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Quantitative determination of cyanoacetic acid content in teriflunomide drug substance by ion chromatography using conductivity detector

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Abstract

Background: The current study focuses on the development and validation of an analytical method for quantifying cyanoacetic acid (CAA) in teriflunomide drug substance using a high-performance ion chromatography (IC) with cation suppressed conductivity detection (TFM). Water was used as the diluent for preparing the sample solution, which was injected into a standard chromatographic device with 250 mm, 4.0 mm ID, and 5.0 µm particle size Metrosep A Supp 5 Ion exchange column and a suppressed conductivity detector. At a flow rate of 0.6 mL min^{-1} and a temperature of 40 °C, the mobile phase was delivered in an isocratic mode.

Results: CAA and TFM had retention times of 12.78 and 15.82 min, respectively. CAA has a limit of detection (LOD) of 33 µg/g and a limit of quantification (LOQ) of 101 µg/g, respectively. For LOD and LOQ accuracy, the percentage RSD of CAA is 1.7 and 1.2, respectively. The average CAA recovery percentage was found to be between 98.6 and 100.1%. With a value of 0.9998, the calibration curve yielded an excellent linear correlation coefficient for CAA. According to the ICH guidelines, all verification parameters are within the range, indicating that the system is stable.

Conclusion: The elution time and run time in the currently developed ion chromatography analytical method have been reduced, demonstrating that the method is cost-effective and generally accepted, as well as simple and functional, and can be used in routine quality control tests in the industry.

Keywords: Cyanoacetic acid, Teriflunomide, Ion chromatography, Validation, Conductivity detector

Background

Chemically, teriflunomide (TFM) (Fig. 1) is known as (2Z)-2-cyano-3-Hydroxy-N-[4-(terifluoromethyl) phenyl]. Butenamide-2-Butenamide-2-Butenamide-2-Butenamide-2-But C12H9 F3N2O2 is its molecular formula, with a molecular weight of 270.21 g/mol [1]. It is the active metabolite of leflunomide [2], and it inhibits pyrimidine synthesis, acting as an immunomodulatory agent. TFM is the primary active metabolite of leflunomide, and it is responsible for teflunomide's in vivo activity [3–6]. It has been studied as a potential treatment for multiple sclerosis (MS). The disease process in MS is stopped swiftly by dividing cells like activated T cells. It can minimize the risk of infection in comparison with medications similar to chemotherapy [7] because of its modest impact on the immune system. The first Sanofi to market was under the Aubagio brand name. Aubagio shall be taken orally at a dose of 14 mg once a day. The adverse effects of TFM include liver disorders,

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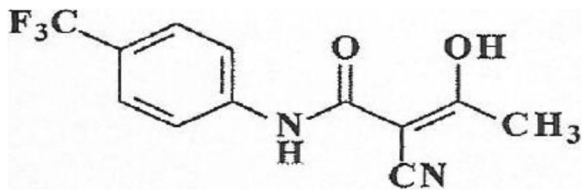

Fig. 1 Chemical structure of teriflunomide

influenza, hair loss or thinning hair, nausea, diarrhea, burning or prickly skin, and numbness or tingling in your hands or feet that is not related to your MS symptoms. The US Food and Drug Administration approved the treatment in 2012 [8] and the European Union in 2013 [9], respectively.

Cyanoacetic acid (CAA) (Fig. 2) is used as a precursor in the production of TFM. CAA is coupled with another beginning material, aniline 4-(tripfluoromethyl) to make intermediate (i.e., 2-cyano-N-(4-trifluoromethyl) phenylacetamide). This intermediate combines with acetyl chloride to produce TFM in the presence of sodium hydroxide and acetone. The synthesis pathway of TFM is shown in Fig. 3. During the entire procedure, CAA traces may be present in TFM. In terms of safety levels, therefore, the CAA control is needed in TFM. CAA is measured by a limit of 500 μg/g much below the respective threshold criterion. Various approaches have been found in the literature review to estimate TFM in API, commercial formulations, and biological fluids. Comprehensive information on the many methods accessible can be obtained by chromatographic methods such as HPLC [10], HPLC [11–14], LC MS [15–19], and CAA material [20–23] by various methods. Ion chromatography (IC) [24–29] has recently emerged as a popular analytical tool for determining inorganic cations and organic acids in a variety of matrices. As

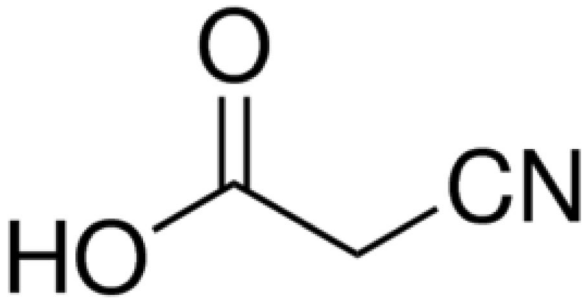

Fig. 2 Chemical structure of cyanoacetic acid

far as we are aware, no IC experiments have been made to date in order to identify CAA content in TFM. In this context, we have developed a Green Ion Chromatography technique for the determination and validation of CAA in TFM in compliance with ICH and the FDA guidelines [30–32] with a reduced run time. CAA content detection process is relatively sensitive to ion-exchange chromatography together with the analytical conductometric detector approach developed and validated that may simply be deployed in routine testing and reporting quality control laboratories.

Methods

Chemicals, reagents, and standards

Teriflunomide and its impurities, which include the 2-Isomer of teriflunomide, the 3-Isomer of teriflunomide, and N-[4-(Trifluoromethyl)phenyl] 2-Cyano-N-[4-(Trifluomethyl)phenyl]-acetamide, 4-(Trifluomethyl)aniline, 2-Cyano-N-[4-(Trifluomethyl)phenyl]-acetamide and ethyl teriflunomide were gifted by Aurobindo Pharma Limited. From Sigma-Aldrich procured acetic and cyanoacetic acids. Analytical reagent grade sodium carbonate, sodium bicarbonate, and sulfuric acid were purchased from Merck India, and HPLC grade water was prepared using Milli Q-Water system.

Chromatographic conditions

An ion chromatograph (Metrohm 930 compact IC Flex) with conductometric detector and Metrohm 863Compact Auto sampler or equivalent with Magic IC Net 3.0 is used. And a Chromeleon 6.8version Ion Chromatograph (Dionex ICS5000) with conductivity detector and AS-AP Auto Sampler or equivalent (for Ruggedness). Metrosep A Supp 5, 6.1006.530, (250 mm × 4.0 mm) 5 μm polyvinyl alcohol particles with quaternary ammonium groups were used in the panel. The mobile phase contains 504 mg sodium bicarbonate and 53 mg sodium carbonate in 1000 mL of water that has been filtered through a 0.45-micron porosity membrane. For the Metrohm system, the suppressor regeneration solution is 2.8 mL of sulfuric acid in 1000 mL of water and for the Dionexsystem and 2.0 mL of sulfuric acid in 4000 mL of water. Mill Q-Water used as diluent. The chromatographic conditions are shown in Table 1.

Analysis performed by using suppressor

Preparation of standard solution

A stock solution (0.0005 mg/mL) was prepared by accurately weighing and transferring about 50 mg of CAA reference standard into a 100 mL clean dry volumetric flask added with 70 mL of diluents and

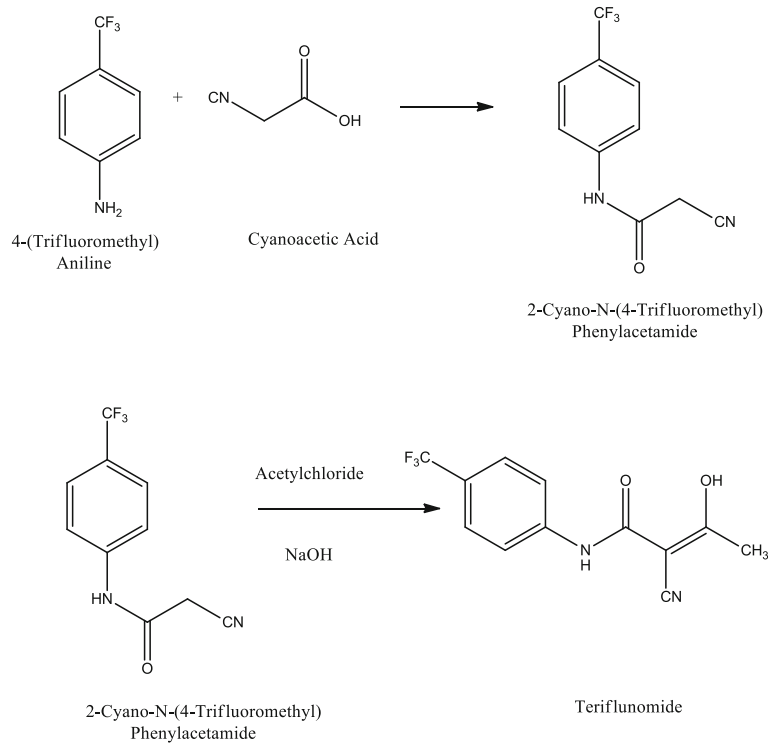

Fig. 3 Typical synthesis scheme of TFM

sonicate to dissolved make up to volume with diluent. Diluted 5 mL of this solution to 100 mL with diluents. Further diluted 2 mL of this solution to 100 mL with diluent.

Preparation of sample solution

Accurately weighed and transferred about 50 mg of sample in 50 mL clean dry volumetric flask added with 30 mL of diluent and sonicated for 3 min make up to volume with diluents. Filtered through 0.45 μm porosity membrane filter.

Method development and optimization

CAA is a precursor in the synthesis of TFM. Method development for quantification of CAA content in TFM started with a solubility of CAA and drug substance, based on the solubility study water, is selected as diluent. Preliminary tryouts were carried out based on the retention of CAA and peak shape with different columns using like Metrosep Super-Sep, Metrosep A Supp 3, Metrosep Anion Dual 2, and Metrosep A Supp 5 column, and different mobile phase combinations were used to investigate the evaluation of the analyte with sodium carbonate, sodium bicarbonate, formic acid, potassium phthalate, and octane-1-sulfonic acid sodium salt. A better chromatographic separation occurred at 504 mg of sodium bicarbonate and 53 mg of sodium carbonate in 1000 ml of water buffer, at a flow rate of 0.6 mL min^{-1} with Metrosep A Supp 5, 6.1006.530, (250 mm × 4.0 mm) 5 μm at 40 °C temperature.

Table 1 Chromatographic conditions

Column	Metrosep A Supp 5, 6.1006.530,(250 mm × 4.0 mm) 5 μm
Flow	0.6 mL/min
Column oven temp	40 °C
Injection Volume	100 μl
Data acquisition time	30 min

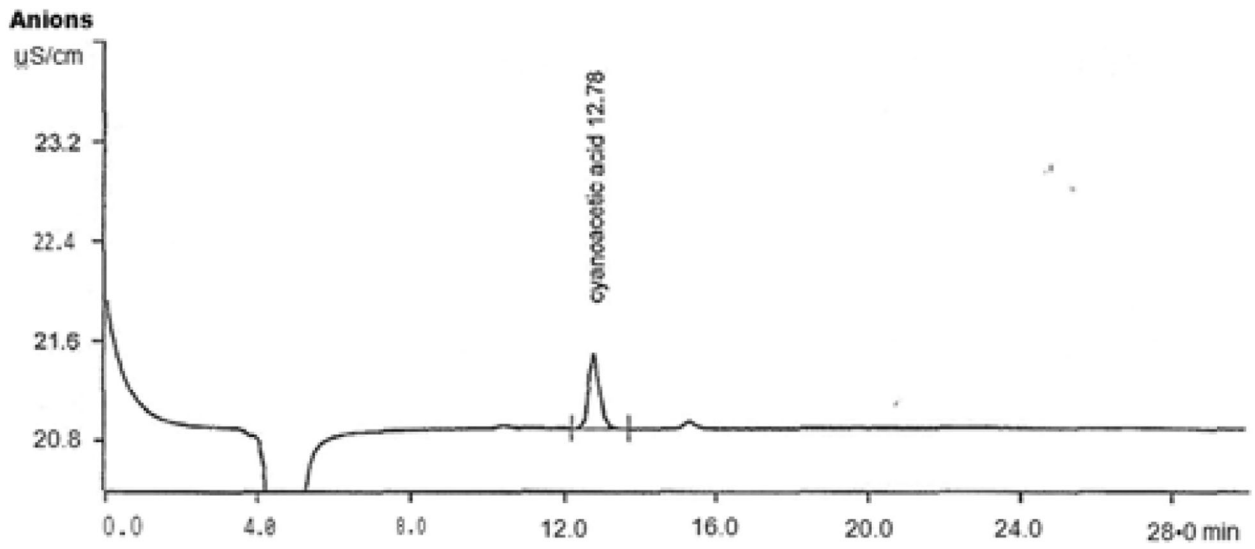

Fig. 4 A typical representative chromatogram of CAA standard

Results

Method validation

The objective of this research work was to quantitatively determine CAA in TFM. According to ICH [31] and FDA [32, 33], the key analytical parameters that require for validation were Accuracy, Precision, Linearity, Recovery, Limit of Detection, Limit of Quantification, and Ruggedness.

Specificity

Specificity's special feature is the method's capacity to quantify analyte in the presence of all possible contaminants. The retention time (RT) of the standard analytical solution and the sample solution (CAA-spiked solution) was identified according to the test technique and injecting into IC according to the methodology. Standard retention time of 12.78 min

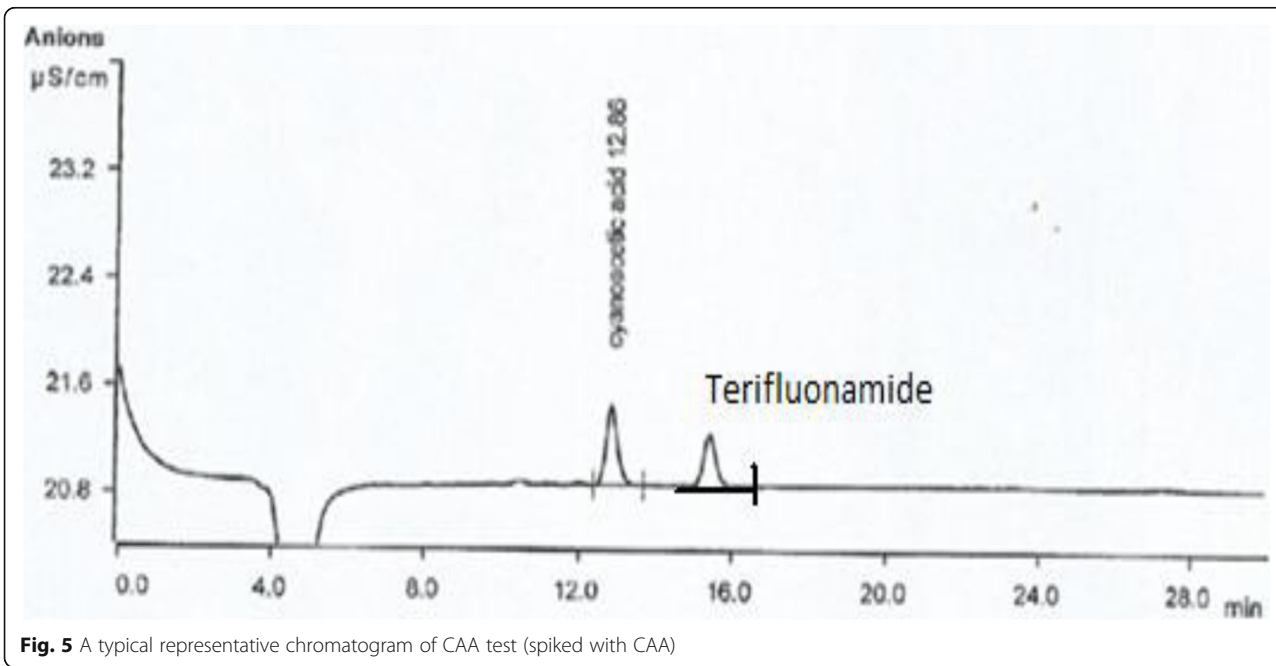

Fig. 5 A typical representative chromatogram of CAA test (spiked with CAA)

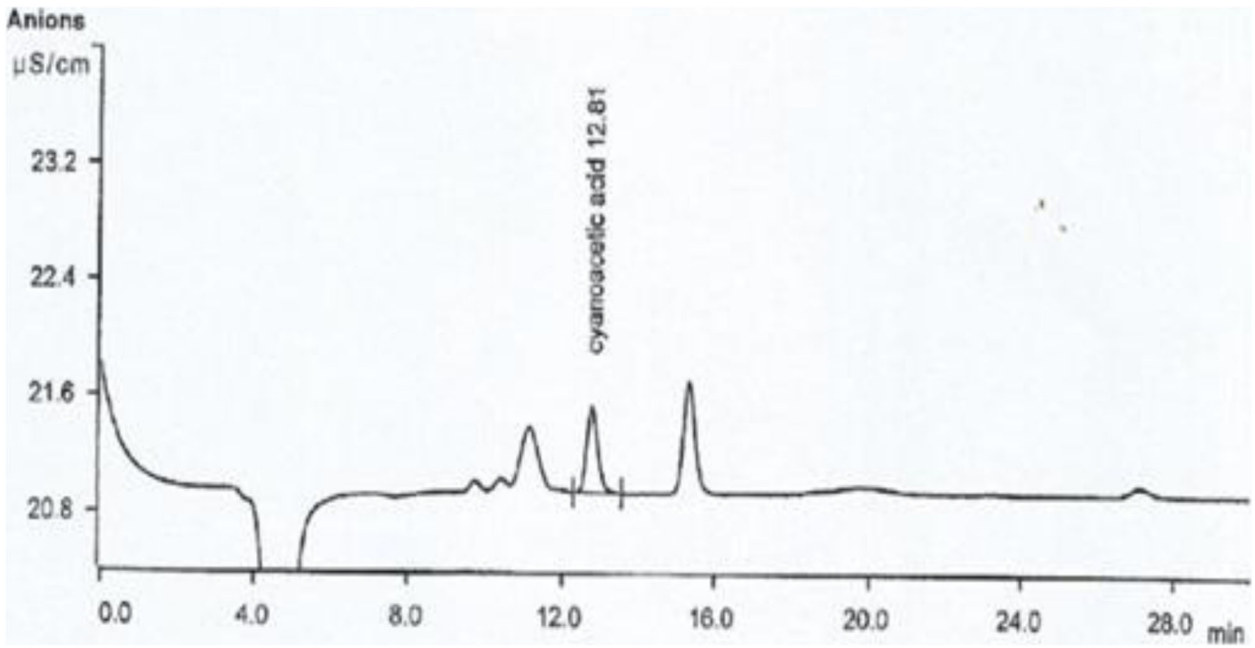

Fig. 6 A typical representative chromatogram of all known related substances

and sample solution of 12.86 min were recorded. For specificity determination, the interference of diluents peaks and determination of CAA were studied. TFM spiked with all known related substances 2-Isomer of terif1unomide (0.15 mg), 3-Isomer of teriflunomide (0.15 mg), N-[4-(Trif1uoro-methy1)phenyl]-acetamide (0.15 mg),4-(Trif1uoromethyl)aniline (0.10 mg),2-Cyano-N-(4-(Trif1uoro-methyl)phenyl]-acetamide (0.10 mg), and Ethylteriflunomide (0.10 mg) and residual impurities which may interfere with cyatioacetic acid [Aceticacid (0.05%)] including CAA at about specification level are prepared in triplicate and injected into IC as per methodology. CAA peak only integrated in chromatograms. Specificity chromatograms have been shown in Figs. 4, 5, and 6 correspondingly. From the above data, there is an absence of interference in the presence of all known related substances and residual impurities with CAA. Hence, it was concluded that the method is specific for the determination of the content of CAA in TFM.

Linearity

Linearity is a requirement in correlation and linear regression analysis. To prove this, the CAA reference standard was used to develop a series of solutions at the concentration levels ranging from 10 to 150% of the specification level injected into IC as per methodology. The linearity was determined from this data

Table 2 Statistical data of linearity

Sample ID (%Level)	Concentration (µg/mL)	Area [(µS/cm × min]	Statistical analysis	
LOQ level	0.101	0.0373	Slope	0.396
25% level	0.126	0.0506	Intercept	− 0.001
50% level	0.252	0.1004		
75% level	0.377	0.1483	STEYX	0.002
100% level	0.503	0.197		
125% level	0.629	0.2478	Correlation coefficient	0.9998
150% level	0.755	0.3000		

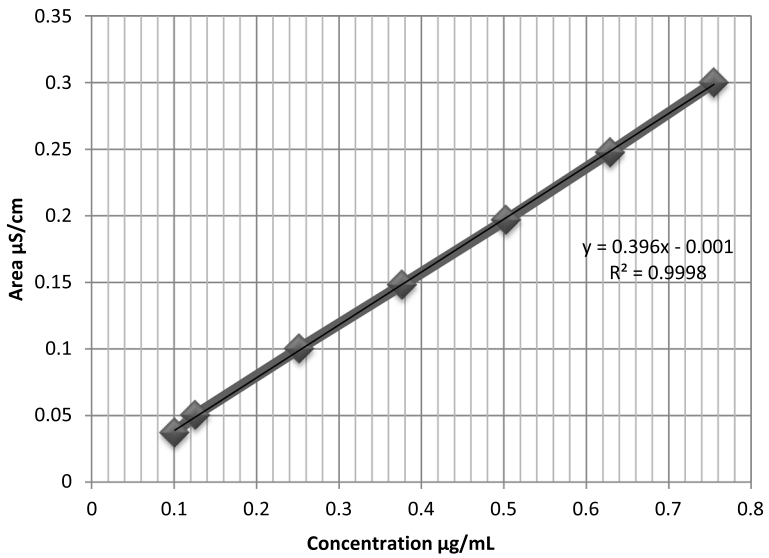

Fig. 7 A typical representative linearity plot (concentration vs area)

following the establishment of the LOQ level from LOQ to 150% of the level and shown below. The correlation coefficient of acceptance criteria must be more than 0.990 and the value achieved was 0.9998. Table 2 represents the linearity data and Fig. 7 shows the linearity graph.

LOD and LOQ

LOD and LOQ are terms used to describe the smallest analysis concentration, which can be detected consistently by an analytical procedure. Linearity data were forecasted to predict the detection limit (LOD) and the quantification limit (LOQ) values for the CAA. By developing solutions for these projected concentrations, each predicted concentration has been precisely validated and each solution has been injected six times into IC, depending on the procedure. The approval requirement for RSD is not more than 10.0% for LOQ and 33.0% for LOD. The LOQ and LODs for CAA listed below are not more than 0.05% below the specified threshold. Therefore, the testing procedure is accurate for measuring CAA at the LOQ and LOD TFM values indicated. LOD and LOQ precision data are made known in Table 3.

Table 3 Representation of LOD and LOQ results

Injection ID	Area {(µS/cm) × min}	
	LOD	LOQ
1	0.0113	0.0372
2	0.0118	0.0374
3	0.0111	0.0372
4	0.0116	0.0371
5	0.0115	0.0382
6	0.015	0.0369
Mean	0.0115	0.0373
SD	0.0002	0.0005
%RSD	1.7	1.3
Conc. (µg/ml)	0.033	0.101
Conc. (µg/g) w.r.t sample	33	101

Accuracy (recovery)

A test method is said to be accurate when it measures what it is supposed to measure. That means it is able to measure the true amount or concentration of a substance in a sample. To demonstrate accuracy, sample solutions were prepared in triplicate using TFM as such and spiked with a known amount of CAA at about LOQ level 50%,l00%, and 150% of specification level as per the test method and injected each solution into IC as per methodology. The acceptance criterion is that recovery should be between 80 and 120%. The data concluded that the average recovery of CAA is 99.3% and in the well in the acceptance limit. Data are shown in Table 4.

Precision

The equipment and the technique were tested for their precision. Precision examination of the system

Table 4 Representation of accuracy results LOQ, 50% to 150% level

% level/sample ID	Amount added (μg/g)	Amount found (μg/g)	% recovery (between 80 and 120%)	Average recovery	%RSD
LOQ	99	97	98	98.7	0.6
	100	99	99		
	98	97	99		
50%	247	246	99.6	100.1	0.6
	245	245	100		
	248	250	100.8		
100%	497	496	99.8	98.6	1.7
	490	474	96.7		
	490	486	99.2		
150%	747	742	99.5	99.6	0.4
	740	734	99.2		
	741	741	100		

with repeatability and reproducibility (ruggedness). The efficiency of the procedure was tested with replicate injections of normal and sample solutions. The efficiency of the ion chromatography system was evaluated six times throughout the day in chromatographic settings with a standard solution (system precision). The relative standard deviation of CAA is 0.5%. The intraday (method accuracy) variance was the repeatability and the relative standard variance for CAA content was 0.8%. The interday variance (roughness) gave intermediate precision and was 1.7% relative standard deviation for CAA content. By assessing six sample solutions independently and adding CAA at a predetermined amount, the reproducibility and the reproduction of the procedure in various settings, the degree of reproductivity gained through the analysis of the same sample (used under the method precise) utilizing separate column series, with a different analyst preparing fresh standards and new mobile phases on different days, has been identified as a robust procedure. Table 5 show the precision (system precision, method precision, and ruggedness) experiment performance.

Table 5 Statistical data of precision for CAA

System precision		Method precision	Intermediate precision
Injection No	CAA area	CAA (μg/g)	CAA (μg/g)
1	0.1993	502	484
2	0.1999	497	488
3	0.1977	504	478
4	0.1986	502	494
5	0.1985	502	485
6	0.1983	509	501
Mean	0.1987	503	488
SD	0.001	3.88	8.12
%RSD	0.5	0.8	1.7

Robustness and system suitability

To demonstrate robustness, a standard solution (for evaluating system suitability) and a sample solution spiked with CAA at specification level were prepared according to the test method and injected into the IC under various intentionally varied conditions to assess system suitability and the method's ability to remain unaffected. The altered conditions include a 10% change in flow rate, a 5 °C increase in column oven temperature, and column variance. The column quality, as calculated by the CAA peak, must be no less than 4000 theoretical plates and the asymmetry must be not more than 2.0. Furthermore, the RSD for six injections of the regular solution in peak areas is less than 5.0%. The outcomes of these experiments are summarized in Tables 6 and 7 below.

The outcomes of the device suitability tests at each of the different conditions met the test procedure's specifications. Also, the chromatograms of TFM spiked with CAA at specification level obtained from the various robustness conditions outlined above show that the RT of CAA obtained

Table 6 Representation of overall statistical data of ruggedness

Parameter	Variation	System suitability		
		Theoretical plates	Asymmetry	%RSD
STP*	–	6371	1.6	1.0
Flow rate*	− 10%	6696	1.5	0.1
	+ 10%	5778	1.6	0.9
Temperature*	− 5 °C	6430	1.5	0.2
	+ 5 °C	6337	1.5	0.2
Column lot variation**	Batch/lot variation	7411	1.3	0.6

*1st column, **2nd column

at each of the varied conditions is not significantly different from that of the STP condition. As a result, the test method is found to be reliable for determining CAA content in TFM across the range of changes investigated for each of the above parameters.

Discussion

Ion chromatography, a form of liquid chromatography, determines ionic group concentrations on the basis of their interplay with a resin. Column components absorb the ions while the solution of the sample passes through a pressured column. When the eluent or ion extraction liquid flows through the column, the separation starts from the column. Different retention durations show various compounds in the sample and simultaneously measure ion concentrations in the sample. A detector result record called a chromatogram comprises of electrical conductance vs. time when the analyte passes through the chromatography apparatus. Suitable and stability signifying Ion chromatography method was developed with Metrosep A Supp 5, 6.1006.530, (250 mm × 4.0 mm) 5 µm polyvinyl alcohol particles with quaternary ammonium groups and with carbonate buffer containing a mixture of sodium carbonate (Na2CO3) and sodium bicarbonate (NaHCO3) dissolved in water to quantitatively determine CAA in TFM. Isocratic elution mode selected at 40 °C temperature with 0.6 mL flow rate.

CAA was determined using several analytical methods in diverse matrices. On the other hand, there is no IC method reported for the determination of CAA in any matrix according to the literature. Since no solvent was needed in this IC approach for estimating CAA material, it was an ecologically benign IC procedure with a quick 30-min running time. This IC technique is suitable for daily analysis. Recorded RT was 12.8 min for CAA and 15.8 min for TFM, which indicated that the retention period was robust and successful. As a result, it is possible to analyze a large number of samples is possible. The approach is linear with a coefficient of correlation (r^2) of 0.9998 (Fig. 7 and Table 2). The intraday and interday relative standard deviations were both less than 1.7% (Table 5). LOD as low as 33 µg/g and LOQ as 101 µg/g were estimated to identify and quantify markers in a resolution sample (Table 3). Excellent recovery was also achieved in acceptable limits for the presented approach (Table 4). The observed validation parameters and statistical data were within the limits of acceptance for ICH and USP [31, 32, and 33]. Table 8 highlights the experimental results compared to other approaches provided in the literature.

Conclusions

A simple and sensitive ion chromatography method was developed and validated for the concurrent

Table 7 Representation of overall statistical data %RSD for ruggedness

Variation		Standard area								
		1	2	3	4	5	6	Mean	SD	%RSD
Standard conditions		0.1593	0.1629	0.1629	0.1628	0.1631	0.1637	0.1625	0.0016	1.0
Flow rate (0.6 mL/min)	− 10%	0.1844	0.1846	0.1843	0.1843	0.1846	0.1849	0.1845	0.0002	0.1
	10%	0.1502	0.1488	0.151	0.1501	0.1515	0.1524	0.1507	0.0013	0.8
Column oven temp (40 °C)	− 5	0.1661	0.1667	0.1664	0.1665	0.1671	0.1667	0.1666	0.0003	0.2
	5	0.1661	0.1657	0.1653	0.1657	0.1653	0.1652	0.1656	0.0003	0.2

Table 8 Comparison of various methods for determination of CAA with published results in the literature

Method	Conditions	Result	Reference
UV-spectrophotometer	Diazosulfanic acid + sodium hydroxide solution, read at 490 nm	Read against reagent blank and test solution	[20]
Calorimetric method	Diazosulfanic acid + sodium carbonate solution	Read against reagent blank and test solution	[22]
Gas chromatography method	Column, 1 m (3–4) mm; sorbent, 10% poly(ethylene glycol) sebacate on Polysorb I; temperature regime: column thermostat, 100 °C; DTP detector, 150 °C; evaporator, 300 °C; detector current, 190 mA; carrier gas (helium) flow rate, 30 ml/min; sample volume 0.4 μl	Recovery ranges from 97.95 to 101.1%	[21]
High-performance liquid chromatography	C18 column(250 mm × 4.6 mm, 4 μm), mixture of 0.02 mol/L potassium dihydrogen phosphate and methanol (volume ratio of 90:10) with pH 2.0, UV detection at 228 nm	Linear correlation of the method was 0.9999, the standard deviation was 0.20 and the average recovery was 99.78%.	[24]
Ion chromatography	The column was a METROSEP A SUPP 5, 6.1006.530, (250 mm × 4.0 mm) 5 μm particles of Polyvinyl alcohol with quaternary ammonium groups. The mobile phase has consists of 504 mg of sodium bicarbonate and 53 mg of sodium carbonate in 1000 mL of water, conductivity detector	LOD and LOQ 33 μg/g and 101 μg/g respectively, average recovery 98.6% to 100.1% and linearity 0.999	Present work

determination of CAA in TFM. The results of various validation parameters demonstrated that the method is specific, stability indicating sensitivity, linear, precise, and accurate. The proposed method is sensitive, simple, and user-friendly for the determination of CAA content in TFM.

Abbreviations

LOD: Limit of detection; LOQ: Limit of quantitation; IC: Ion chromatography; ICH: International Council for Harmonization; FDA: Food and Drug Administration; R^2: Coefficient of determination; TFM: Teriflunomide; CAA: Cyanoacetic acid; RT: Retention time

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Authors' contributions

MK has analyzed samples on IC instrument and completed the experimental work of the drug substance of Interest. He had completed his work under the supervision of KS and TS who helped him to detail the methodology as well as theoretical advance. CS helped him in preparing the manuscript and all authors (MK, TS, CS, and KS) read the manuscript and approved it.

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Availability of data and materials

All data and materials are available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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