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Application of HR CS GF-MAS for Determining the Intracellular Concentration of Fluorinated Drugs and Drug Candidates in Cancer Cells, Using Calcium Monofluoride (CaF) as the Target Molecule

Hubert Descher, Peter Eta Enoh

Abstract: ***Fluorinated drugs and drug candidates are showing signs of becoming a cornerstone in the fight against cancer, a threat to the well-being of humanity. An HR CS GF-MAS method has been developed for quantifying their intracellular concentration in cancer cells, a prerequisite for efficacy and effectiveness. The study determined the intracellular concentration of the fluorinated drug 5 Fluoro uracil (5FU) and a drug candidate, [chlorido (Fe (III)4Fluoro Salophen)], an iron complex. The method presented here is very sensitive with a lower limit of quantitation of 3µg/L equivalent to 3ng/ml, accurate, with an accuracy range of 99.8-102.9%; it is fast, less than two minutes per sample, linear in the range of 12.5-600µg/l (R^2=1), precision was demonstrated by the method, the highest coefficient of variance for inter and intra-day precision test was 5.1. A novel sample preparation approach using citric acid and tetramethyl ammonium hydroxide effectively extracted fluorides with high accuracy and precision. These reagents equally stabilized fluorides and prevented their loss in the form of hydrogen fluoride. These attributes enable the method to generate accurate and reliable data that informs the drug development process. The tested ability of the validated HR CS GF-MAS method to determine the intracellular concentration of both organofluorinated drugs and fluorinated metal complexes makes it a reliable tool for developing and testing fluorinated medications in general. Drug researchers may also use this method to explore the mechanism of fluorinated drug uptake in cells and analyse fluorinated drugs in other matrices.***

Keywords*: *Calcium Monofluoride, Continuum Source, Iron Complex, Molecular Absorption Spectroscopy, Pyrolysis.

Abbreviations:
WHO: World Health Organization
ICP-OES: Inductively Coupled Plasma Optical Emission Spectroscopy
ICP-MS: Inductively Coupled Plasma, Mass Spectrometry
US-FDA: United States Federal Drug Administration
LOD: Limit Of Detection
HF: Hydrogen Fluoride

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I. INTRODUCTION

Cancer is the second leading cause of death and remains one of the significant public health problems facing humanity worldwide, according to the World Health Organization (WHO) [1]. With the increasing incidences of cancer, new cases are estimated to reach 19.3 million in 2025 [2].

Though the types, burden, and incidence vary from region to region, the standard forms experienced worldwide are colorectal, breast, and lung cancers [3]. Targeted radiation therapy, hormone therapy, surgery, and chemotherapy are the general clinical approaches for cancer treatment. Of the options mentioned, chemotherapy is the most commonly employed practice. It is estimated that approximately fifty per cent of patients undergoing anticancer treatment receive therapeutic drugs containing platinum complexes in various forms [4].

Several studies have examined the contribution of chemotherapy in treating cancer compared to other approaches. Some of these studies concluded that chemotherapy was beneficial in minimizing the risk of reoccurrence for patients with early stages of breast cancer [5] and recurrence of stage three colon cancer [6]. Platinum-based drugs like cisplatin have played a dominant role in cancer treatment.

The mode of action is primarily based on their ability to bind to DNA, causing damage to the DNA strands and ultimately leading to the death of cancer cells [7]. Although chemotherapeutic agents are beneficial as treatment options, their use has limitations, including systemic toxicity [8], lack of selectivity that leads to undesirable side effects, and resistance [9]. Approaches undertaken by researchers to circumvent these limitations include targeted delivery using drug carriers, combination therapy, and structural modifications of the drug [11]. Regarding structural change, incorporating fluorine's unique physical and chemical properties as a functional group to therapeutic drugs and drug candidates improves their pharmacokinetic and physicochemical properties [10].

The benefits of incorporating fluorine as an active group of drugs include enhanced stability and less

susceptibility to degradation, improved bioavailability, increased selectivity by increasing the affinity for target receptors or enzymes, and enhanced lipophilicity, which increases drug absorption and distribution [12]. Though incorporating fluorine into drug structures generally imparts improved benefits [13], its effect on cellular uptake is mixed [14]. While some studies show that fluorine incorporation enhances cellular uptake [15], other results show the contrary: fluorine attachment [16], depending on the position of substitution, reduces cellular uptake [17]. Such findings, therefore, make the proof of cellular uptake and internalisation of these compounds an absolute necessity, as most drug targets are located intracellularly, and for drugs to elicit pharmacological responses, they must interact with these targets.

Analytic techniques employed for the determination of fluorides and fluorinated drugs include titrimetric [18], Chromatographic [19], electroanalytical [20], and potentiometric [21] methods. Spectroscopic techniques include Nuclear magnetic resonance spectroscopy (NMR) [22], inductively coupled plasma, mass spectrometry (ICP-MS) [23], and graphite furnace molecular absorption spectroscopy (GF-MAS [24]. The choice of these techniques for developing an assay will depend on several factors, including sensitivity, dynamic range, sample matrix, resolution, and the nature of the assay. As a consideration, GF-MAS is a versatile technique that can be exploited in molecular spectroscopy [25].

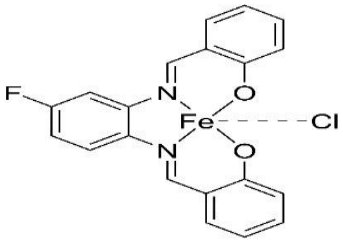
Spectroscopic techniques such as inductively coupled plasma optical emission spectroscopy (ICP-OES) or atomic absorption spectroscopy cannot be used for the determination of fluorides due to their high ionisation potential and the fact that the resonance line of fluorine, at approximately 95 nm, is in the vacuum ultraviolet (UV) region.

However, in the gaseous state, fluorine can form stable monofluorides with metals like aluminium, gallium, strontium, and calcium. These diatomic molecules can absorb distinct or characteristic energy from a Continuum radiation source and create molecular absorption spectra. Examples of these diatomic molecules are GaF, AlF, CaF, and SrF. These diatomic molecules, along with their ability to absorb light energy, are used as targets for detecting fluorinated compounds in samples. Therefore, GF-MAS can be considered an advancement of the traditional AAS, extending the technique's applicability to a broader range of elements, including non-metals, such as halogens. As a sensitive and selective technique, GF-MAS offers a wide dynamic range; it is fast, efficient, cost-effective, and compatible with biological matrices.

Despite the attributes mentioned above and the enormous potential of GF-MAS, it has yet to be extensively utilised for determining fluorinated drugs in cells. The few existing assays that employed the technique are limited to targeting GaF. To our knowledge, no existing method uses CaF for determining the intracellular concentration of fluorinated drugs or drug candidates. Therefore, this project aimed to develop a High-Resolution Continuum Source Graphite Furnace Molecular Absorption Spectroscopy (HR CS GF-MAS) method capable of determining the concentration of fluorinated drugs and drug candidates in cultured cells using CaF as the target molecule. Secondly, to guarantee the reliability and accuracy of the data produced by validating the method.

II. MATERIALS AND METHODS

A. Chemicals and Reagents



Chemical Formula: $C_{20}H_{13}ClFFeN_2O_2$
Molecular Weight: 423.63

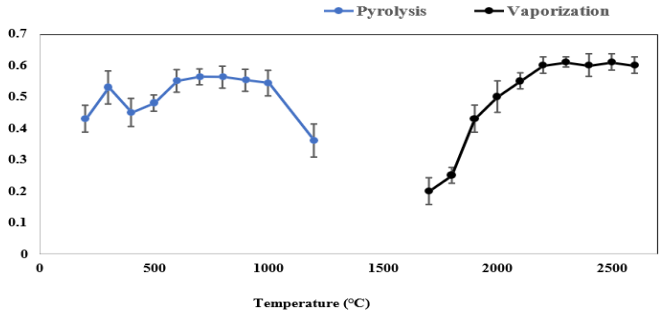
[Fig.1: Structure of Iron Complex Chloride (Fe (III)4 Fluoro Salaphen) (CI Fe^3 4FS)]

[chlorido (Fe (III) 4Fluoro Salophen)] ($ClFe^3$4FS) and 5Fluoro uracil (5FU) were obtained from the Department of Pharmaceutical Chemistry, University of Innsbruck. Zirconium solution (10.018 ppm), fluoride standard solution (1002mg/L), calcium carbonate, citric acid, human serum albumin (HSA), and Coomassie brilliant G-250 were purchased from Sigma Aldrich (Darmstadt, Germany). Phosphate buffer saline (PBS) and Tetramethyl ammonium Hydroxide (TMAH) were ordered from Thermo Fisher (Vienna, Austria). Nitric acid (HNO_3) and Acetic acid were obtained from Merck (Darmstadt, Germany) and VWR (France), respectively. Water used throughout the project was obtained from the Milli Q system (EMD Millipore, Germany). Calcium, used as the molecule-forming reagent in GF-MAS, was prepared from calcium carbonate by dissolving 4g of calcium carbonate with 3 ml of acetic acid and making up the volume to 100 ml with water. This solution was diluted 1:1 with water before use. A citric acid 0.1M solution was prepared by weighing and dissolving.

19.2g in 1 L of water. 0.5% v/v Nitric acid solution was made by adding 2.5ml of HNO3 to water and making the volume to 500ml.

B. Instrumentation

A Contra 700 high-resolution continuum source atomic absorption spectrometer (Analytik, Jena) having a 300-W xenon arc lamp (XBO 301, GLE, Berlin, Germany), a high-resolution double monochromator, with a prism for pre-dispersion, an echelle monochromator for high resolution, and a charge-coupled device.



[Fig.2: Optimization of the Pyrolysis and Vaporization Temperatures for Monitoring CaF. Error Bars Are Standard Deviation (n=3)]

(CCD) An array detector was used for the entire project. A wavelength of 606.4347nm was used to measure the

absorbance of CaF. Pyrolytically coated graphite furnaces with integrated pin platforms ((Analytik Jena part number 407-A81.025) were used for vaporization. The furnaces were coated with zirconium as a permanent modifier following the Donnici et al. [26] procedure before use. The autosampler was an MPE 60 (Analytik Jena, Germany); 20 µL of sample and 10 µL of Ca solution were automatically injected. Argon with ≥ 99.999% purity, obtained from Messer (Gurnpolskirchen, Austria), was used as an inert and purge gas. The pyrolysis and vaporization temperatures of the instrumental method, also known as the temperature program (Table I), were optimized as follows: Cultured cells were spiked with 5fluoro uracil to obtain a fluoride concentration of 10µg/L. The sample was prepared, and 20 µL was injected in triplicate, with each injection increasing the temperature stepwise by 100°C and recording the absorbance at each step. The temperature range for pyrolysis was from 200- 1200°C and 1600-2500°C for vaporization. The pyrolysis and vaporization temperature that produced the highest absorbances were considered optimal and used for the method. The optimal temperatures (Table I) were 700°C for pyrolysis and 2450°C for vaporization.

Table I. Optimized Graphite Furnace Temperature Program for the Monitoring of CaF by HR CS GF-MAS

Steps	Temperature (°C)	Ramp (°C/s)	Hold (s)	Gas Flow
Drying	90	3	20	Max
Drying	110	3	30	Max
Drying	120	5	20	Max
Pyrolysis	700	300	20	Max
Gas Adaptation	700	0	5	Stop
Vaporization	2450	1500	5	Stop
Cleaning	2600	1000	5	Max

C. Cell Culture, Drug Exposure, and Uptake

The human epithelial breast cancer cell line, MDA-MB-231, was grown in 75 cm3 cell culture flasks with RPMI-1640 culture medium, supplemented with 10% bovine serum albumin (BSA) in a humid atmosphere at 37°C and 5% CO_2. Cells were allowed to grow to a confluency of approximately 80-85% before passaging. The passaging and other procedures were done under sterile conditions. The spent medium was carefully removed from culture flasks by suction. The cells were washed two times, each with 5 mL of ice-cold phosphate-buffered saline (PBS) to remove dead cells and other possible contaminants. After removing the PBS, 2 mL of accutase (a cell detachment solution of proteolytic and collagenolytic enzymes) was added, and the flask was incubated for five minutes at 37°C to detach the cells. Upon detachment, the cells were transferred to a centrifuge tube, and 2 mL of culture medium was added. The mixture was then centrifuged for 5 minutes at 2000g at room temperature. The supernatant was removed, leaving behind the cell pellets. 1 mL of cell culture medium was mixed with the pellets, and the cells were split 1:4 into new culture flasks. This procedure was done twice a week for three weeks to obtain enough cells for method validation, drug exposure, and cellular uptake experiments.

For exposure and uptake experiments, cells were counted and seeded at a density of 500,000 cells per well in six-well plates, with each well containing 3 mL of cell culture medium. The plates were incubated for 24 hours for cell attachment, and after that, the culture medium was removed by suction. 5FU prepared in a cell culture medium at a concentration of 20µM was added to one set of cells, and the other set was exposed to a cell culture medium containing 0.5µM of the fluorinated drug candidate (Cl Fe34FS). The cells were grouped into six sets, each with three replicates, based on the duration of exposure. The time points of exposure were 0,1,2,3,4,6,8, and 24 hours, respectively. At the end of each time point, the medium was removed, cells were washed, detached with accutase, transferred to an Eppendorf tube, centrifuged, the supernatant removed, and the cell pellets stored at -20°C. It is worth noting that each time point had a corresponding blank, used to correct for fluorine contributions from labile fluoride and fluorides from other sources, like the reagents used. The cells at time 0 were pelleted and stored without exposure to the drug.

D. Preparation of Quality Control (QC) Samples

Quality control standards were prepared at three concentration levels: 25 µg/L, 200 µg/L, and 400 µg/L, designated as low, medium, and high concentrations.

i. Respectively.

The samples were made in three 50 mL Greiner centrifuge tubes. For low concentrations, 1.25 mL of a stock solution at 1000 µg/L was transferred to the tube; the medium and high levels were prepared from a 10,000 µg/L stock. For the medium, 1 mL, and the high concentration, 2 mL, were transferred to their respective tubes. All the tubes were filled to the 50 mL mark with solutions of diluted cells in water, vortexed for 5 minutes, and sonicated for 10 minutes. 300µl aliquots from each tube were pipetted into 1.5 ml Eppendorf tubes, dried by lyophilisation, and stored at -20°C. The stock solutions were prepared with 5-fluorouracil, and the concentrations were calculated based on the amount of fluorine in the solution.

E. Sample Preparation

Cell pellets exposed to drugs were prepared by pipetting 20µl of 0.1M citric acid, vortexed, and sonicated for 5 minutes. Then, 20µl of 25% (v/v) tetramethyl ammonium hydroxide was added, and the samples were again sonicated for 10 minutes. After that, 260 µL of 0.5% (v/v) HNO3 was added, and the samples were centrifuged for 5 minutes at 2000g and room temperature. 20 µL of the supernatant was reserved for protein determination, and the rest was transferred to autosampler vials for drug analysis. The same sample preparation approach without protein determination was applied to quality control standards for method validation.

F. Protein Determination

The protein concentration of the samples was determined by the Bradford method [27], an established method used for determining low protein concentration in samples. Human serum albumin was used for plotting the calibration curve. 2mg of protein was weighed and dissolved in 1 ml of water. This solution was used to prepare calibrators with the following concentrations: 0, 0.015, 0.03, 0.13, 0.25, and 1mg/ml. 10µl of calibrators and samples diluted 1:4

were pipetted in triplicate into the wells of a microtiter plate with flat bottoms. 190µl of Bradford reagent diluted 1:4 was added to the wells and mixed for 10 minutes, and the absorbance was measured at 595nm by a microplate reader. Three wells with water were used to correct for background absorbance. The calibration curve was plotted and used to determine the protein concentration of the samples.

G. Method Validation

The method was validated following the recommendations of the United States Federal Drug Authority (US-FDA) [10], based on the following components or parameters: the linearity, the limit of detection (LOD), the lower limit of quantitation (LLOQ), the upper limit of quantitation (ULOQ), matrix effect, precision, accuracy, stability, recovery, and system suitability test.

Linearity of calibration curve: The calibrators were prepared from a stock solution of 1200µg/l. The stock solution was prepared as follows: 100 µL of a 0.1 M citric acid solution was pipetted, followed by the addition of 60 µL of a 1 mg/mL fluoride standard solution. The mixture was then sonicated for 5 minutes. Next, 100 µL of 25% (v/v) tetramethyl ammonium hydroxide was added, and the mixture was sonicated again for 10 minutes. The volume was then adjusted to 50 mL with a 0.5% (v/v) HNO_3 acid solution. Each calibrator was prepared separately from the stock solution as opposed to a serial dilution to avoid the possibility of propagating errors at one point to the rest of the calibrators. The calibrators comprised zero and six concentrations (0, 12.5, 25, 75, 150, 300, and 600µg/l), respectively. The calibrators were injected in triplicate, and their absorbance was recorded to plot the calibration curves. The linearity was determined by the coefficient of determination (R^2). To ascertain the goodness of fit of the regression line, the residuals of the calibrators were plotted and tested for the normality of their distribution. The homogeneity of the residuals was checked by visual inspection. Therefore, the acceptance criteria for linearity were a coefficient of determination ≥ 0.99, a residual plot with a normal distribution, and the homogeneity of residuals.

LOD, LLOQ, and ULOQ: The LOD and LLOQ were determined based on the standard deviation (SD) of blanks and the slope (S) of the calibration curve. 20µL of ten sample blanks were injected, and the standard deviation of the responses was determined. The LOD was calculated as three times the standard deviation divided by the slope of the calibration curve, and the LLOQ as ten times the standard deviation divided by the slope. The LOD and LLOQ values obtained following this procedure were 2.56 and 9.57µg/l, respectively. Since these values are theoretical, matrix-matched samples were prepared and analysed to confirm the LLOQ experimentally. Samples with the following concentrations: 3, 6, 10, and 12.5µg/l, were analysed in triplicate, and the accuracy and coefficient of variance (CV) were determined.

The acceptance criteria for LLOQ (CV≤ 20) and 85- 115% accuracy. The ULOQ was considered the highest point of calibration, where the detector was not saturated and the instrumental response remained linear.

Matrix Effect: Six replicates of quality control samples were analyzed, and the average result at each level was compared to those of standard solutions without cell matrix. The matrix effect was determined by the difference in concentration between standard solutions and quality controls in the matrix, divided by the analyte concentration in the standard solutions, and then multiplied by 100. The results are expressed in percentages.

Precision: Precision was evaluated based on intra-day and inter-day variation. Low, medium, and high-quality control standards were analyzed in six replicates in a day for intra-day precision and three different days for inter-day precision. The coefficient of variance was calculated for each level to determine the precision of the results. For the method to be precise, the CV was to be ≤ 20 for low, ≤ 15 for medium, and < 10 for high QC samples, respectively.

Accuracy and Recovery: Six replicates of quality control samples were analysed, and the average result at each level was compared to its nominal value. The results of accuracy were expressed in percentages. The coefficient of variance of the accuracy was calculated at each level, representing the method's recovery. The accuracy should be between 85% and 115%, and the coefficient of variance should be ≤ 20 for recovery.

Stability: Stability was evaluated under three conditions. Stability of compounds in samples at room temperature (Benchtop), stability of prepared samples in the autosampler, and freeze/thaw stability of analytes in the matrix. The three levels of quality control samples were used to evaluate each condition. For stability at room temperature, samples were left at room temperature for six hours before preparation and analysis. Autosampler stability was assessed by preparing samples and allowing them to stay in the autosampler for six hours before analysis. Free/thaw stability was achieved by subjecting a set of samples, low, medium, and high, to one freeze/thaw cycle, the second set to two, and the third to three. The samples were dried down by lyophilization, prepared, and analyzed to determine the effect each of these conditions had on the stability of the compound. The results were compared to their nominal concentrations, and the stability was expressed as a percentage of the nominal concentration. Acceptance criteria were 85-115%.

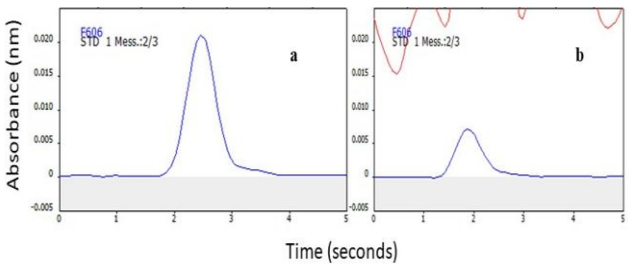
System suitability Test: The system's suitability was checked before every analysis. The lowest calibrator was injected six times, and the reproducibility was determined by calculating the coefficient of variance of the results. The shape of the absorption peak was also examined. CV values above 20 or an unsymmetrical peak shape indicated that troubleshooting was necessary, and the problem had to be solved before the analysis. Familiar sources of the issues discovered during troubleshooting were the calcium solution, which was to be prepared and used for no more than a week, and the age of the graphite furnaces.

III. RESULTS AND DISCUSSION

Method development is a process that involves selecting an appropriate method and optimising conditions to achieve the intended analytical performance. The development of the HR CS GF- MAS method for determining the intracellular concentration of fluorinated drugs and drug candidates in cells began

Optimising 1) the temperature program, 2) using a permanent matrix modifier, and 3) sample preparation.

The temperature program is an essential instrumental parameter that needs to be optimised for an HR CS GF-MAS method. Of the four stages of the temperature program (drying, pyrolysis, vaporization, and cleaning), the most crucial are the pyrolysis and vaporization stages. The pyrolysis temperature must be high enough to destroy all organic matter in the matrix; at the same time, it must not be so high as to begin the process of analyte vaporization prematurely. According to Eugen et al. [28], a low pyrolysis temperature produces a substantial amount of matrix in the vaporisation stage, vaporising as smoke and resulting in excessive background absorption. On the other hand, the vaporization temperature should be high enough to ensure all the analytes in the samples vaporize. Therefore, the pyrolysis and vaporization temperatures were carefully optimized; 700 and 1450°C were considered optimal for the pyrolysis and vaporization temperatures (Fig. 2 and Table I). This careful optimization resulted in a smooth and symmetrical absorption peak (Fig. 3a).



[Fig.3: Comparison of the Time-Resolved Molecular Signals Recorded at the CaF Wavelength Transition Showing the Effect of the Permanent Matrix Modifier (Zirconium) on the Sensitivity of CaF Absorbance. (a) Increased Sensitivity with the Modifier, and (b) less Sensitivity Without the Modifier; Red Line Traces are Background Signals. The Same Sample was Injected in Both Situations]

Matrix interference is a significant drawback in HR CS GF-MAS assays. The adverse effects of the matrix are generally corrected using matrix modifiers. Though matrix modification was not applied by Nil et al. [29], and the authors even suggested that it was not necessary for their analysis. Others have established that the use of zirconium as a permanent modifier reduces fluoride interaction with the graphite layer of the furnace, and as a result, increases sensitivity [30]. These two suggestions were tested, and the results showed that using zirconium as a permanent modifier increases the method's sensitivity, making it necessary. A comparison of the two situations (Fig. 3) reveals an increase in sensitivity with the use of Zirconium as a permanent matrix modifier (Fig. 3a) and a reduction in sensitivity in the presence of background absorption (red traces) without the use of a matrix modifier (Fig. 3 b).

Proper sample preparation is a crucial component of method development. Sample preparation enables the analytes to be extracted and converted into a suitable form for instrumental analysis. For this method, two extraction reagents, citric acid and tetramethyl ammonium hydroxide (TMAH), were employed for sample preparation. Citric acid chelates fluorides to form fluorocitrate complexes, which solubilize and retain the fluorides in solution. Formation of these complexes also prevented the loss of fluorides in the form of hydrogen fluoride (HF) during the sample preparation and pyrolysis stages. TMAH completely solubilized the sample matrix, and the positive charge in the solution enabled it to complex with fluorides, thus enhancing their solubility. The solubilization of the sample matrix was very beneficial because it prevented the deposition of carbonaceous material in the graphite. Furnace. Prevention of such deposition extends the life span of the graphite furnace [30].

A. Method Validation

An additional aim of the study was to validate the method. The validation process involved testing the method using several samples to demonstrate that it meets the required specifications for linearity, limit of detection LOD), lower limit of quantitation (LLOQ), matrix effect, accuracy, precision, and stability.

Linearity is the ability of an analytical method to produce a linear response over a range of concentrations. Calibration curves are essential for procedures that require quantifying analytes in samples. It is used to determine the instrumental response to analytes and to predict the concentration of analytes in unknown samples. To test for linearity, zero and six calibrators with known analyte concentrations were analysed (n = 3), their absorbance was recorded, and the data were used to plot the calibration curve. The calibration curve exhibited good linearity, as evident by the coefficient of determination (R^2) (Fig. 4). However, since the coefficient of determination primarily indicates the relationship between the analyte concentration and the instrumental response, it cannot be considered the sole measure or proof of linearity. Next, residual plots were used to assess the validity of the linear regression by testing for the homoscedasticity (Homogeneity of the residuals) and the normality of the residual distribution. For the curve to be considered linear, the residuals should be independent of each other, with no visible pattern existing in their distribution. Secondly, the plot of residuals is to have a normal distribution.

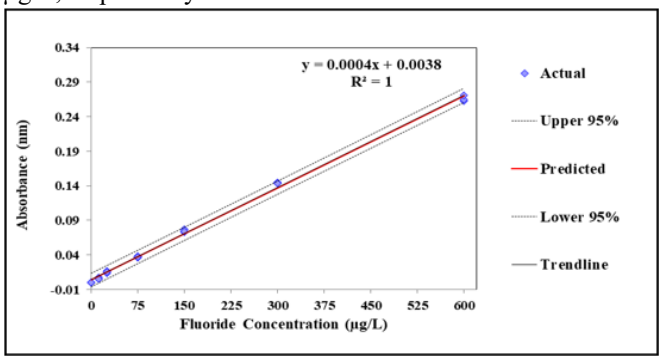
Table II. Experimental Evaluation and Confirmation of LLOQ. Acceptance Criteria: Coefficient of Variation (CV) ≤20 and Accuracy Between 85-115%

Target Concentrations (µg/L)	Obtained Concentrations (µg/L)	Accuracy (%)	CV
3	3.19	106.4	16.8
6	6.45	107.5	12.8
10	9.22	92.2	13.8
12.5	11.93	95.6	6.2

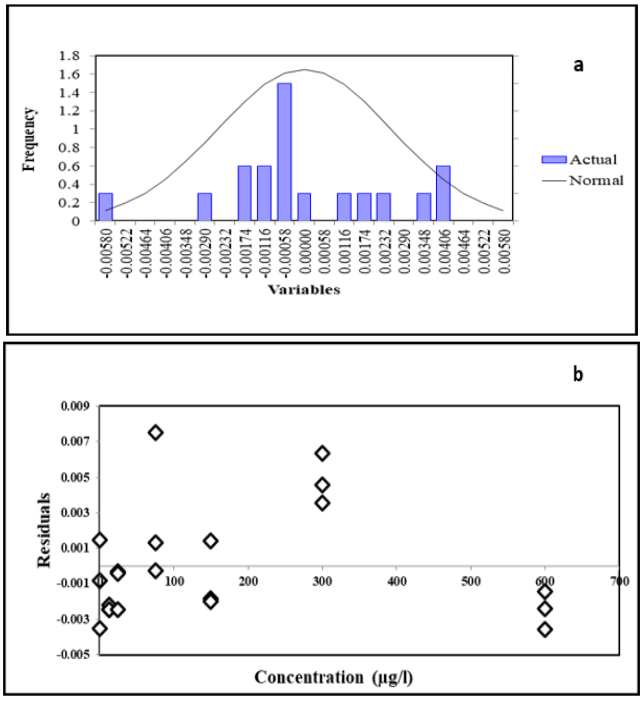
Figure 5 shows that the calibration curves met these additional requirements. The residuals were normally distributed (Fig. 5a). In addition, the Anderson-Darling test for normality confirmed the normality of the distribution (A-D*) p> 0.05, and (Fig. 5 b) shows that the residuals were consistent, the degree of variance was minimal and therefore homogenous. The superimposed 95% confidence interval of the calibrators (Fig. 4) indicates

that the calibrators fall within the upper and lower 95% confidence levels, meaning that the concentration of analytes in samples determined by this calibration curve will be 95% sure to be their actual values. Meeting the above requirements indicates that the calibration curve was linear, and a linear regression model was appropriate for the method.

LOD is the lowest analyte concentration in samples that can be detected but not necessarily quantified under the stated experimental conditions. LLOQ is the lowest analyte concentration in samples which can be quantified with a degree of precision by a method. Their determination was based on three times the standard deviation of ten blank injections, divided by the slope of the calibration curve for LOD, and ten times the standard deviation of ten blanks, divided by the slope for LLOQ. The concentrations obtained by this calculation for LOD and LLOQ were 2.6 and 9.7 µg/L, respectively.



[Fig.4: The Calibration Curve Employed for Monitoring CaF in Samples in the Working Range of 12.5-600µg/l, (n=3), Including the Superimposed 95% Upper and Lower Confidence Level of Calibrators]



[Fig.5: Plot of the Histogram of Residuals Showing Their Normal Distribution (a). The Anderson-Darling test for Normality rejects the Hypothesis of Normality When the p-value is ≤ 0.05. Normality test (A-D*): p >0.05. Residual Plot of the Calibrator Residual Showing their Homogeneity (b)]

The obtained values were theoretical; quality control samples with known concentrations ranging from 3 to 12.5 µg/L were prepared and analysed to confirm the validity of the concentration value received in the LLOQ experiment. Interestingly, the experimental results were reproducible down to 3 µg/L, as indicated by the coefficient of variance (Table II). The LLOQ of the method was therefore assigned as 3µg/l. The results of this experiment highlight the importance of experimental evaluation or confirmation of the theoretically obtained concentration values of LLOQ. The upper limit of quantitation (ULOQ) was the highest level of calibrators. The detector was not saturated at this level, and thus, the response remained linear. The implication of these three concentrations, concerning

The method is that any concentration values in µg/L obtained from the limit of detection (LOD) and below are not to be considered. Values between the LOD and the limit of quantification (LLOQ) are considered semi-quantitative until they are proven experimentally reproducible. In contrast, those from the LLOQ to the upper limit of quantification (ULOQ) are accurate and quantitative.

The precision determines the closeness and agreement among obtained or determined values of concentrations or responses when the method is repeatedly applied to multiple samples. The method's precision was evaluated by analysing six replicates of quality control samples at three concentration levels and calculating the coefficient of variance.

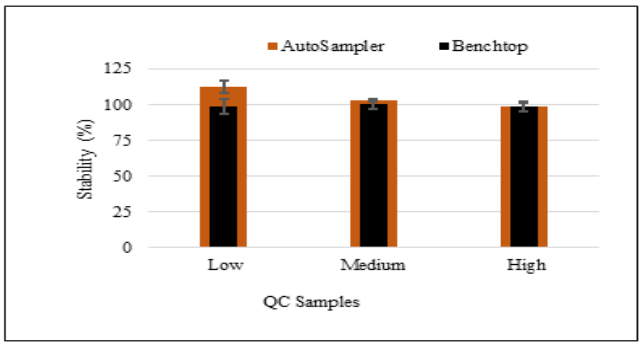
Table III. Results of Validation Showing Intra- and Inter-Assays Precisions, Accuracy, Matrix Effect, Recovery, LOD, LLOQ and ULOQ. Values in Brackets are the Coefficient of Variance (CV). The Number of Replicates Are six for all Experiments Except for Intraday Precision with 18 Replicates for the Three Different Days of Evaluation

Samples	Interday Precision (CV)	Intraday Precision (CV)	Accuracy (%)	Matrix Effect (%)	Recovery CV
QC Low	4.6	5.1	100.9	3.4	7.5
QC Medium	3.2	2.95	102.9	2.1	1.5
QC High	2.1	1.42	99.8	0.98	1.3
		Sensitivity (µg/L)			
LOD	2.6 (26.8)				
LLOQ	3.0 (16.8)				
ULOQ	600 (1.3)				

The results for inter-day and intraday precision are shown in Table III. The excellent precision, with low coefficients of variance obtained (1.42-5.1) for inter-day and intraday precision, demonstrates that the method is precise.

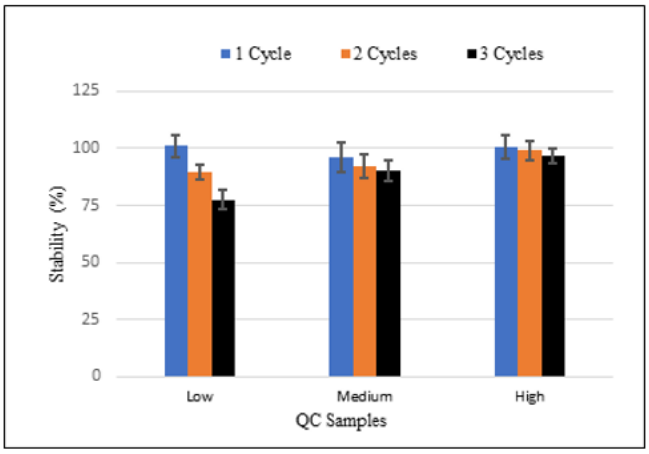
Accuracy measures the difference between the concentrations obtained by an analytical method and the nominal values. The accuracy was assessed after analysing samples at three concentration levels (low, Medium, and high). These results were compared with the nominal concentration, and the results were expressed in percentages. The results, as depicted in Table III, show an accuracy range of 99.8%

to 102.9%. On the other hand, recovery measures how effectively a method extracts an analyte of interest from its matrix. The extent of extraction in a recovery experiment must not be 100%; the extraction must be consistent. Recovery experiments were performed to assess the degree of extraction at three concentration levels of the quality control samples, each measured in six replicates. The evaluation of recovery was based on the reproducibility of the results. Therefore, the coefficient of variance was calculated for each concentration level, as shown in the Table. III, CV ranges (1.3-7.6).



[Fig.6: Stability of Analyte After Keeping Samples at Room Temperature and Prepared Samples in the Autosampler for Six Hours Before Analysis. Error Bars are Standard Deviation (n=6)]

The test of analyte stability is a crucial component of the method validation process. The test evaluates whether analytes remain stable under specific handling or storage conditions. This is important because the stability of an analyte directly impacts the reliability of the analytical method. and accuracy, including the validity of the obtained results.



[Fig.7: The Effect of Three Freeze and Thaw Cycles on Analyte Stability. Error Bars are Standard Deviation (n=6)]

The stability of the analyte was determined after subjecting the samples to three conditions: room temperature (benchtop), autosampler, and freeze/thaw cycles. Stability experiments were done at three levels of concentration, each with six replicates. Results were compared with their nominal values, and stability was expressed as a percentage of the nominal value. The stability between 85% and 115% is acceptable [31]. Therefore, the duration of samples at the benchtop and autosampler did not affect the stability of the analyte (Fig. 6). The slight increase in concentration observed for the low concentration in the autosampler could be attributed to solvent evaporation since the autosampler had no thermostatic control. However, the difference falls within the range of acceptable variation.

The freeze and thaw cycles, however, affected the stability of the analyte, especially at the low concentration level (Fig. 7), where stability decreased with each additional freeze/thaw cycle [32]. This same effect of freeze-thaw on analyte stability was observed in other studies [33]. Given this finding [34], it is recommended that samples with low analyte concentration be analysed fresh or lyophilised before storage.

B. Method Application

MDA-MB 231 cells were exposed to 5-fluorouracil and a drug candidate [chlorido (Fe (III)4Fluoro Salophen)] in a time-dependent uptake experiment. After method optimisation and validation, the method was used to determine the intracellular concentration of these drugs. The results were normalized with the samples' protein concentration and expressed in µg/mg protein content. Time-dependent uptake analysis is relevant for determining the pharmacokinetics of drugs, which define their dose and dosing regimen [35]. Three factors that could significantly influence the results — namely, cell density, the volume of cell culture medium, and drug concentrations — were optimised to ensure that the experiment's results were reliable and reproducible. A high cell density is characterised by pronounced paracrine signalling and contact inhibition, reducing cell proliferation [36]. Both events influence cell metabolism, affecting the cellular response to pharmaceutical and toxicological compounds, as well as cell viability. On the other hand, a smaller volume of cell culture medium will affect the composition of the medium [37], reducing the amount of nutrients and the accumulation of waste material like lactic acid and ammonia [38] Both of which will adversely affect results.

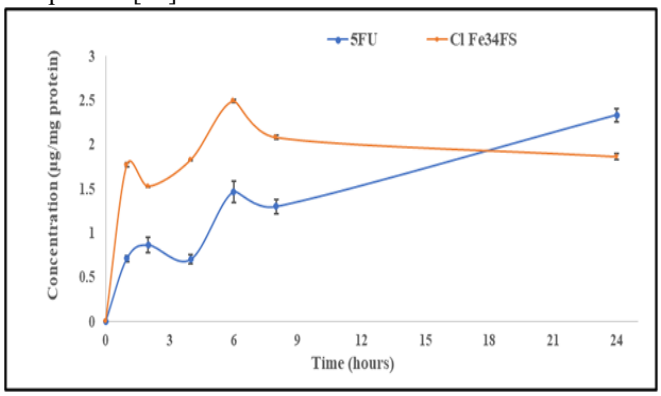
The concentration of drugs to which cells are exposed influences their viability and integrity. High concentrations will be toxic and lethal to the cells. Dead cells do not maintain cell membrane integrity; the drugs initially taken up are eventually lost to the medium when they die. Such events, therefore, falsify the quantity of medications cells take. The optimised conditions were a density of 500,000 cells per well in a six-well plate, 3 mL of culture medium, and sublethal concentrations of 20 µM (5-FU) and 0.5 µM (ClFe34FS).

The results of drug uptake (Fig. 8) show an initial unsteady increase in the uptake of both drugs from time zero to 6 hours. The uptake rate of the iron complex (Cl Fe3 4FS) was initially higher than that of (5FU) up to six hours. After six hours, the uptake of the iron complex entered a steady state, while that of 5FU increased continuously for up to 24 hours. This trend in uptake is consistent with the results of other studies [16]. MAS spectroscopy determines fluorine concentration in samples. Therefore, the authors of the above studies [14] all report the concentration of fluorine in samples, which suffices as in vivo proof of cellular drug uptake

However, this limits the application of their results for this purpose only because the fluorine concentration, although contributed by the drugs, is not equivalent to the actual concentration of the drugs themselves in the samples. A report of the exact concentration of drugs in a sample is essential for several reasons. 1) In vitro results are usually used to predict clinical pharmacokinetic parameters (In vitro/in vivo correlations). To obtain such a correlation, determining the actual in vitro concentration of drugs is critical [39]. 2) The active entities in cells are the drugs, and results that do not reflect their actual concentrations may compromise their efficacy. 3) It is significant for in vivo testing, such as therapeutic drug monitoring (TDM). Reports of actual drug concentrations will be used in both in vivo and in vitro testing. In this study, the molar ratios of fluorine and the compounds were used as factors to convert the obtained fluorine concentrations to the actual concentrations of the active substances 5FU and ClFe34FS. The conversion factors were 6.846 and 22.296 for 5FU and ClFe34FS, respectively. Other researchers used a similar approach to determine cisplatin in biological samples [40]. Results that reflect the actual concentration of drugs are more representative of the actual situation.

Although much of the discussion has focused on the uptake and intracellular concentration, it is worth mentioning that one of the objectives of this experimental design was to test the method's ability to determine the intracellular concentration of both fluorinated organic compounds (5FU) and fluorinated metal complexes (ClFe34FS). Given that the technique passed this test, it extends its applicability and benefit to a broad audience and sectors.

Since 50% of the recently approved drugs are fluorinated [41], the method is probably applicable and beneficial to pharmaceutical industries and regulatory agencies, and university communities, especially faculties interested in or involved in drug development [42]. For clinicians, the method may benefit the therapeutic monitoring of fluorinated drugs. Most persistent organic compounds are fluorinated [45]; therefore, the procedure may also be beneficial to environmental scientists interested in monitoring these compounds [43].



[Fig.8: Direct Determination of the Intracellular Concentrations of 5FU, and ClFe34FS Following a Time-Dependent Uptake of MDA-MB-231 cell line Exposed to 20 µmol L-1 5-FU and 0.5 µmol L-1 ClFe3 4FS. Exposure Times, 0, 1, 2, 4, 6, 8, and 24 Hours Respectively. Error Bars Are the Standard Deviations of Three Replicates]

This method, like any other, has unavoidable flaws or shortcomings. The limitation of the technique is the use of calcium as the molecule-forming reagent. It has been reported that calcium reacts with the furnace's graphite layer, reducing the graphite furnace's life span [44]. This effect is, however, compensated by the novel and optimal sample preparation procedure that effectively extracts these analytes and minimises the deposition of carbonaceous matter in the furnace. The adverse effect of calcium is also compensated for by the reliable results produced by the method.

IV. CONCLUSION

A significant requirement for therapeutic drugs, concerning their efficacy, is the ability to interact primarily with intracellular targets. An HR CS GF-MAS method targeting CaF was developed and used to determine the intracellular concentration of the fluorinated drugs (5FU) and drug candidate (ClFe34FS) in cancer cells exposed to these drugs in a time-dependent uptake experiment. The method was validated to ensure its reliability and accuracy in determining the intracellular concentrations of these fluorinated drugs. The excellent linearity, precision, and sensitivity demonstrate the method's suitability for its intended application.

The application of the validated HR CS GF-MAS method has significant and practical implications for anticancer drug development. The ability of the process to determine the intracellular concentration of fluorinated drugs and drug candidates taken up by cells will provide pertinent insights that will be helpful in optimising dosing regimens and enhancing drug efficacy. The method's ability to accurately and sensitively determine the intracellular concentration of fluorinated drugs makes it a valuable tool for generating reliable data that informs the development of effective therapies for combating cancer.

DECLARATION STATEMENT

After aggregating input from all authors, I must verify the accuracy of the following information as the article's author.

- **Conflicts of Interest/ Competing Interests:** Based on my understanding, this article has no conflicts of interest.
- **Funding Support:** This article has not been funded by any organizations or agencies. This independence ensures that the research is conducted with objectivity and without any external influence.
- **Ethical Approval and Consent to Participate:** The content of this article does not necessitate ethical approval or consent to participate with supporting documentation.
- **Data Access Statement and Material Availability:** The adequate resources of this article are publicly accessible.
- **Author's Contributions:** The authorship of this article is contributed equally to all participating individuals.

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