chemistry proceedings

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Proceeding Paper

Development and Validation of a Stability of *p-SCN-Bn-Df* via RP-HPLC Method: Practical Experiences †

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† Presented at The 28th International Electronic Conference on Synthetic Organic Chemistry (ECSOC 2024), 15-30 November 2024; Available online: https://sciforum.net/event/ecsoc-28.

Abstract: The DFO, a special hexadentate chelator with three hydroxamate moieties, is a bifunctional para-thiocyanatobenzyl-decafluorobenzoate (*p*-SCN-Bn-Df), a significant next-generation ligand. The presence of the thiocyanate (-SCN) group, which is capable of hydrolysis and protonation process. Something is missing. This study aims to optimized the HPLC protocol for 1-(4-isothiocyanatophenyl)-3-[6,17-dihydroxy-7,10,18,21-tetraoxo-27-(*n*-acetylhydroxylamino)-6,11,17,22-tetraazaheptaeicosine] thiourea (*p*-SCN-Bn-Df) via Reverse PhaseChromotography (RP-HPLC) method. A variety of mobile phases were tested in various ratios of solvent constituents such as methanol: water, acetonitrile: water and phosphate buffer along with at variable pH lconcentrations. However, when employing a mobile phase consisting of water to acetonitrile containing 0.1% TFA (05:95, *v*/*v*) in an isocratic manner, satisfactory separation and symmetric peaks were observed. This method utilized an Eclipsed C-18 column (5 µm, 4.6 × 250 mm) column with a flow rate of 0.5 mL/min. The maximum absorption of *p-SCN-Bn-Df* at 254 nm wavelength was selected as the detection wavelength. The retention time (t_R) of *p*-SCN-Bn-Df was found at 5.205 min. The ICH guideline was used to evaluate linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), specificity and system appropriateness criteria to validate the optimized chromatographic and spectrophotometric procedures. For accurate compound separation in pharmaceutical and environmental analyses, this phase is adaptable and often used. This study is useful for the evaluation of *p*-SCN-Bn-Df QC parameters and chelation rates with different radioisotopes e.g., Zirconuim-89 (Zr-89).

Keywords: *p-SCN-Bn-Df*; analytical methods; RP-HPLC; mobile phase; validation; stability indicating

Citation: Shrivastav, A.; Faheem, M.; Pandey, V.; Dixit, M. Development and Validation of a Stability of *p-SCN-Bn-Df* via RP-HPLC Method: Practical Experiences. *Chem. Proc.* **2024**, *6*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s): Name

Published: 15 November 2024

1. Introduction

The *p*-SCN-Bn-Df, or *p*-Isothiocyanatobenzyl-desferrioxamine, is a bifunctional chelator widely used in the field of radiopharmaceuticals, particularly in the labelling of biomolecules with radiometals for diagnostic and therapeutic applications [1]. The molecule consists of a benzyl group functionalized with an isothiocyanate (-SCN) group and desferrioxamine (Df), a high-affinity chelator for various radio metal ions, notably gallium-68 (Ga-68) and zirconium-89 (Zr-89) [2]. The isothiocyanate group (-SCN) allows for facile conjugation to primary amines on proteins, peptides or other biomolecules, forming stable thiourea linkages [3]. This capability makes *p*-SCN-Bn-Df highly versatile for labelling a range of targeting vectors, such as monoclonal antibodies, peptides and small molecules, enabling their use in positron emission tomography (PET) imaging and radio-immunotherapy.

Desferrioxamine (Df) is a siderophore with a strong affinity for metal ions, forming stable and inert complexes [4–6]. The incorporation of Df into the *p*-SCN-Bn-Df structure allows it to sequester radiometals efficiently, ensuring high radiochemical purity and stability of the radiolabeled conjugates in biological systems [7]. This property is crucial for minimizing non-specific binding and enhancing the targeting specificity and sensitivity of the radiopharmaceuticals.One of the prominent applications of *p*-SCN-Bn-Df is in the development of Ga-68 and Zr-89 labelled antibodies and peptides for PET imaging. Ga-68, with its favourable half-life and positron emission properties, is suitable for rapid imaging protocols, while Zr-89, with a longer half-life, is ideal for tracking the biodistribution of monoclonal antibodies over extended periods [7].

In summary, *p*-SCN-Bn-Df is a critical component in the toolkit of radiopharmaceutical chemistry, enabling the creation of highly specific and stable radiolabeled compounds for advanced diagnostic and therapeutic applications in nuclear medicine. Its ability to conjugate to a wide range of biomolecules and form stable complexes with radiometals underpins its widespread use and importance in the field employed in pharmaceutical [9,10]. It functions based on differential interactions between the components of the sample, a mobile phase and a stationary phase. The separation of components in a sample occurs when the mobile phase flows through the stationary phase at various rates. In this research, the RP-HPLC method for *p*-SCN-Bn-Df was successfully developed under optimized chromatographic conditions. This method demonstrated excellent resolution, symmetric peak shapes and linearity across a broad concentration range, confirming its suitability for quantitative analysis.

2. Materials and Methods:

Chemical and Instrument

The compound 1-(4-isothiocyanatophenyl)-3-[6,17-dihydroxy-7,10,18,21-tetraoxo-27-(*N*-acetylhydroxylamino)-6,11,17,22-tetraazaheptaeicosine]thiourea (*p*-SCN-Bn-Deferoxamine, B-705, ≥94%) was obtained from Macrocyclics, Inc. (USA). The DMSO as a solvent and trifluoroacetic acid (TFA) were sourced from Sigma Aldrich India Pvt. Ltd. The mobile phase was prepared using HPLC-grade acetonitrile and water, both procured from Merck India Pvt. Ltd. All the chemicals were of analytical grade and used without further modification.

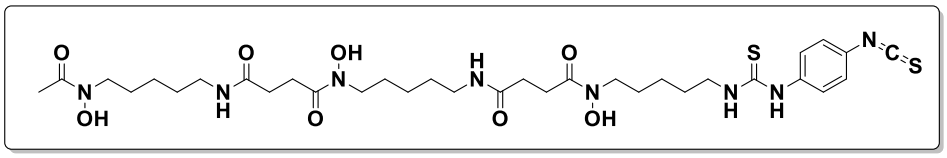

Figure 1. Chemical structure of *p*-SCN-Bn-Df.

The RP-HPLC was performed on Agilent Technologies 1260 Infinity equiped with UV detector) and quaternary pump G1311C (S/N DEAB813498), VWD detector G1314B (S/N DEAAU03726). Automated sample injector G1329B (S/N DEAAC25753) with (100 µL) injector loop and Eclipse Plus C18 (4.6 × 250 mm, 5 µm) column. The pH measurements were carried out using a pH meter (EUTECH INSTUMENTS 2700 pH/mV/°C/°F meter (S/N 2364439)). The Nylon membrane filters of pore size 0.45 µm diameter 25 mm (Cat No. SFNY25XB, from AXIVA) were used for the filtration of the mobile phase. The analytes were fully separated in under 15 min. Several solvents were tested to achieve good symmetric UV peaks and the optimal solvent system was determined to be water:acetonitrile (5:95, *v*/*v*) containing 0.1% TFA, with a flow rate of 0.5 mL/min. Using this solvent system, the resolution and peak symmetry were satisfactory, with well-resolved peaks displaying good symmetry and sharpness. The mobile phases were degassed using an ultrasonic power 120W bench top ultrasonic bath (Model: HBS-30A, Helix Biosciences).

3. Results and Discussion

A compound *p-SCN-Bn-Df* of 2 mg/mL in DMSO was taken in an eppendorf tube and the mixture was sonicated for 10 min for complete dissolution. After that compound was filtered through a 0.45 µm membrane filter and make a solution of 2 mg/mL. This solution (corresponding to 100%) was used for linearity, precision and sensitivity tests and the sequence was run starting from 100 µL from each stock sample (0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, 1 mg/mL, 1.2 mg/mL, 1.4 mg/mL, 1.6 mg/mL) injected into the HPLC system. The resulting solutions were analysed in triplicate and the peak areas were normalized and treated to a 6-point linearity regression curve as shown in Figure 3. The slope, y-intercept and coefficient of determination (r^2) were used as measures of linearity. The optimized method for the HPLC as follows:

Equipment	Agilent High performance liquid chromatography equipped with Auto Sampler and VWD detector.
Column	Eclipse Plus C18 (4.6 × 250 mm, 5 µm)
Flow rate	0.5 mL/min
Wavelength	254 nm
Injection volume	100 µL
Column oven	Ambient
Run time	15.0 min

The prepared homogeneous samples were injected in triplicate into the HPLC using the previously described technique to assess system suitability. The retention time for *p*-SCN-Bn-Df was approximately 5.1 min. The mean peak area was 18,848.5, with a standard deviation of 877.6, resulting in a relative standard deviation (RSD) of 4.656%. The peak areas of the triplicate injections were recorded. A chromatogram demonstrating the system's suitability is shown in Figure 2.

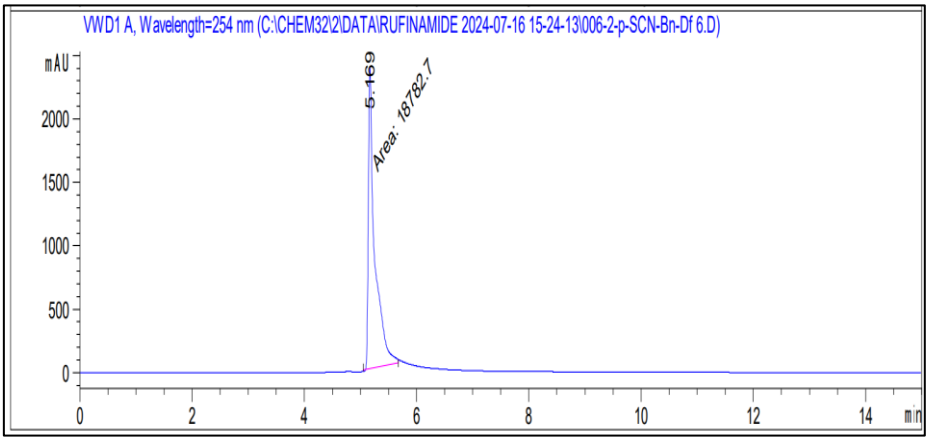

Figure 2. Chromatogram of p-SCN-Bn-Df along with retention time at 254 nm.

Precision was evaluated by assessing the method's intraday variations, and HPLC repeatability was determined by analyzing the standard solution on the same day. The precision study involved injecting the standard solution twice at four different concentrations: 10, 20, 30, and 40 µg/µL.

Table 1. Determination of Precision for *p*-SCN-Bn-Df at 254 nm.

Sample No.	Conc. (µg/µL)	Mean Peak Area ± SD	RSD %
1	10	5276.3 ± 58.12	1.102
2	20	11,998.3 ± 108.82	0.907

3	30	18,070.45 ± 91.56	0.507
4	40	25,065.3 ± 110.24	0.440

The standard calibration curve was generated using eight standard solutions with concentrations ranging from 0.2 to 1.6 mg/mL. Under optimized chromatographic conditions, each standard solution was injected three times with a run time of 15 min per injection. The method's linearity was assessed by performing least squares linear regression analysis on the average peak area plotted against concentration.

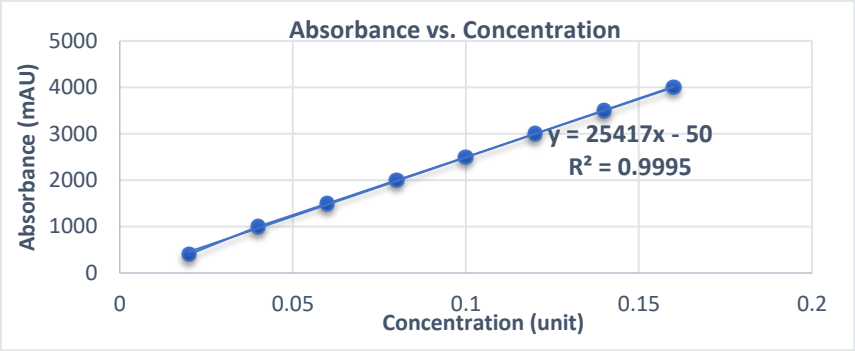

Figure 3. Linearity curve for p-SCN-Bn-Df at 254 nm HPLCchromatography.

The representative chromatogram obtained for *p*-SCN-Bn-Df is shown in Figure 2 and those of marketed formulations. The calibration curve was linear over the concentration range 0.2–1.6 mg/mL (Table 2) and the regression equation was found to be y = 25417x − 50 with a correlation coefficient of 0.9995 (See Table 2). The RSD in precision studies was 0.44–1.10% (Intra-day) (Table 2). The LOQ was found to be 36.87 mcg/mL and the LOD was found to be 12.16 mcg/mL.

Table 2. Optimum conditions, Optical characteristics and Statistical data of the Regression equation of *p*-SCN-Bn-Df.

Parameter	UV Method
λmax (nm)	254
Beer's law limits (mcg/mL)	20–40
Molar extinction coefficient (L mol-1 cm-1)	25
Sandell's sensitivity (mcg/cm2 −0.001 absorbance units)	0.0004
Regression equation (Y *)	y = 25417x − 50
Slope (b)	25
Correlation coefficient (r2)	**0.9995**
Precision (% RSD **)	0.739
Limit of detection (mcg/mL)	12.16
Limit of quantitation (mcg/mL)	36.87

4. Conclusions

In conclusion, the development and validation of a stability-indicating RP-HPLC method for *p*-SCN-Bn-Df was successfully achieved using optimized chromatographic conditions. The method exhibited satisfactory resolution, symmetric peaks, and linearity over a wide concentration range, demonstrating its effectiveness for quantitative analysis. Additionally, the method was validated by ICH guidelines, ensuring its precision and accuracy. This method proves to be highly suitable for evaluating the stability of *p*-SCN-Bn-Df in pharmaceutical formulations, as well as for its quality control and chelation efficiency with radiometals like Zr-89, which are crucial for radiopharmaceutical applications. The RP-HPLC method described here provides a reliable analytical tool for both

routine quality control and research purposes in the field of radiopharmaceutical chemistry.

Author Contributions: A.S.: Investigation, methodology, original draft, data correction, M.F.: original draft, editing, communication., V.P.: data correction, editing, M.D.: Supervision, review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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