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Coupling $MgSO_4$-Assisted SALLE with a Fluorimetric Turn-Off Strategy for the Determination of Cinacalcet HCl in Pharmaceutical and Human Matrices

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Abstract

An innovative, sustainable analytical approach was developed by coupling an Epsom salt, E518 (magnesium sulfate)–facilitated salting-out assisted liquid–liquid extraction (SALLE) system with a safe molecular fluorescent probe, Celfia Pink B (CPB), employing a fluorescence turn-off sensing strategy. $MgSO_4$ acts as a green salting-out agent, enhancing phase separation efficiency and producing cleaner extracts with higher analyte recovery. CPB, a safe, food-grade dye, serves as a selective fluorogenic probe whose emission at 553 nm is quenched upon electrostatic ion-pair complex formation with Cinacalcet HCl under mildly acidic conditions. The method exhibited excellent linearity (0.08–1.3 µg/mL) with high sensitivity (LOD = 0.024 µg/mL; LOQ = 0.075 µg/mL). Validation according to ICH guidelines demonstrated robustness, precision, and accuracy across pharmaceutical formulations and biological matrices. Sustainability assessment using the WAC framework categorized the platform as "white," highlighting low environmental impact, safe operation, and simplicity. By minimizing organic solvent use and eliminating hazardous reagents, this $MgSO_4$-assisted SALLE/fluorescence turn-off approach provides a green, highly sensitive, and versatile tool for trace-level determination of Cinacalcet HCl in pharmaceutical and biological matrices.

Keywords

Epsom salt–facilitated SALLE; CPB molecular probe; fluorescence turn-off sensing strategy; Trace-level Cinacalcet HCl determination in pharmaceutical and biological matrices; sustainable chemistry and human Health considerations

1. Introduction

Magnesium sulfate (E518, commonly known as Epsom salt) is a highly versatile and safe compound with wide applications in food, pharmaceutical, and analytical sciences. In salting-out assisted liquid–liquid extraction (SALLE), it plays a dual role as an effective phase-separation agent and drying reagent, efficiently removing residual water from organic phases such as acetonitrile to ensure cleaner extracts and enhanced analyte recovery. Comparative studies have shown its superiority to sodium sulfate, making it especially valuable in trace-level pharmaceutical assays. As a non-toxic, registered food additive (E518) with high water solubility, environmental compatibility, and lack of bioaccumulation, magnesium sulfate represents a cost-effective and sustainable option that aligns with green and sustainable chemistry principles [6-1].

However, there is a continuous demand for more efficient, versatile, rapid, and environmentally friendly extraction systems in modern pharmaceutical and bioanalytical chemistry. Salting-out assisted liquid–liquid extraction (SALLE) has gained increasing popularity due to its simplicity, cost-effectiveness, and alignment with green chemistry principles. In SALLE, the addition of an appropriate salt induces phase separation between an aqueous sample and a water-miscible organic solvent, resulting in selective partitioning of target analytes into the organic phase [7]. The method is remarkably fast, safe, and economical, and the obtained extracts can be directly integrated into subsequent spectrofluorimetric measurements. Various organic solvents (acetonitrile, acetone, ethyl acetate, isopropanol) and salting-out agents (magnesium sulfate, ammonium sulfate, calcium chloride, potassium carbonate, calcium sulfate) have been successfully employed [8].

In the present work, magnesium sulfate (E518) was employed as the salting-out agent, providing efficient phase separation and enhanced extraction performance. To the best of our knowledge, this is the first attempt to couple SALLE with a fluorimetric "switch-off" strategy using Celfia Pink B (CPB), a safe food dye repurposed as a fluorescent probe for the determination of cinacalcet HCl. The quenching of CPB's intrinsic fluorescence occurs through electrostatic or ion-pair complexation with the target analyte, offering a highly selective and sensitive platform. Such a hybrid methodology not only extends the application of SALLE in pharmaceutical analysis but also establishes a novel, sustainable, and cross-disciplinary analytical approach for ultra-trace determination of the target analyte, cinacalcet HCl.

Cinacalcet (CCT.HCl, Fig. 1a), chemically designated as N-[1-(R)-(−)-(1-naphthyl)ethyl]-3-[3-(trifluoromethyl)phenyl]-1-aminopropane, functions as a calcimimetic moderator by selectively modulating calcium-sensing receptors in parathyroid tissues [9]. This mechanism enhances receptor sensitivity to extracellular calcium, effectively suppressing parathyroid hormone secretion [10, 11]. CCT.HCl is clinically approved for managing secondary hyperparathyroidism in chronic kidney syndrome patients undergoing dialysis [12] and hypercalcemia associated with diagnosed parathyroid carcinoma [13].

Fluorometric quantification of CCT.HCl has employed aromatic fluorogenic reagents such as 5-nitrobenzofuran derivatives [14, 15], 1,2,3-indantrione [16], aminonaphthalene analogs [17], fluorescamine [18], diformylbenzene [19], and *o*-phthalaldehyde/2-mercaptoethanol [20]. These reagents form stable, fluorescent covalent adducts upon reaction with primary amine groups of the analyte.

Prior methodologies for CCT.HCl quantification in pharmaceutical and biological matrices encompasses spectrophotometric [21-23], spectrofluorometric [24, 25], HPTLC [26], HPLC [27-29], and LC-MS [30, 31] techniques. However, these approaches have been associated with operational limitations, including reliance on sophisticated instrumentation, labor-intensive protocols, and high costs.

To date, few spectrofluorometric derivatization strategies have been reported for CCT.HCl analysis [24]. While demonstrating superior sensitivity to the current method, this methodology demands a harsh derivatization procedure with 4-chloro-7-nitro-1,2,3-benzoxadiazole, involving 20-minute heating at 60°C followed by acidic quenching with HCl. The procedure is complicated by the requirement to control multiple variables, such as NBD-Cl reagent concentration, pH, buffer composition, temperature, reaction duration, cooling conditions, HCl strength, and solvent effects. Furthermore, NBD-Cl degradation under heating releases corrosive vapors, posing risks of respiratory and dermal irritation. In contrast, the proposed methodology eliminates hazardous reagents, streamlines workflow through a single-step reaction, and aligns with green chemistry principles while retaining sufficient sensitivity to quantify CCT.HCl within therapeutic plasma concentrations.

The predominant methodologies for cinacalcet (CCT.HCl) analysis have historically depended on high-performance liquid

chromatography (HPLC), which is often constrained by prolonged analysis durations, substantial consumption of high-purity organic solvents during sample purification, elevated operational costs, and adverse environmental implications [32-35]. While spectrophotometric techniques offer simplicity, their limited sensitivity restricts their utility in biological fluid analysis. In contrast, spectrofluorimetry emerges as a superior alternative owing to its rapidity, cost efficiency, operational simplicity, minimal sample preparation, and inherent methodological straightforwardness.

celfia pink B (CPB, Fig. 1b), a disodium salt of 2,4,5,7-tetraiodofluorescein, functions as both a biological stain and an ion-association reagent, enabling its application as a fluorogenic probe. The protonatable amino group within CCT.HCl facilitates electrostatic interaction with CPB's anionic moieties under acidic conditions, forming a stable ion-paired complex. Although alternative dyes such as merbromin and Rhodamine 6G have been utilized in spectrofluorimetric assays, their applicability is limited: merbromin's mercury content raises toxicity worries, while Rhodamine 6G exhibits structural incompatibility with CCT.HCl, hindering effective electrostatic interactions.

CPB was selected for its exceptional sensitivity, robust negative charge (promoting stable ion-pair formation with cationic analytes), aqueous solubility, cost-effectiveness, and alignment with green chemistry ethics. These attributes render it ideal for quantifying amine-containing pharmaceuticals, including antidepressants, antihypertensives, and anticancer agents [36, 37], without necessitating hazardous reagents or derivatization.

Prior fluorometric method [24] for CCT.HCl quantification relied on a harsh derivatization procedure involving heating and using hydrochloric acid, a corrosive and hazardous medium, underscoring the novelty of the current approach, which prioritizes safety and sustainability. This study introduces an innovative, eco-friendly spectrofluorimetric strategy for CCT.HCl determination in bulk materials and dosage forms, validated through comprehensive green metrics. The method's simplicity further permits its adaptation to content uniformity testing in tablet formulations, ensuring batch-to-batch consistency.

By leveraging CPB's intrinsic fluorescence quenching upon interaction with CCT.HCl in a mildly acidic aqueous buffer, this methodology achieves high sensitivity (detection limits surpassing conventional techniques), accuracy, and operational safety. The elimination of toxic solvents and complex instrumentation positions this approach as a paradigm shift in pharmaceutical analysis, combining analytical rigor with environmental stewardship.

Analytical methods for trace-level quantification of pharmaceuticals in complex matrices demand high sensitivity, selectivity, and minimal environmental impact.

Conventional chromatographic techniques (e.g., HPLC, GC–MS) demonstrate robust performance but typically involve expensive instrumentation, extensive purification protocols, and substantial organic solvent volumes.

Salting-out assisted liquid–liquid extraction (SALLE) has emerged as a facile and efficient sample-cleanup technique in pharmaceutical analysis, where an inorganic salt—such as magnesium sulfate—induces rapid phase separation of a water-miscible organic solvent from an aqueous matrix, enriching the target analyte while reducing matrix interferences [38, 39]. This approach offers significant advantages over conventional liquid–liquid extraction, including reduced consumption of hazardous organic solvents and simplified operational procedures. Fluorescence "switch-off" tagging strategies complement SALLE by converting target drugs into non-fluorescent ion-pair complexes with food-grade dyes (e.g., Celfia Pink B), enabling ultra-trace detection via quenching measurements at specific wavelengths with exceptional sensitivity.

The present work introduces a novel hybrid analytical platform for the ultra-trace quantification of cinacalcet HCl, a clinically significant calcimimetic agent used in the management of parathyroid disorders. The novelty lies in the synergistic integration of magnesium sulfate-based SALLE with a fluorescence quenching detection strategy, providing both efficient sample clean-up and highly selective signal transduction. Unlike conventional extraction methods, magnesium sulfate ensures superior phase separation, enhanced recovery of cinacalcet, and minimal interference from matrix components, representing a greener and safer alternative that aligns with sustainable analytical chemistry principles.

The second innovative dimension is the strategic repurposing of Celfia Pink B (CPB), a food-grade dye, as a selective fluorescent probe. While CPB is typically recognized as a food colorant, its intrinsic

fluorescence properties are exploited here for sensitive detection of cinacalcet through electrostatic or ion-pair complex formation. The resulting fluorescence quenching ("switch-off" signal) offers a unique analytical response with high sensitivity and selectivity. This dual-function platform combines the sample purification advantages of SALLE with the analytical precision of fluorescence quenching, utilizing safe reagents ($MgSO_4$ and CPB), reduced solvent consumption, and an eco-friendly workflow as a sustainable, cost-effective, and innovative tool for pharmaceutical quality control and clinical sample analysis.

2. Experimental Section

2.1. Instrumentation

Fluorimetric measurements were conducted using an FS-2-SCINCO spectrofluorometer (Korea) fitted with a xenon arc lamp (150 W). The spectra of emission and excitation were recorded at a scan rate of 571 nm/min. Sample sonication was performed using an SC-101TH SONICOR ultrasonic bath (USA) to enhance solubility. pH adjustments were carried out with an AD11P pH measuring equipment (ADwa, Romania).

2.2. Materials and Reagents

Cinacalcet (CCT.HCl) reference standard was generously provided by the Egyptian Group for Pharmaceutical Industries (EGPI). Cinacalcet® F.C. Tablets (30, 60, and 90 mg/tab) were procured from local pharmacies. Celfia pink B (CPB; 2,4,5,7-tetraiodofluorescein disodium salt, 4.34×10^{-2} mM) was prepared by dissolving 15 mg in 50 mL deionized water. Analytical-grade reagents—phosphoric acid, sodium hydroxide, hydrochloric acid, anhydrous magnesium sulphate, acetic acid, methanol, ethanol, acetonitrile, dimethylformamide (DMF), and dimethyl sulfoxide (DMSO)—were sourced from El-Naser Co. (Egypt). Buffers (Britton-Robinson [40], Teorell-Stenhagen [41, 42], McIlvaine [43], acetate [44]) were employed for pH optimization.

2.3. Standard Solution Preparation

A primary CCT.HCl stock solution (100.0 µg mL^{-1}) was prepared by dissolving 10.0 mg CCT.HCl in distilled water and diluting to 100 mL. Working standards (80–1300 ng mL^{-1}) were derived via serial dilution. Anhydrous magnesium sulphate (2.0 M) solution was prepared by dissolving about 24.05 g dissolved in 100 mL water. Solutions were stored at 4°C to ensure stability.

2.4. Calibration Protocol

Aliquots of CCT.HCl working standards (800–13000 ng mL^{-1}) were taken into 10 mL standard volumetric flasks. Teorell-Stenhagen (1.80 mL, pH 4.2) and CPB solution (1.4 mL, 3.41×10^{2} µM) were added, followed by dilution to volume with deionized water. Fluorescence production intensity (λ_{ex} = 526 nm; λ_{em} = 553 nm) was measured. A calibration graph was created by plotting Δ_{RFI} ($F_o - F$) against CCT.HCl concentration, where F_o and F represent the fluorescence intensities of free CPB and the CCT.HCl -CPB complex, respectively.

2.5. Analytical Applications

2.5.1. Pharmaceutical applications

2.5.1.1. Tablet Assay

Ten Cinacalcet® tablets (90 and 60 mg) were pulverized. A portion equivalent to 10 mg CCT.HCl was sonicated in water, filtered, and diluted. CCT.HCl content was quantified using the calibration curve, with triplicate measurements per concentration.

2.5.1.2. Content Uniformity Testing

Compliance with USP guidelines [45] was verified by individually analyzing ten Cinacalcet® tablets (30 mg). Each tablet was processed as per Section 2.5.1 and CCT.HCl content was assessed against label claims.

2.5.2. Biological Sample Investigation

2.5.2.1. Plasma Sample Preparation

Ethical approval for human plasma collection was secured from the institutional review board (Al-Azhar University blood store, Assiut branch), and informed consent was obtained from participants prior to sample acquisition. Venous blood (5.0 mL) was drawn from a fasting healthy volunteer into heparinized tubes to prevent coagulation. Plasma separation was achieved via centrifugation at 5000 rpm for 5 minutes. Aliquots of the supernatant were spiked with known CCT.HCL concentrations and subjected to Salting-out $MgSO_4$-assisted liquid-liquid extraction (SALLE) using acetonitrile for plasma purification and analyte isolation.

This involved sequential addition of 500 µL plasma, 1000 µL acetonitrile (ACN), and 250 µL saturated $MgSO_4$ solution (2.0 M) to the sample in 10 mL working volumetric flask. The mixture underwent vortex-mixing (5 minutes) followed by centrifugation (5000 rpm, 5 minutes). The cinacalcet HCl-enriched acetonitrile layer was retrieved and reconstituted in working flasks for fluorimetric analysis. The validated methodology was subsequently applied to pretreated plasma specimens. A blank measurement was acquired by executing identical processing on plasma matrices lacking cinacalcet.

2.5.2.2. Urine Sample Preparation

Fresh urine specimens were collected from a fasting healthy donor and filtered twice over a 0.45 µm membrane to retain particulate

matter. Filtrate aliquots were conveyed to 10 mL standard flasks and analyzed by the same spectrofluorimetric protocol applied to plasma. Method specificity was confirmed by comparing spiked urine samples with unspiked controls.

3. Results and Discussion

3.1. Spectral Characteristics of the CCT.HCl-CPB Complex System

Amino-containing analytes, such as cinacalcet (CCTs.HCL), can be quantified through spectrofluorometric analysis by forming binary ion-pair complexes with acidic fluorophores like (CPB). In this study, electrostatic interactions between the protonated amino group of CCT.HCl and the anionic species of CPB (HL^-) facilitated complex generation in the Teorell-Stenhagen buffer (pH 4.2). This interaction induced structural modifications in CPB's conjugated π-electron system, redistributing electron density and leading to fluorescence quenching. The resultant "turn-off" fluorescence mechanism enabled precise CCT.HCl detection, as evidenced by the linear reduction in CPB's intrinsic emission intensity at 553 nm (λ_{ex} = 526 nm) with increasing analyte concentration (**Fig. 2**).

3.2. Mechanistic Insights into Complex Formation

3.2.1. pH-Dependent Ionization of CPB

The ionization state of CPB, critically influenced by pH, governs its interaction with CCT.HCl. With dissociation constants (pKa1 = 3.9 and pKa2 = 5.0 [46]), CPB predominantly exists as the monovalent anion at pH 4.2, as confirmed by maximal fluorescence quenching within the pH range of 3.8–4.6. Iodine substituents in the xanthene structure reduced electron density at the hydroxyl group, enhancing its reactivity relative to the carboxyl group [37, 47]. Quantum chemical computations (AM1 method) revealed hydroxyl ionization released 120.3 kJ/mol more energy (−285.8 kJ/mol) than carboxyl dissociation (−165.5 kJ/mol), favoring HL^- stability [48].

3.2.2. Protonation Behavior of CCT.HCl

CCT.HCl compound's chemical structure contains only one nitrogen atom, and exhibits sufficient basicity for protonation under acidic conditions. Protonation generated a cationic species ($CCT.HClH^+$), which interacted electrostatically with CPB^- to form a stable 1:1 ion-pair complex (**Fig. 3**). Hydrophobic interactions further stabilized the complex, underscoring its analytical robustness.

3.3. Mechanistic Analysis of Fluorescence Quenching

The fluorescence attenuation of CPB upon interaction with cinacalcet (CCT.HCl) was investigated to elucidate the underlying quenching

mechanism. Potential pathways, including dynamic collision, energy transfer, and static complexation, were evaluated using the Stern-Volmer relationship:

$$f_o/f = 1 + k_{sv}[Q] \quad \text{(Equation 1)}$$

Here, F_o and F denote the fluorescence powers of CPB in the absence and presence of CCT.HCl (quencher, [Q]), respectively, while K_{SV} represents the Stern-Volmer constant. A linear correlation between F_o/F and [Q] (See Supplementary Materials**, Fig. S1a**) confirmed quenching dependence on CCT.HCl concentration. The Stern-Volmer constant (K_{sv}) as calculated from the plot was 775.76 M^{-1}. The bimolecular quenching constant (K_q) was derived using $K_{SV} = K_q\ \tau_0$, where τ_o (CPB's intrinsic fluorescence lifetime (τ_0) = 89 ps [49]) yielding $K_q = 8.72 \times 10^{12}\ M^{-1}\ s^{-1}$. This value significantly exceeds both the theoretical maximum for dynamic quenching ($2 \times 10^{10}\ M^{-1}\ S^{-1}$) [50] and thediffusion-controlled limit ($\sim 10^{10}\ M^{-1}\ s^{-1}$), conclusively strongly supporting a static quenching mechanism in which a stable ground-state ion-pair complex forms between CPB and cinacalcet HCl, rather than quenching occurring solely through dynamic collisions.

3.4. Thermodynamic and Binding Affinity Studies

The binding stoichiometry (n) and association constant (K_a) were determined via the modified Stern-Volmer equation (double-log Stern–Volmer plot) [51]:

$$\text{Log}(f_o - f)/f = n\ \log[Q] + \log k_a \quad \text{(Equation 2)}$$

In applying the modified Stern–Volmer (double-log) plot for erythrosine B fluorescence quenching by cinacalcet HCl, the linear equation obtained was (**Fig. S1b**):

$$\log\frac{(f_o - f)}{f} = 1.0889\ \log[Q] + 6.4072$$From this, the slope (n) is 1.0889, which signifies approximately one binding site per CPB molecule, indicative of a 1:1 stoichiometry, consistent with Job's method. The intercept, representing $\log K_a$, is 6.4072, which corresponds to a binding constant $K_a \approx 2.55 \times 10^6\ M^{-1}$, reflecting strong affinity between the dye and cinacalcet HCl.

These findings support the development of a static quenching mechanism, where a stable non-fluorescent ion-pair complex forms in the ground state. The value of n ≈ 1 confirms one site involved in

complexation, and the high K_a highlights strong binding. Collectively, this confirms that cinacalcet HCl effectively quenches CPB via complex formation, validating the use of this binding model in the analytical platform.

3.5. Optimization of Methodological Parameters

The experimental variables influencing the fluorescence quenching efficiency of the CCT.HCl-CPB system were systematically investigated to refine the spectrofluorimetric protocol. Each parameter was meticulously adjusted to maximize analytical performance while adhering to sustainability principles.

3.5.1. Influence of pH

The pH of the system medium was evaluated using Teorell-Stenhagen buffer across a 2.2–6.5 range. Optimal fluorescence attenuation was observed at pH 4.2 (**Fig. 4**), where CCT.HCl undergoes protonation to form a cationic species ($HCCT.HCl^{+}$), while CPB predominantly exists in its monovalent anionic state (CPB^{-}). This pH facilitates robust electrostatic interactions and hydrophobic stabilization between the drug and dye, promoting the construction of a stable electrostatic-based complex. Deviations from this range resulted in reduced quenching efficiency due to suboptimal ionization states of the reactants.

3.5.2. Selection of Buffer System

Four buffer systems—Britton-Robinson, McIlvaine, Toerell-Stenhagen, and acetate—were compared at a fixed volume (1.8 mL). The Teorell-Stenhagen buffer demonstrated superior performance (**Fig. 4**), attributed to its broad buffering capacity and compatibility with the reaction milieu, ensuring stable complex formation and maximal signal attenuation.

3.5.3. Effect of CPB Volume

Volumes of CPB reagent ranging from 0.1 to 2.8 mL were tested to fix the optimal quantity for maximal fluorescence suppression. A volume of 1.4 mL (**Fig. S2**) was identified as ideal, yielding consistent quenching without signal saturation. Volumes below 1.2 mL provided insufficient reagent for complete complexation, while quantities exceeding 1.8 mL induced self-quenching due to dye aggregation, diminishing the analytical response.

3.5.4. Buffer Volume Optimization

The Teorell-Stenhagen buffer volume (in a 0.2–3.0 mL range) impact on signal intensity was assessed. A volume of 1.8 mL was found to balance pH stabilization and minimize competitive anion interference (e.g., phosphate). Excessive buffer (>2.4 mL) introduced competing

ions that disrupted CPB-CCT.HCl interactions, whereas insufficient volumes (<1.2 mL) failed to maintain consistent pH, leading to variability in quenching efficiency (**Fig. S2**).

3.5.5. Solvent Compatibility Assessment

Aqueous and organic solvents (ethanol, acetonitrile, methanol, and acetone) were evaluated as dispersion media. Aqueous solutions achieved maximal quenching due to water's high polarity of value = 9.0, dielectric constant = 80.2) [52] and hydrogen-bonding capacity, which stabilized the excited-state orientation of the complex (**Fig. S3**). Organic solvents, particularly short-chain alcohols, disrupted hydrophobic interactions and complex assembly at raised concentrations [53]. Water's dual role as a hydrogen bond donor and acceptor further enhanced proton transfer, ensuring amino group protonation and complex stability [54]. Additionally, the elevated polarity of aqueous systems suppressed π-π* electronic transitions while promoting n-π* interactions, stabilizing the excited state, and amplifying the quenching effect [55, 56].

3.5.6. Temporal Stability of the Complex

The CCT.HCl-CPB complex exhibited rapid formation at ambient temperature, with signal stabilization achieved within 4 minutes post-reaction (**Fig. 4**). Prolonged incubation beyond this period showed no significant variation, confirming the method's suitability for high-throughput analysis.

3.5.7. Stoichiometric Determination via Job's Method

Job's continuous variation analysis [57] was used to establish the binding ratio between CPB and CCT.HCl. Fluorescence intensities were measured for solutions containing fixed total molar concentrations of CPB and CCT.HCl, with mole fractions varying between 0.1 and 0.9. Corrected fluorescence values ($\Delta F = F_0 - F$) were plotted counter to the drug's mole fraction, yielding a peak value at 0.5 (**Fig. S4**), confirming a 1:1 stoichiometry for the CPB:CCT.HCl complex. This finding aligned with prior mechanistic and thermodynamic analyses.

3.5.8. Application of Scatchard model for Binding Site(s) and association affinity Estimations

The Scatchard model (**Fig. 5**) is a graphical technique used in analytical chemistry to study binding interactions between molecules,

such as dyes and drugs, by determining the binding affinity constant (K) and the number of binding sites (n) [58].

It applies the equation: $r/[L] = nKa - rKa$

Here, (r) is the number of bound ligands, ($[L]$) is the concentration of free ligands, (n) is the number of binding sites, and (Ka) is the association constant [59].

In the investigation of the interaction between CPB dye and Cinacalcet, Scatchard plot analysis yielded a linear relationship. The slope represents $-K_a$, thus $K_a = 7.58 \times 10^5$ M^{-1}, reflecting a very strong binding affinity. The y-intercept represents nKa, yielding n ≈ 0.94 (approximately 1), which confirms that each molecule of the dye binds to one molecule of the drug in a stable 1:1 stoichiometric interaction [60, 61]. This strong binding constant (K_a) indicates a robust interaction between CPB and Cinacalcet HCl, reducing the amount of free dye available and altering its fluorescence properties through ground-state complex formation. This phenomenon supports the system's efficiency as a fluorescence quenching platform and can be leveraged to develop precise analytical methods for drug detection or to study interaction mechanisms [61].

3.6. Validation of the Developed Approach

Following comprehensive optimization of all experimental variables, the proposed spectrofluorimetric method was rigorously validated in compliance with International Council for Harmonization (ICH) strategies [62]. Critical validation considerations, including linearity, sensitivity, accuracy, precision, robustness, selectivity, and applicability to pharmaceutical formulations, were systematically evaluated.

3.6.1. Linearity and Range

The linear relationship between CCT.HCl concentration and fluorescence quenching response (ΔRFI) was established under standardized experimental conditions. Serial dilutions of CCT.HCl standard solutions spanning 80–1300 ng mL^{-1} were analyzed, with ΔRFI values demonstrating proportional attenuation relative to analyte concentration. Regression analysis yielded a correlation coefficient (r) approaching unity (**Table 1**), confirming exceptional linearity across the specified range.

3.6.2. Sensitivity Limits (LOD and LOQ)

Method sensitivity was quantified via statistical determination of the limit of detection (LOD) and limit of quantification (LOQ). These parameters were derived via the formulas LOD = 3.3σ/S and LOQ = 10σ/S, where σ symbolizes the standard deviation of the intercept and S signifies the calibration curve slope. Calculated values of 24.8 ng mL^{-1} (LOD) and 75.2 ng mL^{-1} (LOQ) (**Table 1**) underscored the method's capability for trace-level CCT.HCl detection.

3.6.3. Accuracy Assessment

Accuracy was assessed through recovery readings at four CCT.HCl concentrations (200, 700, 1000, 1200 ng mL^{-1}), with triplicate measurements per level. Percent recoveries ranged from 99.78% to 101.98%, accompanied by %RSD (relative standard deviation) values below 2.0% (**Table 2**). The proximity of recoveries to 100% validated the method's accuracy in quantifying CCT.HCl across its linear range.

3.6.4. Precision Evaluation

Intra-day and inter-day precision were assessed to determine method reproducibility. Intra-day precision was evaluated via triplicate analyses of three CCT.HCl concentrations (300, 700, 1000 ng mL^{-1}) within a single analytical run. Inter-day precision involved replicate measurements across three consecutive days. %RSD values remained below 1.75% for both precision tiers (**Table 2**), confirming minimal variability under repeatability and intermediate precision conditions.

3.6.5. Robustness Testing

Method resilience was examined by introducing minor modifications to three critical parameters: buffer pH (±0.2), buffer solution volume (±0.2 mL), and CPB solution volume (±0.2 mL). Recovery percentages (99.91–101.93%) and %RSD values (<1.85%) remained consistent across all variations (**Table 3**), demonstrating insensitivity to controlled procedural deviations.

3.6.6. Selectivity and Interference Analysis

Selectivity was verified by analyzing CCT.HCl in the presence of common pharmaceutical excipients and structurally analogous compounds. Recovery rates exceeding 98% with %RSD <2% (**Table S1**) confirmed negligible matrix interference. The absence of reactive aliphatic amine groups in excipients prevented competitive complexation with CPB, ensuring specificity for CCT.HCl.

3.7. Applications of the approach

3.7.1. Pharmaceutical Application

The validated approach was used to quantify CCT.HCl in Cinacalcet® tablets (60 and 90 mg/tablet). Results were statistically compared with a reference method [24] using Student's t-test and F-test. Calculated t- and F-values fell below critical thresholds (2.78 and 6.39 at 95% confidence), confirming equivalence in accuracy and precision (**Table 4**).

3.7.2. Content Uniformity Evaluation

Content uniformity testing was performed on ten Cinacalcet® tablets (30 mg) per USP criteria [63]. The AV, acceptance value, calculated as $AV = |R - \bar{A}| + kS$ (where k = 2.4, S = standard deviation, R = reference value, $\bar{A}$ = mean content), yielded values below the maximum allowable limit (L1 = 15) (**Table 5**). This confirmed homogeneous drug distribution within the tested batch.

3.7.3. Application to Spiked Human Plasma and Urine Specimens

Salting-out assisted liquid–liquid extraction (SALLE)—a streamlined sample preparation approach—employs water-miscible organic solvents (e.g., acetonitrile) and salting-out agents (e.g., MgSO4) to induce phase separation. This technique yields purified extracts while diminishing matrix interference relative to classical liquid–liquid extraction or protein precipitation methods [38, 39].

The enhanced sensitivity of the developed methodology enabled its successful implementation for quantifying CCT.HCl spiked in biological matrices (human plasma and urine) within the validated calibration range. For plasma samples fortified with CCT.HCl, an average recovery of 99.60-101.98% ± 1.43% was achieved (**Table 6**), demonstrating high accuracy and minimal matrix interference. Similarly, urine samples spiked with CCT.HCl yielded a mean recovery of 99.38-101.86% ± 1.76%, confirming the method's reliability and suitability for biofluid analysis.

4. Sustainability Evaluation of the Method

An environmental sustainability assessment is characterized by minimal or zero utilization of hazardous reagents, complete waste

mitigation, and low energy demands. The ecological compatibility of the planned spectrofluorometric technique was systematically examined through multiple modern green chemistry metrics.

Analytical professionals in chemical and pharmaceutical disciplines bear significant responsibility for safeguarding human and environmental health from harmful substances [16, 19, 64-69]. Continuous emphasis on advancements in green chemistry principles remains essential. The current spectrofluorimetric methodology was rigorously evaluated using Green Analytical Chemistry (GAC) rating criteria.

As environmentally conscious methodologies gain prominence, diverse qualitative and quantitative metrics have been established by researchers to assess compliance with GAC standards [70-72]. Notably, theoretical indicators of ecological sustainability have been widely implemented. These include the Eco-Scale [73], AGREE [74], GAPI [75], NEMI [76], the AGREEprep tool for material preparation [77], the RGB12 platform, and the BAGI tool (**Table S2**).

4.1. Analytical Eco-Scale Assessment

The Eco-Scale quantifies ecological impacts by measuring penalty scores built on chemical quantities, occupational hazards, waste output, and energy consumption. The total penalty points are subtracted from 100 to yield the Eco-Scale score [73]. Methods scoring above 75 qualify as sustainable. This fluorometric method achieved a score of 95 (compared to 74for conventional methods), confirming its environmental safety (**Table S2**).

4.2. National Environmental Methods Index (NEMI)

The NEMI framework was employed to evaluate the procedural sustainability [76]. This metric evaluates solvent persistence, bioaccumulation potential, and toxicity. Methanol, employed in this spectrofluorometric procedure, is classified as a non-PBT (persistent, bioaccumulative, toxic) solvent. The Teorell-Stenhagen buffer system (pH 4.2) used in the procedure was non-caustic, and overall waste generation was kept less than 50 mL. Findings confirmed minimal solvent consumption and waste production, fulfilling all four NEMI criteria (**Table S2**), thereby designating the method as eco-friendly.

4.3. Application of the complexGAPI Tool

In analytical chemistry, the complexGreen Analytical Procedure Index (complexGAPI) is a widely used tool for evaluating the environmental impact of analytical techniques based on green chemistry principles. The complex GAPI, an advanced version, provides a detailed assessment by breaking down the evaluation into multiple criteria, often represented visually as a pentagonal or hexagonal diagram with color-coded sections. Each color—green (little impact), yellow (reasonable impact), red (great impact), and white (unevaluated)—indicates the level of environmental friendliness for specific criteria, such as the usage of renewable resources, energy efficiency, waste generation, and toxicity of reagents [78, 79].

The analysis involved examining the color distribution in each image to assess the green chemistry criteria. The complex GAPI tool typically evaluates 15 sub-indicators grouped into categories like sample treatment, reagent use, energy consumption, and waste management [75]. Colors are interpreted as follows: green for little impact, yellow for reasonable impact, red for great impact, and white for unevaluated criteria. The images were compared based on the percentage of each color to determine which method is greener [69, 79-81].

The GAPI pictogram dissects the analytical lifecycle into 15 stages, color-coded as follows:

- **Green (10 stages)**: Sample preparation (aqueous dilution), detection (non-destructive fluorescence), and waste management (low-volume disposal).
- **Yellow (4 stages)**: Offline analysis (manual data collection) and derivatization (CBP complexation).
- **Red (1 stage)**: Limited automation and use of organic solvents (It's the small amount of acetonitrile in SALLE for biological samples). The predominance of green zones (**Table S2**) underscored the method's alignment with GAC principles.

4.4. AGREE Framework Analysis

The AGREE metric, a software-driven GAC tool, was implemented to assess the methodology's sustainability [74]. Twelve GAC-aligned criteria are graded on a 0.0 (red) to 1.0 (green) scale, generating a radial chart with a central color indicator (aggregate score: 0.78) [82-84]. Key evaluations included:

Criterion 1 (Sample Handling): Yellow (offline analysis requirement).

Criterion 2 (Sample Volume): Light green (minimal consumption).

Criterion 3 (In Situ Analysis): Red (offline instrumentation).

Criterion 4 (Preparation Steps): Green (simplified workflow).

Criterion 5 (Automation): Yellow (manual operation).

Criterion 6 (Derivatization): Light green.

Criterion 7 (Waste): Green (low volume).

Criterion 8 (Throughput): Green (high hourly analyte capacity).

Criterion 9 (Energy): Green (low-energy detection).

Criterion 10 (Renewables): Green (water-based solvents).

Criterion 11 (Solvent Safety): Green (low toxicity).

Criterion 12 (Operator Safety): Green (safe practices).

The high summative score (0.78) reflects distilled water implementation, reducing processing time and ecological burden (**Table S2**).

4.5. AGREEprep Tool Implementation

AGREEprep evaluates the sustainability of the sample preparation via a circular diagram with 10 segmented areas signifying Green Sample Preparation (GSP) principles [85]. The proposed method scored 0.80, attributed to streamlined workflows, elimination of thermal cycling steps (reducing energy use), and enhanced safety via aqueous solvents (Table 6).

The following analysis details how the method aligns with each principle, supported by the AGREEprep

Here’s a concise summary of how the CCT.HCl spectrofluorimetric method aligns with the 10 Green Sample Preparation (GSP) principles:

1. **Favor in situ sample preparation**
   - Lab-based, not in situ (yellow AGREE rating).
   - *Not met.*
2. **Use safer solvents and reagents**
   - 1 mL methanol (minimal, not PBT), 9 mL water, safe CPB.

- *Met.*

3. **Target sustainable, renewable, reusable materials**
   - Water is renewable, no organic solvents, and low waste (<50 mL).
   - *Met*
4. **Minimize waste**
   - <50 mL waste (green AGREE score).
   - *Fully met.*
5. **Minimize sample, chemical, and material amounts**
   - Small volumes (0 mL organic solvent, 10 mL water).
   - *Met.*
6. **Maximize sample throughput**
   - High hourly rates (green AGREE rating).
   - *Fully met.*
7. **Integrate steps, promote automation**
   - Manual, not automated (yellow AGREE score).
   - *Not fully met.*
8. **Minimize energy consumption**
   - Ambient temperature, no heating/cooling (green AGREE rating).
   - *Fully met.*
9. **Select the greenest post-sample preparation**
   - Low-energy spectrofluorimetry.
   - *Met.*
10. **Confirm safe procedures for the worker**
    - Safe solvents/reagents (green AGREE rating).
    - *Fully met.*

Therefore, the planned CCT.HCl method excels in safety, waste reduction, and energy efficiency (AGREEprep 0.80, Eco-Scale 95), but falls short in in situ preparation and automation, suggesting areas for future enhancement (**Table S2**).

4.6. Green Solvent Selection Tool (GSST)

GSST identifies sustainable solvents using Hansen Solubility Parameters (HSP) to assess dispersion, polarity, and hydrogen bonding interactions [86, 87]. Solvents are graded (G-score: 1–10), with higher values indicating sustainability. Methanol (5.8) and water (7.3) in the method outperformed prior spectrophotometric solvents (Figure S2). GSST's rapid online evaluation facilitates solvent comparisons [88].

GSST prioritized solvents with optimal Hansen Solubility Parameters (HSPs):

- **Dispersion Forces (δD)**: Water (15.5 $MPa^{1/2}$), methanol (15.1 $MPa^{1/2}$).
- **Polarity (δP)**: Water (16.0 $MPa^{1/2}$), methanol (12.3 $MPa^{1/2}$).
- **Hydrogen Bonding (δH)**: Water (42.3 $MPa^{1/2}$), methanol (22.3 $MPa^{1/2}$).
  High G-scores for water (7.3) and methanol (5.8) confirmed their environmental suitability.

4.7. Whiteness Evaluation via RGB12

The RGB12 framework integrates red (analytical efficiency), green (ecological compatibility), and blue (economic feasibility) metrics to compute a whiteness score [89]. The method demonstrated:

Red (Performance): Broad linear range (0.08–1.3 µg/mL), low LOD (24.8 ng/mL), precision (RSD <1.75%), accuracy (99.78–101.98%).

Green (Ecology): Water-dominated reagents, no organic solvent, low energy, Eco-Scale 95.

Blue (Economics): Cost-effective, simple, solvent-efficient.

The composite whiteness score (**Table S3**) confirmed superior sustainability, efficiency, and practicality.

4.8. Blueness Assessment via BAGI

The Blue Applicability Grading Index (BAGI) is a relatively new tool designed to grade the applicability of analytical methods in practical settings (25–100 scale). Research suggests it provides a scoring system to evaluate how well a method can be implemented, considering aspects like safety, cost-effectiveness, and operational simplicity [90, 91]. It seems likely that BAGI uses a color-coded or

numerical scoring system, with higher scores indicating better applicability, making it useful for quick assessments in laboratories. The evidence leans toward BAGI being particularly valuable in resource-limited environments, where cost and safety are critical, but it may lack depth in environmental impact assessment compared to other tools. [92]

BAGI focuses on applicability, with criteria likely including safety (e.g., handling hazards), cost (e.g., reagent and equipment expenses), and ease of use (e.g., training requirements). These criteria indirectly relate to green chemistry, as lower costs and safer methods often align with reduced environmental impact, but BAGI does not explicitly evaluate detailed environmental factors like waste or energy use. The method scored 72.5, reflecting high applicability and ease of comparative analysis (**Table S2**).

5. Conclusion

A green hybrid analytical platform combining $MgSO_4$-SALLE with "switch-off" fluorescence detection has been successfully developed for the ultra-trace quantification of Cinacalcet HCl in pharmaceutical and biological matrices. The method relies on charge-mediated ion-pair formation between Cinacalcet HCl and Celfia Pink B (CPB), a food-grade fluorescent dye, in a weakly acidic environment, with quantification achieved through spectrofluorimetric monitoring of CPB fluorescence quenching. Excellent analytical performance was demonstrated, including linearity over 80 – 1300 ng mL^{-1}, with detection and quantification limits of 24.8 ng mL^{-1} and 75.2 ng mL^{-1}, respectively. The method was successfully applied to tablet homogeneity assessments and dosage-form analysis, confirming its practical utility in pharmaceutical quality control.

Key advantages of this approach include simplified implementation through aqueous-based sample preparation, direct analysis without chromatographic separation, elimination of volatile organic solvents, and reduced procedural complexity, resulting in faster analysis and enhanced cost efficiency. The methodology's environmental sustainability was rigorously evaluated using multiple contemporary metrics, yielding an exceptional holistic sustainability profile across integrated greenness, whiteness, and blueness frameworks.

Beyond immediate analytical performance, this work presents several broader implications for pharmaceutical analysis and green analytical chemistry. The hybrid SALLE-fluorescence platform represents a versatile framework that can be extended to other basic or cationic drugs capable of forming ion-pair complexes with anionic fluorescent dyes. The successful use of food-grade CPB as a fluorescent probe establishes a precedent for developing safe, sustainable sensor systems applicable across pharmaceutical quality control, bioanalytical testing, and environmental monitoring. By demonstrating that high sensitivity and selectivity need not compromise environmental responsibility, this methodology contributes to advancing eco-conscious analytical practices and addresses critical sustainability challenges in routine pharmaceutical laboratories.

The compatibility with both pharmaceutical and biological matrices, combined with minimal reliance on sophisticated instrumentation, underscores the practical scalability of this approach. Future applications may include adaptation for multi-analyte detection, integration with portable spectrofluorimeters for point-of-care testing, and expansion to other therapeutic classes where ion-pair formation mechanisms are feasible. The simplicity, cost-efficiency, and minimal

ecological footprint make this hybrid methodology particularly suitable for resource-limited settings and large-scale quality control operations. Ultimately, this work supports the global transition toward sustainable analytical practices in pharmaceutical sciences, positioning the SALLE-fluorescence platform as a model for future method development that harmonizes analytical excellence with environmental stewardship.

Declarations section

Conflict of interest

There is no conflict of interest to declare.

Availability of data and material

All data are available in the submitted manuscript and its supplementary file.

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Ethics approval

All experimental procedures, including analyses performed on spiked human plasma and urine samples, were reviewed and approved by the Institutional Ethics Committee for Scientific Research, Faculty of Pharmacy, Al-Azhar University, Assiut, Egypt. All methods were performed in accordance with the relevant guidelines and regulations. The investigators affirm that the work complied with the ethical principles outlined in the Declaration of Helsinki (1975) and its 2008 amendment.

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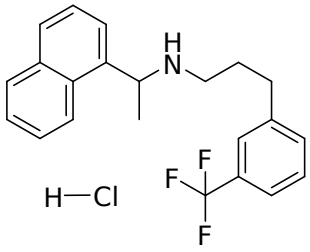
Figures

A- Cinacalcet hydrochloride (CCT.HCl) drug



B- celfia pink B (CPB) dye

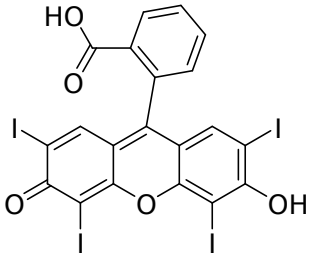


Fig. 1: The drug (A) and dye (B) chemical structures.

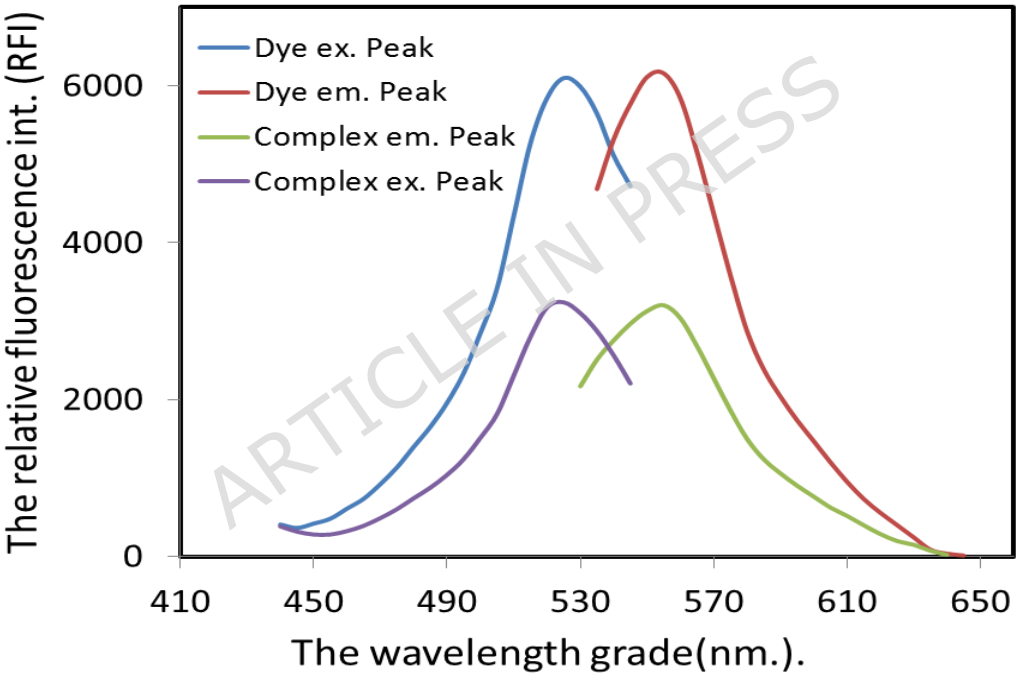


Fig. 2: The fluorescence spectra (excitation and emission) of the developed complex between the dye (CPB) and the target drug (CCT.HCl).

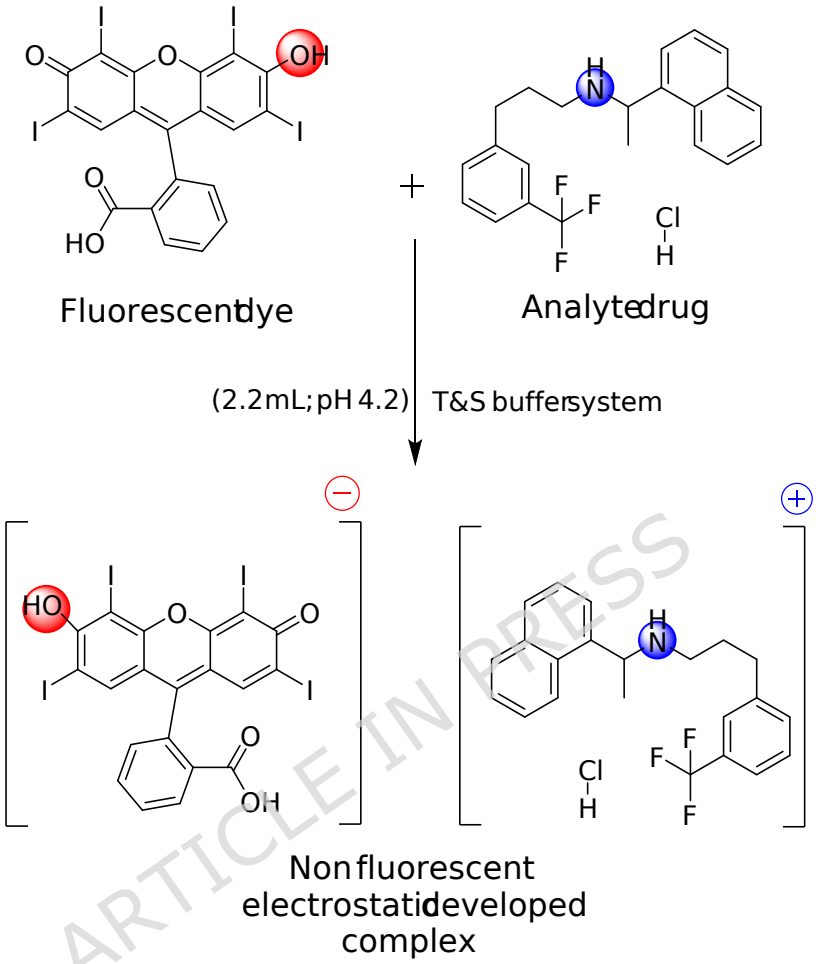


Fig.3: The suggested complex formation pathway between the dye (CPB) and the studied drug (CCT.HCl).

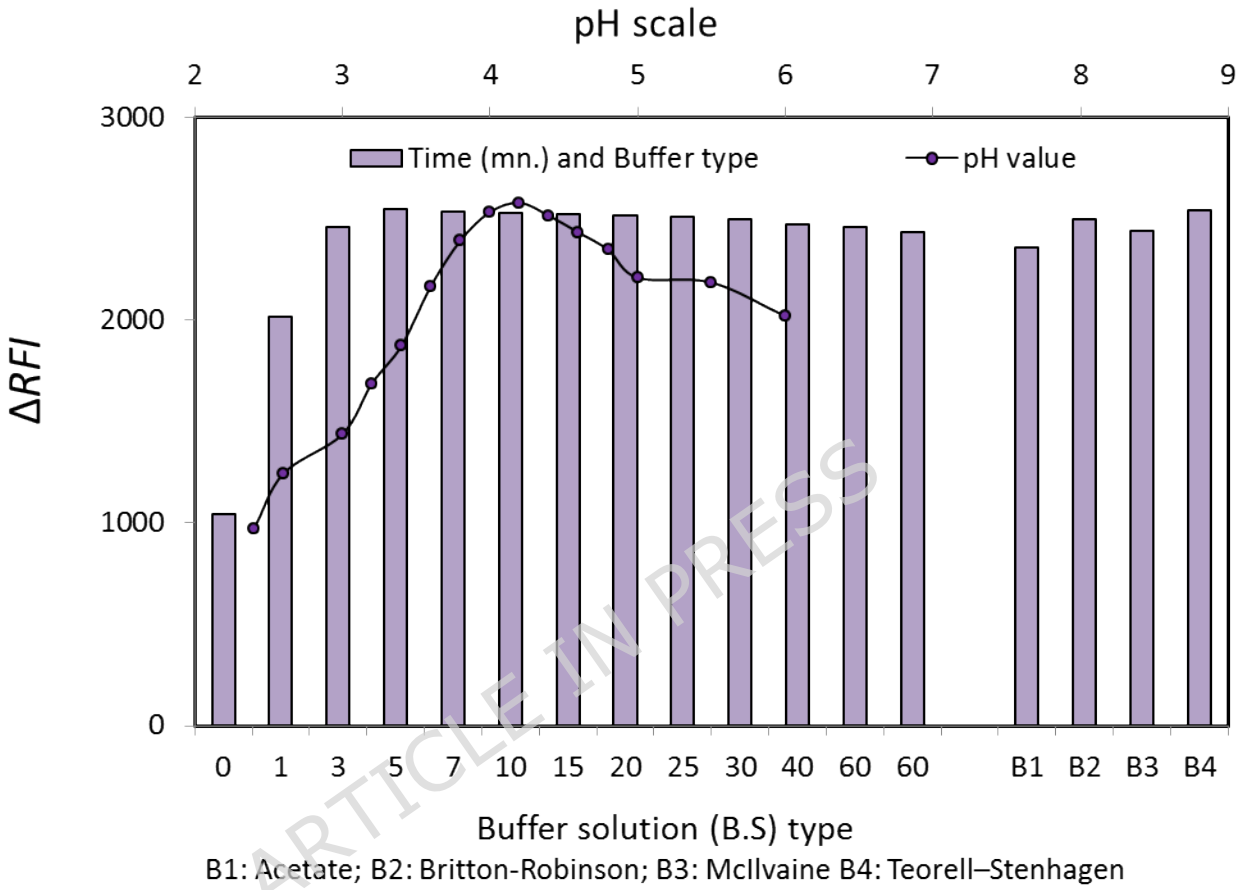


Fig. 4: The study of the medium pH, buffer system type used, and the reaction required for the complex formation between the dye (CPB) and the investigated drug (CCT.HCl).

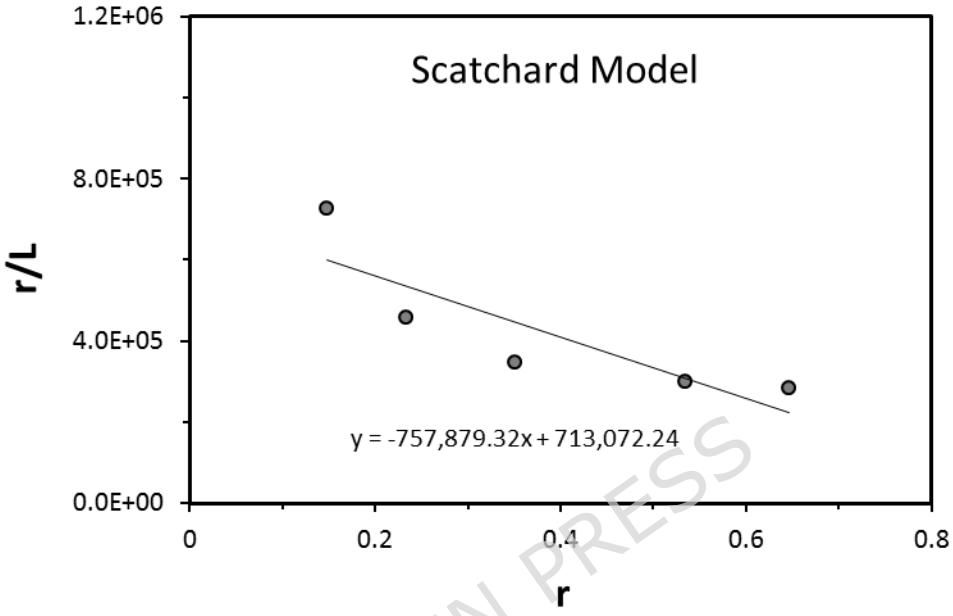


Fig. 5: Application of the Scatchard model for the binding site(s) and association affinity estimations.

Tables

Table 1: The statistical analytical factors for the developed approach.

Parameter	Value
Linear range*	80-1300
Slope	3.69
Standard deviation of slope (S_b)	0.035
Intercept	652.31
Standard deviation of intercept (S_a)	27.788
Determination coefficient (r^2)	0.9996
Correlation coefficient (r)	0.9998
Standard deviation of residuals ($S_{y/x}$)	39.59
Limit of quantitation*	75.27
Limit of detection*	24.84

* Concentration (ng mL^{-1})

Table 2: Precision and accuracy data for the current method.

parameter	ng mL^{-1}	% Rec[a]	±	SD	±	%RSD	±	%Er
Accuracy	200.00	99.78	±	1.76	±	1.77	±	0.22
	700.00	101.61	±	1.32	±	1.30	±	-1.61
	1000.00	101.92	±	1.57	±	1.54	±	-1.92
	1200.00	101.98	±	0.96	±	0.94	±	-1.98
	ng mL^{-1}	**% Rec[a]**	**±**	**SD**	**±**	**%RSD**	**±**	**%Er**
Inter-day precision	300.00	100.97	±	1.63	±	1.62	±	-0.97
	700.00	102.45	±	1.77	±	1.73	±	-2.45
	1000.00	100.68	±	1.25	±	1.24	±	-0.68
Intra-day precision	300.00	99.88	±	1.74	±	1.74	±	0.12
	700.00	101.11	±	1.15	±	1.13	±	-1.11

	1000.00	99.11	±	1.26	±	1.27	± 0.89

a: Mean of three determinations, SD: Standard deviation, RSD: Relative standard deviation, Er: relative error.

Table 3: Robustness testing of the current system for the assay of CCT.HCl drug.

Parameters	**±Value**	**% Rec[a]**	**±**	**SD**	**% RSD**
pH value	0.2	99.95	±	1.74	1.74
	0.2	100.14	±	1.46	1.45
Acetate Buffer vol. (mL)	0.2	101.05	±	1.38	1.37
	0.2	99.91	±	1.36	1.36
CPB dye vol. (mL)	0.1	100.72	±	1.85	1.83
	0.1	101.93	±	1.04	1.02
Time (min.)	1	101.32	±	1.27	1.25
	1	100.42	±	1.53	1.53

a: Average of five determinations; SD: Standard deviation; % RSD: Relative standard deviation percentage

Table 4: Analysis of CCT.HCl active constituent in different products containing the drug.

Dosage form	% recovery[a] ± SD		t-Value[b]	F-Value[b]
	Proposed	Reported		
Cinacalcet® (30 mg/ tab	102.02 ± 1.03	100.27 ± 1.86	1.83	3.28
Cinacalcet® (60 mg/ tab)	102.50 ± 1.94	100.15 ± 1.31	2.25	2.20
Cinacalcet® (90 mg/ tab)	101.27± 1.04	99.32 ± 1.84	2.05	3.17

[a]: Mean of five determinations.
[b]: Tabulated value at 95% confidence limit; F=6.338 and t =2.306.

Table 5: Results of the CU test of CCT.HCl in Cinacalcet® tablets by the developed method.

Parameter	Result
Ā	102.30
SD	3.09
RSD	3.02
AV	7.41
L1	15

Ā, Average of % recoveries; L1, maximum acceptable acceptance value.

Table 6: An application of the developed method to human plasma and urine for the analysis of the investigated drug, CCT.HCL.

Drug added[a]	Spiked plasma			Spiked urine		
	% Rec[b]	± SD	% RSD	% Rec[b]	± SD	% RSD
300	101.60	± 1.63	1.61	101.98	± 1.16	1.13
700	99.38	± 1.92	1.93	100.63	± 1.87	1.85
1100	101.86	± 1.74	1.71	99.60	± 1.26	1.26

[a] Concentration in ng mL^{-1}.

[b]Average of three determinations.