intended quantitative analysis (Table 2). Citicoline and piracetam eluted at 3.197 min and 10.723 min, respectively, and were baseline-separated, with a

resolution (Rs) of 9.42, well above the minimum acceptable value of 2.0.

Parameter	Citicoline sodium	Piracetam	ICH/USP acceptance limit
Retention time (min)	3.197	10.723	—
Peak area (%RSD)	1.359	0.921	≤ 2.0%
Retention time (%RSD)	0.314	0.654	≤ 2.0%
Tailing factor	1.24	1.37	< 2.0
Theoretical plates (N)	6745	14632	> 2000
Resolution (Rs)	9.42	—	> 2.0

Table 2: System suitability results of the proposed method (n = 6).

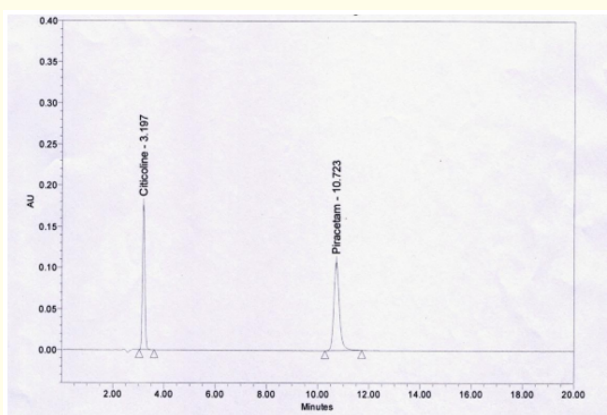


Figure 2: Representative optimized chromatogram of citicoline sodium and piracetam under the final chromatographic conditions.

Linearity

Calibration curves constructed by plotting peak area against concentration were linear over the ranges 25-75 µg/mL for citicoline sodium and 40-120 µg/mL for piracetam, with correlation coefficients of 0.9997 and 0.9996 and linear regression equations $y = 22248x - 20842$ and $y = 20098x - 41035$, respectively (Table 3, Figure 3).

Limit of detection and limit of quantitation

The LOD (S/N = 3.3:1) and LOQ (S/N = 10:1) were 0.06 µg/mL and 0.18 µg/mL for citicoline sodium, and 0.09 µg/mL and 0.26 µg/mL for piracetam, respectively, indicating that the method is sufficiently sensitive for the intended application.

Analyte	Concentration (µg/mL)	Peak area*	Regression equation
Citicoline sodium	25	539179	$y = 22248x - 20842$ $R^2 = 0.9997$
	37.5	817204	
	50	1081136	
	62.5	1364138	
	75	1656238	
Piracetam	40	769741	$y = 20098x - 41035$ $R^2 = 0.9996$
	60	1166923	
	80	1557189	
	100	1954091	
	120	2385915	

Table 3: Linearity data for citicoline sodium and piracetam.

*Mean of six replicate injections.

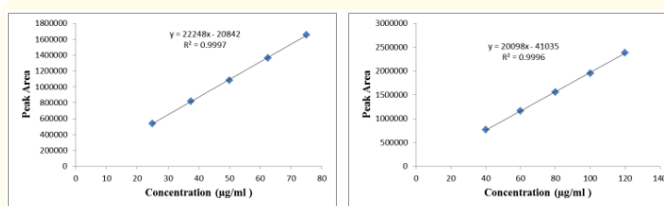


Figure 3: Calibration curves for citicoline sodium (left) and piracetam (right).

Accuracy

Recovery studies performed at 50%, 100% and 150% spiking levels gave mean percentage recoveries in the range of 99.01-99.64% for citicoline sodium and 98.92-99.18% for piracetam,

with %RSD values below 0.4% at all levels, confirming that the method was accurate (Table 4). The overall average recoveries were 99.52% for citicoline and 98.98% for piracetam.

Analyte	Spiking level (%)	Mean recovery (%)	%RSD
Citicoline sodium	50	99.64	0.12
	100	99.01	0.15
	150	99.09	0.13
Piracetam	50	99.18	0.20
	100	98.92	0.10
	150	99.11	0.39

Table 4: Recovery (accuracy) data for citicoline sodium and piracetam.

Precision

Injection repeatability (%RSD) values were 1.55 for citicoline sodium and 1.36 for piracetam. Intra-day and inter-day (two analysts) precision, evaluated at three concentration levels, gave

%RSD values within 2.0% for both analytes at all levels, confirming that the method is precise and reproducible (Table 5).

Analyte	Concentration (µg/mL)	Intra-day precision	Inter-day precision	Reproducibility (Analyst 1 / Analyst 2)
CTS	37.5	0.51	1.28	0.82 / 1.34
CTS	50	0.85	0.58	0.54 / 1.49
CTS	62.5	0.91	1.76	1.58 / 1.45
PIC	60	1.58	1.53	1.42 / 1.80
PIC	80	1.53	1.26	1.21 / 0.59
PIC	100	0.75	1.52	1.29 / 0.81

Table 5: Precision data (%RSD) of the proposed method.

Robustness

Deliberate small variations in flow rate (± 0.1 mL/min), detection wavelength (± 5 nm) and buffer pH (± 0.2 units) did not significantly affect peak area (%RSD $\leq 0.36\%$), tailing factor or theoretical plate

count for either analyte, and assay results remained close to the true value under all tested conditions, confirming that the method is robust (Table 6).

Variable	Level	CTS peak area %RSD	PIC peak area %RSD
Flow rate (mL/min)	0.9	0.11	0.12
	1.0 (original)	0.15	0.34
	1.1	0.11	0.19
Buffer pH	2.8	0.11	0.15
	3.0 (original)	0.15	0.34
	3.2	0.15	0.36

Wavelength (nm)	205	0.11	0.25
	210 (original)	0.15	0.34
	215	0.06	0.15

Table 6: Robustness data for citicoline sodium and piracetam (peak area %RSD).

Specificity

The peak-purity data confirmed that citicoline and piracetam peaks were homogeneous and free from co-eluting degradation products under acid, base, peroxide, thermal and UV-light stress

conditions, and no interference from tablet excipients was observed at the retention times of either analyte, confirming that the method is stability-indicating and specific (Table 7).

Condition	Citicoline recovered (mg)	Piracetam recovered (mg)
Control (unstressed)	53.72	80.64
Thermal (105 °C, 8 h)	53.37	80.50
Alkali (0.1 N NaOH)	53.68	80.53
Acid (0.1 N HCl)	53.55	80.56
Peroxide (3% H ₂ O ₂)	53.41	80.47
UV light (4 h)	53.28	80.42

Table 7: Specificity (forced degradation) data.

Assay

Application of the validated method to the marketed tablet formulation (label claim: 500 mg citicoline and 800 mg piracetam per tablet) gave a mean drug content of $99.78 \pm 0.62\%$ for citicoline and $99.23 \pm 0.74\%$ for piracetam ($n = 6$), with %RSD values of 0.62% and 0.75%, respectively, confirming the suitability of the method for routine quality-control assay (Table 8).

Label claim per tablet	% Drug found $\pm$ SD ($n = 6$)	%RSD
Citicoline - 500 mg	99.78 ± 0.62	0.62
Piracetam - 800 mg	99.23 ± 0.74	0.75

Table 8: Assay results for the marketed tablet formulation.

Discussion

The chromatographic behaviour observed during method optimization is consistent with the differing acid-base properties of the two analytes. Citicoline sodium ($pK_a \approx 4.4$) exists predominantly in a protonated, more polar form at pH 3.5, whereas piracetam, a neutral pyrrolidinone lactam with no ionizable group in this pH range, is unaffected by pH-driven changes in charge state. Operating the mobile phase at pH 3.5 — within about one pH unit of

the citicoline pK_a — provided sufficient control over the ionization equilibrium of citicoline to achieve a consistent, symmetrical peak shape (tailing factor 1.24) while leaving the retention behaviour of piracetam essentially governed by hydrophobic partitioning alone. This mechanistic interpretation is consistent with the empirical observation that shifting the buffer pH away from 3.5 in the exploratory trials (pH 3.0 and pH 4.5) reintroduced baseline humps and peak-purity failures, most likely reflecting a shift in the citicoline ionization equilibrium and consequent co-elution with a closely related species.

All eight validation parameters examined — system suitability, specificity, linearity, accuracy, precision, robustness, LOD/LOQ and assay — met the acceptance criteria recommended in the ICH Q2(R2) guideline [19-21], with correlation coefficients exceeding 0.999, recoveries within the 98-102% range specified for assay methods, and %RSD values below 2% throughout. The method's tolerance to small, deliberate variation in flow rate, detection wavelength and mobile-phase pH (peak-area %RSD $\leq 0.36\%$ in all cases) indicates adequate ruggedness for transfer between analysts, instruments and laboratories, a property that is particularly relevant given the ICH Q14 emphasis on lifecycle robustness as part of the analytical target profile [20]. The absence

of any co-eluting peak from tablet excipients or from degradation products generated under acid, base, peroxide, thermal and photolytic stress further substantiates the specificity and stability-indicating nature of the method, extending its applicability beyond routine assay to forced-degradation and shelf-life stability studies of the combined formulation.

Relative to previously reported HPLC and spectrophotometric methods for this drug combination [6-18], the present method uses a single, readily available aqueous buffer mobile phase without an organic modifier, which reduces solvent cost and environmental burden while retaining adequate resolution ($R_s = 9.42$) between citicoline (3.197 min) and piracetam (10.723 min) within a practicable total run time of 20 min, features that are advantageous for routine, high-throughput quality-control testing.

Limitations

This validation was performed in a single laboratory on a single instrument, and inter-laboratory reproducibility as defined by ICH (collaborative-study precision) was not assessed; intermediate precision was instead evaluated using two analysts within the same laboratory as a partial surrogate. Forced-degradation studies were limited to product formation/loss at fixed time points under the five standard ICH stress conditions, and full degradation kinetics and mass-balance closure were not established. These aspects, along with multi-laboratory validation, represent appropriate directions for future work.

Conclusion

A simple, selective, accurate, precise, robust and stability-indicating RP-HPLC method was developed and validated for the simultaneous estimation of citicoline and piracetam in bulk drug and tablet dosage form, in full accordance with the ICH Q2(R2) guideline. Mechanistically, resolution of the pair was governed principally by pH-dependent control of citicoline ionization at a mobile-phase pH close to its pK_a , while piracetam retention remained governed by hydrophobic partitioning. The method demonstrated linearity ($R^2 > 0.999$), accuracy (recovery 98-102%), precision (%RSD < 2%), robustness and specificity within the numerical limits recommended by ICH, and was successfully applied to the assay of a marketed tablet formulation. On this basis, the method is recommended for adoption in routine quality-control estimation of citicoline and piracetam in bulk drugs and

pharmaceutical dosage forms, and as a starting point for future stability-indicating and forced-degradation kinetic studies of the combination.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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