Journal of Chromatographic Science, 2026, **64**(9), bmag030
https://doi.org/10.1093/chromsci/bmag030
Published: 18 October 2026
Article

Glycopyrronium bromide, formoterol fumarate dihydrate and their related compounds with UPLC/PDA-MS for simultaneous determination in pressurized metered-dose inhaler product

Serdar Seckin[1,2,*] **and Serap Saglik Aslan**[3]

[1]Analytical Chemistry, Institute of Health Sciences, Istanbul University, 34126, Beyazıt, Istanbul, Türkiye
[2]DEVA Holding A.S., R&D Center, Karaağaç Mh. Fatih Blv. No: 26, Kapaklı, 59510, Tekirdağ, Türkiye
[3]Department of Analytical Chemistry, Faculty of Pharmacy, Istanbul University, Beyazıt, 34116, Istanbul, Türkiye
*Author to whom correspondence should be addressed. Email: serdarseckin59@gmail.com

An ultra-performance liquid chromatography (UPLC) technique with photodiode array-mass spectrometry (PDA-MS) detectors has been developed and validated for the determination of glycopyrronium bromide (GB), formoterol fumarate dihydrate (FFD) and their related substances in pressurized meter dose inhalers. This marks the first time the method has been implemented. Chromatographic analyses have been carried out on a UPLC system with a single quadrupole mass spectrometer (QDa) and a PDA detector. A C18 column has been used prior to the injection valve. The column temperature was set to 40°C. For each analysis, 1 μL of sample was injected at a flow rate of 0.1 mL/minute. The mobile phases used were A (0.5% formic acid in methanol) and B (0.5% formic acid in water). The total run time of the method was 30 minutes. The PDA detector was employed at 215 nm while the mass detector was set to extracted ion chromatography (XIC) mode. The range for detecting impurities of GB was found to be between 0.28 and 0.30 ng/mL, with a limits of 0.92–1.01 ng/mL for quantification. GB and FFD demonstrated linear accuracy. We studied the accuracy of related substances within the limit of quantification value range of corresponding impurities and up to 400% of the limit value. The method we used was linear, rapid, reproducible and accurate. Our proposed method is fast, linear, accurate and reproducible. The UPLC-MS method, which utilized QDa with XIC mode, was developed to ascertain the related substances and assay of GB and FFD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a serious group of diseases involving chronic and recurrent airflow obstruction in the lungs (1–4). The main symptoms are shortness of breath, cough and sputum production. There are four main types of COPD: chronic bronchitis, bronchiectasis, emphysema and bronchial asthma (5). Breathlessness in asthmatics is due to narrowing of the airways, and emphysema in bronchiectasis is due to deformation of the elastic structure of the lungs (6, 7).

The use of tobacco and tobacco products is the main cause of the disease. There are also less potent causes such as genetics (8). In most developing countries, smoke from cooking and heating fires without proper ventilation causes air pollution. Prolonged exposure to such irritating and corrosive substances causes exacerbations in the lungs, further narrowing the existing small airways and causing tissue destruction (9). Diagnosis can be made by simple lung function tests that can measure airflow (10).

Glycopyrronium bromide (GB) is a long-acting antimuscarinic agent and generally has a similar affinity for the anticholinergic M1 and M5 subtypes of muscarinic receptors. It exerts its pharmacological action by inhibiting the M3 receptor in the airways, causing bronchodilation in smooth muscle. Preclinical *in vitro*/*in vivo* studies have shown that the bronchoconstrictive effects of acetylcholine are dose-dependent and last longer than 12 hours. Bronchodilation is a predominantly site-specific effect following glycopyrronium inhalation. Glycopyrronium is a long-acting muscarinic receptor antagonist that works by blocking the bronchoconstrictive effect of acetylcholine on airway smooth muscle cells. Of the five known muscarinic receptor types, M1–M5 has a defined physiological function only in the M1–M3 human lung. Glycopyrronium has been shown to reduce the frequency of attacks and improve symptoms in moderate and severe COPD patients. Formoterol fumarate dihydrate (FFD) is a long-acting selective beta2-adrenergic agonist with a rapid onset of action. Inhaled formoterol fumarate has bronchodilator properties in the lungs. The pharmacology of beta2-adrenoceptor agonists, in which formoterol fumarate is included, may be the stimulation of adenyl cyclase, the enzyme that catalyzes the conversion of adenosine triphosphate to cyclic 3′, 5′-adenosine monophosphate. With increasing frequency, adenosine monophosphate levels cause relaxation of the smooth muscles in the bronchi. Immediately, the release of mediators is inhibited. When inhaled, it becomes a potent

Received: 24 May 2024. **Revised:** 7 September 2026. **Accepted:** 14 September 2026
© The Author(s) 2026. Published by Oxford University Press.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (https://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact reprints@oup.com for reprints and translation rights for reprints. All other permissions can be obtained through our RightsLink service via the Permissions link on the article page on our site—for further information please contact journals.permissions@oup.com.

and selective beta2-adrenergic receptor stimulant. It has a bronchodilator effect in patients with reversible airway obstruction. Its effect is rapid (within 1–3 minutes) and long lasting. It is still effective 12 hours after inhalation. A well-known combination drug on the market containing GB and FFD in one strength is Bevespi Aerosphere™ (11).

Many analytical methods have been published in the literature for the determination of GB and FFD and their related substances or metabolites in drug products or biological matrices using high performance liquid chromatography (HPLC) and different types of detectors such as electrochemical, Ultra Violet (UV) and coulometric. It was found that there are many published articles suggesting the HPLC methods with UV detector for single or combined determinations of GB or FFD in pressurized meter dose inhaler (pMDI) products or active ingredients by using reversed-phase stationary phase and different types of mobile phases (12–22). In these methods, active ingredients (GB and FFD) and some of their corresponding related substances or metabolites were mainly analyzed. There are also some groups using coulometric or electrochemical detectors integrated with HPLC to analyze these compounds (23). Mass spectrometry (MS) has been used to achieve lower detection levels for compounds of interest by using fast gradients with relatively short columns for detection in biological compounds (24, 25).

Products containing multiple active ingredients in a pMDI formulation present a challenge due to the potential for a large number of impurities to arise from each active ingredient, thereby increasing the number of excipients in the matrix. It is therefore important to use a single, reliable method to quantitatively analyze the active ingredients and their associated impurities in a given pharmaceutical product. As determining impurity levels in pharmaceutical products is crucial, there is a need for rapid, accurate and reliable analytical methods to analyze active ingredients and their impurities simultaneously. Of the methods mentioned above, only one has been developed and validated in accordance with global regulations. Compared to the published Reversed Phased (RP)-ultra-performance liquid chromatography (UPLC) method, this study offers a 10-minute advantage in run time for a single injection while enabling the detection of 23 impurities at lower concentrations—a capability not found in any previous method. Using UPLC-quadrupole mass spectrometer (QDa) has many advantages over other techniques, particularly the ability to analyze multiple compounds simultaneously with high sensitivity [low limit of quantification (LOQ) values] and selectivity. Currently, none of the UPLC-QDa methods simultaneously monitor GB, FFD and their impurities (see Tables I and II). The method presented here enables the simultaneous analysis of active pharmaceuticals and their impurities using a single system. This allows for efficient use of time, which is critical in the pharmaceutical industry.

The aim of the present study was to develop a faster, more sensitive and accurate UPLC-QDa method for the quantification of GB, FFD and their corresponding impurities in drugs. The proposed method showed improved performance in terms of sensitivity, selectivity and efficiency due to shorter run time with simplicity of sample preparation. Furthermore, this method has advantages over other similar methods in terms of environmental concerns as it has a lower mobile phase flow rate and organic phase ratio.

Experimental

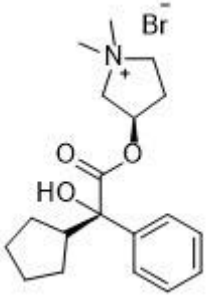
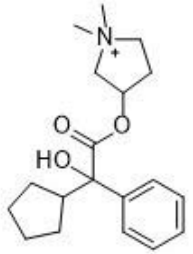
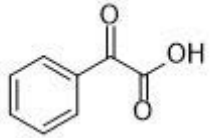
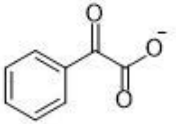
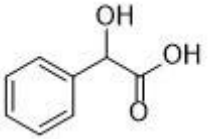
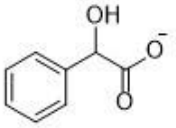
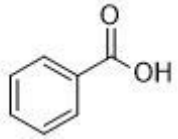
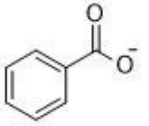
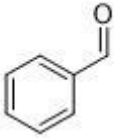
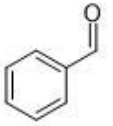
Reagents

All chemicals used were appropriate for MS. We used high-quality methanol (MERCK GMBH, Germany), formic acid American Chemical Society (ACS) reagent ≥97%) from Sigma-Aldrich in Steinheim, ultra-gradient acetonitrile (Sigma-Aldrich, Steinheim, Germany) and Milli-Q (Millipore, Billerica, MA) water of UPLC grade to create the mobile phases and diluents. GB RS and FFD RS compounds are both available with certified purity levels of 100%. GB RS is composed of (RS)-[3-(SR)-hydroxy-1, 1-dimethylpyrrolidinium bromide] α-cyclopentylmandelate, while FFD RS is made up of a dihydrate salt of (±)-2′-hydroxy-5′-[(R*)-1-hydroxy-2-[[(R*)-p-methoxy-α-methylphenethyl] amino] ethyl] formanilide fumarate in a 2:1 ratio. Impurities are present in substances used for pharmaceutical purposes in the European Pharmacopeia (EP) 9.0. The impurities present are GB B, C, D, E (with unknown structure), F, G, H, I, J, K, L, M, N and O. For substances used for pharmaceutical purposes in the EP 9.0, FFD impurities A, B, C, D, E, G, H and I have been used (12, 13). Standards of GB impurities B, C, D, G, H, J, K, M and O RS certified by LGC GmbH, Luckenwalde, Germany, GB impurity F RS certified by Synzeal, Gujarat, India, GB impurity I and GB impurity L RS certified by United States Pharmacopeia (USP) Reference Standard (RS), USA and GB impurity N RS certified by EP RS, Strasbourg, France. Standards of FFD impurities A, B, C, D, E, G and H RS certified by Analytica Chemie Inc., Bengaluru, India and FFD impurity I RS certified by EP RS, Strasbourg, France. Bevespi Aerosphere® inhaler, containing 9/4.8 μg per puff (made by AstraZeneca, USA), was acquired from the UK.

Instruments and analytical conditions

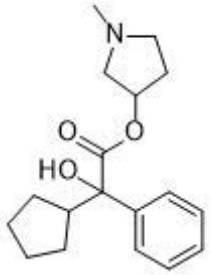
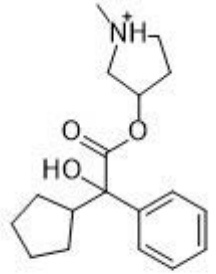
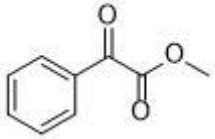
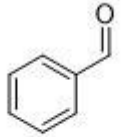
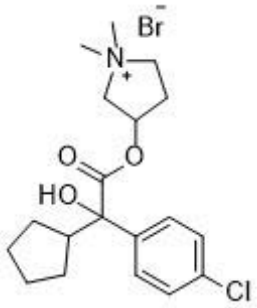
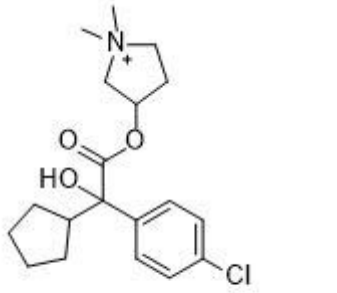
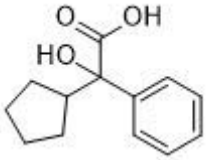
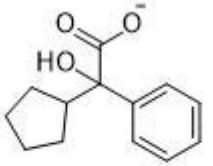
The separation process used a Waters UPLC system from MA, USA. This system was connected to the quaternary solvent manager Acquity Liquid Chromotography (LC) pumps, Acquity sample manager, Acquity QDa detector and Acquity photodiode array (PDA) detector, all from MA, USA. Additionally, the separation process used an Acquity column oven from MA, USA. The mobile phase A used a 0.5% (v/v) solution of formic acid in methanol. The mobile phase B used a 0.5% solution of formic acid in water. The system's gradient programs were as follows: from 0 to 12 minutes, there were 5–10% of A; from 12 to 18 minutes, there were 10–90% A; from 18 to 22 minutes, there were 90% A constantly; from 22 to 26 minutes, there were 90–50% A and from 26 to 30 minutes, there were 50–5% A, at a flow rate of 0.1 mL/minute. The analytes were separated on the mobile phase of an Acquity Ethylene Bridged Hybrid (BEH) C18 column (100 × 2.1 mm; particle size: 1.7 μm). The column was operated at 40°C, and 1 μL was the injection volume. The Waters Mass Spectrometer, based in MA, USA and fitted with an Electrospray Ionization (ESI) source working through positive and negative ionization modes, was deployed for QDa detection. Analysis was conducted in the extracted ion chromatography (XIC) mode, with a retention time (RT) of 50–100 ms. The Empower 3 software, MA, USA, was implemented for data acquisition and processing while the optimized ion source parameters comprised probe temperature at 600°C, 2.0 L/minute nitrogen gas flow, 5 kV capillary voltage and drying gas flow of 10 L/minute; heating block

Table 1 GB and its related substances; structure, formula and mass spectral data.

Name	Structure	Chemical name and formula	MS m/z
GB		(3*R*)-3-(2-cyclopentyl-2-hydroxy-2-phenylacetoxy)-1,1-dimethylpyrrolidin-1-ium bromide Chemical Formula: $C_{19}H_{28}BrNO_3$ Molecular Weight: 398.33	 Chemical Formula: $C_{19}H_{28}NO_3^+$ Exact Mass: 318.21
GB impurity B		2-oxo-2-phenylacetic acid Chemical Formula: $C_8H_6O_3$ Molecular Weight: 150.13	 Chemical Formula: $C_8H_5O_3^-$ Exact Mass: 149.02
GB impurity C		2-hydroxy-2-phenylacetic acid Chemical Formula: $C_8H_8O_3$ Molecular Weight: 152.15	 Chemical Formula: $C_8H_7O_3^-$ Exact Mass: 151.04
GB impurity D		benzoic acid Chemical Formula: $C_7H_6O_2$ Molecular Weight: 122.12	 Chemical Formula: $C_7H_5O_2^-$ Exact Mass: 121.03 Glycopyrronium Impurity D
GB impurity F		benzaldehyde Chemical Formula: C_7H_6O Molecular Weight: 106.12	 Chemical Formula: C_7H_6O Exact Mass: 106.04

(*Continued*)

Table 1 Continued

Name	Structure	Chemical name and formula	MS m/z
GB impurity G		1-methylpyrrolidin-3-yl 2-cyclopentyl-2-hydroxy-2-phenylacetate Chemical Formula: $C_{18}H_{25}NO_3$ Molecular Weight: 303.40	 Chemical Formula: $C_{18}H_{26}NO_3^+$ Exact Mass: 304.19
GB impurity H		methyl 2-oxo-2-phenylacetate Chemical Formula: $C_9H_8O_3$ Molecular Weight: 164.16	 Chemical Formula: C_7H_6O Exact Mass: 106.04
GB impurity I		3-(2-(4-chlorophenyl)-2-cyclopentyl-2-hydroxyacetoxy)-1,1-dimethylpyrrolidin-1-ium Chemical Formula: $C_{19}H_{27}BrClNO_3$ Molecular Weight: 432.78	 Chemical Formula: $C_{19}H_{27}ClNO_3^+$ Exact Mass: 352.17
GB impurity J		2-cyclopentyl-2-hydroxy-2-phenylacetic acid Chemical Formula: $C_{13}H_{16}O_3$ Molecular Weight: 220.26 Glycopyrronium Impurity J	 Chemical Formula: $C_{13}H_{15}O_3^-$ Exact Mass: 219.10

(*Continued*)

Table I Continued

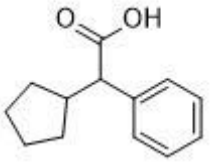
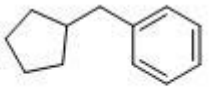
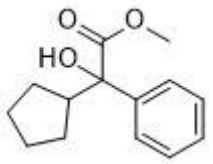
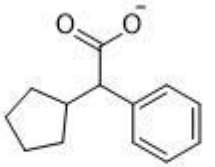
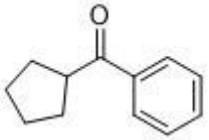
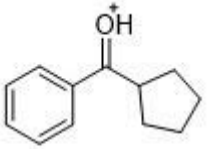
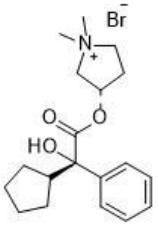
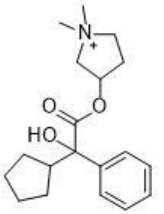
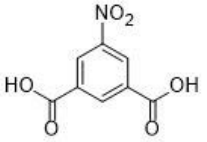
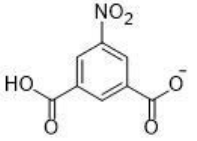
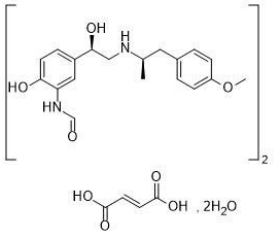
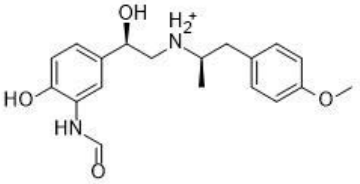
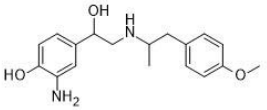
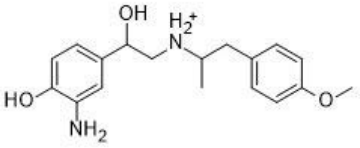
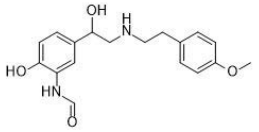
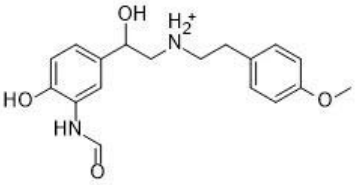
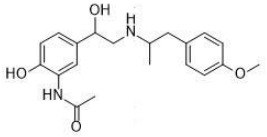
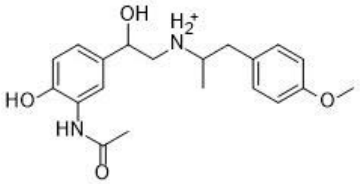
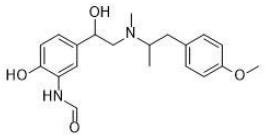
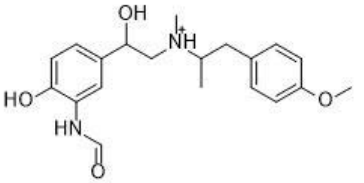
Name	Structure	Chemical name and formula	MS m/z
GB impurity K		2-cyclopentyl-2-phenylacetic acid Chemical Formula: $C_{13}H_{16}O_2$ Molecular Weight: 204.26	 Chemical Formula: $C_{12}H_{16}$ Exact Mass: 160.13
GB impurity L		methyl 2-cyclopentyl-2-hydroxy-2-phenylacetate Chemical Formula: $C_{14}H_{18}O_3$ Molecular Weight: 234.29	 Chemical Formula: $C_{13}H_{15}O_2^-$ Exact Mass: 203.11
GB impurity M		cyclopentyl(phenyl)methanone Chemical Formula: $C_{12}H_{14}O$ Molecular Weight: 174.24	 Chemical Formula: $C_{12}H_{15}O^+$ Exact Mass: 175.11
GB impurity N		(3*S*)-3-(2-cyclopentyl-2-hydroxy-2-phenylacetoxy)-1,1-dimethylpyrrolidin-1-ium Chemical Formula: $C_{19}H_{28}BrNO_3$ Molecular Weight: 398.33	 Chemical Formula: $C_{19}H_{28}NO_3^+$ Exact Mass: 318.21
GB impurity O		5-nitroisophthalic acid Chemical Formula: $C_8H_5NO_6$ Molecular Weight: 211.13	 Chemical Formula: $C_8H_4NO_6^-$ Exact Mass: 210.00

Table II Formoterol fumarate and its related substances; structure, formula and mass spectral data.

Name	Structure	Chemical name and formula	MS m/z
Formoterol fumarate dyhidrate		*N*-[2-Hydroxy-5-[(1*RS*)-1-hydroxy-2-[[(1*RS*)-2-(4-metoxy-phenyl)-1-methylethyl]amino]ethyl]phenyl]formamide (*E*)-butenedioate dihydrate Formotereol fumarate dihydrate Chemical Formula: $C_{42}H_{56}N_4O_{14}$ Molecular Weight: 840.91	 Chemical Formula: $C_{19}H_{25}N_2O_4^+$ Exact Mass: 345.18
Formoterol impurity A		2-amino-4-(1-hydroxy-2-((1-(4-methoxyphenyl)propan-2-yl)amino)ethyl)phenol Chemical Formula: $C_{18}H_{24}N_2O_3$ Molecular Weight: 316.39	 Chemical Formula: $C_{18}H_{25}N_2O_3^+$ Exact Mass: 317.19
Formoterol impurity B		*N*-(2-hydroxy-5-(1-hydroxy-2-((4-methoxyphenethyl)amino)ethyl)phenyl)formamide Chemical Formula: $C_{18}H_{22}N_2O_4$ Molecular Weight: 330.38	 Chemical Formula: $C_{18}H_{23}N_2O_4^+$ Exact Mass: 331.17
Formoterol impurity C		*N*-(2-hydroxy-5-(1-hydroxy-2-((1-(4-methoxyphenyl)propan-2-yl)(methyl)amino)ethyl)phenyl)formamide Chemical Formula: $C_{20}H_{26}N_2O_4$ Molecular Weight: 358.43	 Chemical Formula: $C_{20}H_{27}N_2O_4^+$ Exact Mass: 359.20
Formoterol impurity D		*N*-(2-hydroxy-5-(1-hydroxy-2-((1-(4-methoxyphenyl)propan-2-yl)amino)ethyl)phenyl)acetamide Chemical Formula: $C_{20}H_{26}N_2O_4$ Molecular Weight: 358.43	 Chemical Formula: $C_{20}H_{27}N_2O_4^+$ Exact Mass: 359.20

(*Continued*)

Table II Continued

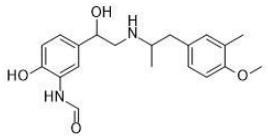
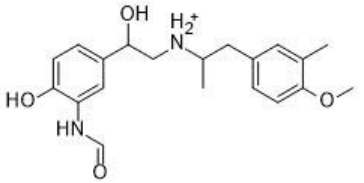
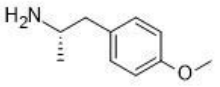
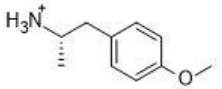
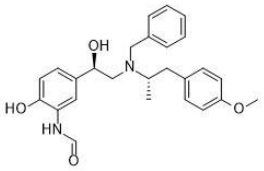
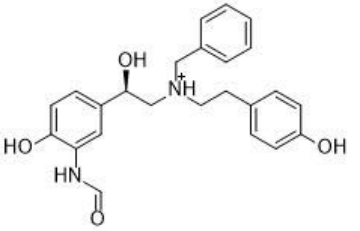
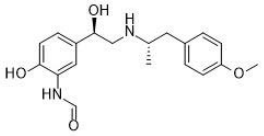
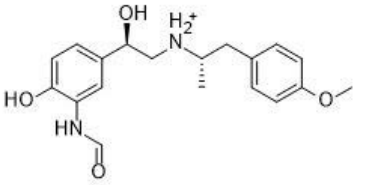
Name	Structure	Chemical name and formula	MS m/z
Formoterol impurity E		*N*-(2-hydroxy-5-(1-hydroxy-2-((1-(4-methoxy-3-methylphenyl)propan-2-yl)amino)ethyl)phenyl)formamide Chemical Formula: $C_{20}H_{26}N_2O_4$ Molecular Weight: 358.43	 Chemical Formula: $C_{20}H_{27}N_2O_4^+$ Exact Mass: 359.20
Formoterol impurity G		(*S*)-1-(4-methoxyphenyl)propan-2-amine Chemical Formula: $C_{10}H_{15}NO$ Molecular Weight: 165.23	 Chemical Formula: $C_{10}H_{16}NO^+$ Exact Mass: 166.12
Formoterol impurity H		*N*-(5-((*R*)-2-(benzyl((*S*)-1-(4-methoxyphenyl)propan-2-yl)amino)-1-hydroxyethyl)-2-hydroxyphenyl)formamide Chemical Formula: $C_{26}H_{30}N_2O_4$ Molecular Weight: 434.53	 Chemical Formula: $C_{24}H_{27}N_2O_4^+$ Exact Mass: 407.20
Formoterol impurity I		*N*-(2-hydroxy-5-((*R*)-1-hydroxy-2-(((*S*)-1-(4-methoxyphenyl)propan-2-yl)amino)ethyl)phenyl)formamide Chemical Formula: $C_{19}H_{24}N_2O_4$ Molecular Weight: 344.40	 Chemical Formula: $C_{19}H_{25}N_2O_4^+$ Exact Mass: 345.18



Figure 1 Photographs of apparatus for sampling (A: Sample dropping apparatus into the liquid nitrogen tank; B: Tank with liquid nitrogen; C: Cannister cutting apparatus; D: Compression apparatus).

temperature 450°C. Table I summarizes the optimized QDa settings for each compound. A nitrogen generator (PEAK Scientific, Chicago, IL, USA) produced high purity nitrogen and air. High purity argon cylinders (Linde Industrial Gases, Istanbul, Turkey) were utilized as the collision gas source.

Preparation of the standard solutions

Stock standard solutions of GB and FFD were prepared separately using a mixture of 0.5% formic acid in water, acetonitrile and methanol in a 65:20:15 (v/v/v) ratio. The concentration of the GB solution was 180 μg/mL, while the FFD solution was 100 μg/mL. Dilutions were carried out with the same solvent mixture to achieve final concentrations of 1.8 μg/mL and 1.0 μg/mL for GB and FFD, respectively.

Preparation of the sample solutions

To make the sample solution, put the canister in the tube shown in Figure 1A with the valve facing up. Then, put the canister in the nitrogen tank seen in Figure 1B, where it is filled with liquid nitrogen. Wait for 3–4 minutes and then remove the canister. Use the apparatus shown in Figure 1C and D to quickly cut the canister. Wait until the hydrofluoroalkane gas is completely removed from the container. Cut open the canister and wash it with the solvent mixture (25). Final concentrations of 1.8 and 1.0 μg/mL for GB and FFD, respectively, were achieved through gradual dilution.

Preparation of the standard solution for impurities quantifications

Using stock solutions of GB and FFD were prepared at a concentration of 0.02 and 0.01 μg/mL each in solvent mixture, respectively.

Validation of the method

After the ideal separation conditions and MS parameters were determined, validation was performed to prove the reliability and applicability of the method.

Accuracy and precision

Accuracy for active ingredients was determined by analyzing samples prepared in triples at 50, 100 and 200% of the corresponding target concentrations on testing. Accuracy for impurity measurement was determined by scrutinizing samples arranged in three repeats at the respective LOQ, 100% and 400% of the target concentration on quantification. Accuracy was evaluated as recovery (Rec): Rec (%) = experimental concentration/theoretical concentration $\times$ 100. The acceptable range is between 98.0 and 102.0% of the normal value for recovering active substances on assay analysis. For impurities, the acceptable range is between 80.0 and 120.0% for 400 and 100% levels, and between 70.0 and 130.0% for the LOQ level. The relative standard deviation (RSD%) of the active substance assay should not surpass 2.0%. The precision study indicates that the RSD% between the peak areas of individual compounds should be lower than 10.0%.

Solution stability

Solution stability refers to determining how long the prepared sample and standard solution areas can remain fixed within a specified range without altering the lab conditions. To achieve this, we prepared and analyzed the sample and standard solutions at specific time intervals while keeping the laboratory conditions constant. The prepared solutions were then injected into the autosampler at 5°C at predetermined time points.

Results

Method development

Develop a new accurate and precise method to measure GB, FFD and their impurities together. No one has reported an LC–MS method for detecting two active ingredients (GB and FFD) and 21 impurities (GB imp B, C, O, F, D, N, G, I, H, J, L, K, M and FFD imp A, G, B, I, D, C, E, H) in a pMDI drug product simultaneously. First, to ensure proper ionization, we chose the diluent and mobile phase before optimizing the chromatographic conditions. The strength of each substance was assessed by injecting standard solutions (180 μg/mL GB and 100 μg/mL FFD) directly, which were made by dissolving and diluting in a mixture of solvents. Various mixtures of

Table III QDa parameters for active substances.

Name of active substance	RT (minute)	Scan mode	Precursor ion (m/z)
GB	19.50	(+)	318.24
FFD	17.51	(+)	345.25
GB impurity B	6.987	(+)	149.14
GB impurity C	10.936	(−)	151.06
GB impurity D	18.492	(+)	121.05
GB impurity F	18.438	(−)	106.04
GB impurity G	19.484	(+)	304.20
GB impurity H	21.531	(+)	105.10
GB impurity I	20.721	(+)	352.23
GB impurity J	22.370	(−)	219.18
GB impurity K	23.433	(−)	159.14
GB impurity L	22.370	(+)	203.17
GB impurity M	24.811	(+)	175.13
GB impurity N	19.437	(+)	318.27
GB impurity O	17.171	(−)	210.09
FFD impurity A	12.485	(+)	317.27
FFD impurity B	16.593	(+)	331.21
FFD impurity C	17.701	(+)	359.30
FFD impurity D	17.675	(+)	359.28
FFD impurity E	18.564	(+)	359.28
FFD impurity G	12.871	(+)	166.14
FFD impurity H	19.667	(+)	407.49
FFD impurity I	17.458	(+)	345.24

Dwell time: 50 ms and collision energy: +15 V

formic acid ammonium acetate and ammonium formate were also tried out. Formic acid is generally used to create ions for determining these substances. The initial stage in improving the chromatographic conditions involved selecting a diluent and mobile phase to achieve favorable ionization and peak responses. The best peak signals for all the compounds were noted using 0.5% formic acid as a mobile phase additive.

The various substances were measured as ions with negative and positive charges using mass channels listed in Table III in XIC mode. The detector's capillary voltage was set to 5 kV and the cone voltage to 15 V. The probe temperature was kept at a steady 600°C, and the nitrogen flow was maintained at 2.0 L/minute. We gathered scan data in both positive and negative modes in the ESI probe to achieve the maximum ion current. Product ions were obtained using specific ions when a collision voltage of +15 V was applied. The mass spectra for GB and FFD are illustrated in Figures 2 and 3.

Discussion

Proper column selection played a crucial role in optimizing the chromatographic conditions. In order to obtain the optimal separation for the peak pairs of GB and FFD, a thorough evaluation of various UPLC columns with different characteristics, pore size, specific surface area and carbon loading was performed. The Acquity BEH C18, Acquity High Strength Silica (HSS) Pentafluorophenyl (PFP) and Acquity BEH C8 UPLC columns were tested, and the Acquity BEH C8 UPLC was found to provide the most effective separation pattern in the chromatograms.

Since FFD impurity C, FFD impurity D and FFD impurity E all have the same m/z ratio when ionized, the goal was to achieve chromatographic separation. During development, we aimed to separate peaks using both the PDA and QDa detectors set at 215 nm. Our goal was to detect and measure GB and FFD compounds in the same sample preparation, eliminating the need for a second impurity sample. Our chromatographic development studies used 0.5% formic acid in water and 0.5% formic acid in methanol as mobile phases in the gradient study. The mobile phase was selected from easily prepared solutions that promote ionization and do not leave residue in the mass detector. Additionally, choosing a gradient program was aimed at reducing the running time. The study used a gradient, which enabled rapid injection times for molecules with varying polarity, not achievable with an isocratic separation. Twenty-three different substances, comprising GB, 13 GB impurities, FFD and 8 FFD impurities were examined together for 30 minutes using the progressive gradient study. Three distinct gradient programs were tested and assessed, with the most appropriate one selected.

The assay and impurity analysis outcomes for Bevespi Aerosphere® 9/4.8 mcg per puff for use by mouth only (Astra Zeneca), which are obtainable, were evaluated using the final techniques. The findings from the European marketplace were examined for Bevespi Aerosphere® with Batch No. 6030052A00, 6030011A00 and 6030007A00. The previous products' assay findings were recorded as 99.5/97.3%, 100.1/98.9%, 101.2/100.1% for GB/FFD%, respectively. Impurities from GB were not detected in previous products. Impurity A for FFD was at 0.1%, and impurity D was below the LOQ. Impurities B, C, E, G, H and I for FFD were not detected in three batches.

Validation

The method was validated for specificity, limit of detection (LOD), LOQ, accuracy, linearity, precision and solution stability according to guideline of the International Council for Harmonization (ICH) (26).

Specificity

Specificity was assessed by injecting blank solution, standard and sample solutions, impurities separately spiked at the corresponding limit concentrations.

No interference from the ambient matrix (impurity, placebo and solvent) was observed in the RTs of both active ingredients, which demonstrates the method's specificity.

The absence of interference from the placebo, impurities and blank peaks in the chromatographic RT of the glycopyronium and formoterol peaks proves the method's specificity.

Selectivity was enhanced by mass filtering through the QDa detector. The specificity of the method was ensured by monitoring the analyte using XICs at the target m/z value with the Waters QDa mass detector. Evaluation of blank, placebo and sample chromatograms revealed no interfering peaks at the RT of the analyte in the extracted ion channel. The XICs for all injections are included in the supplementary materials section of the article.

Linearity, LOD and LOQ

Calibration curves were created at three concentration levels to confirm linearity for both assay and impurity quantification. Each compound utilized the calibration curve to ensure a linear correlation between analyte concentration and analyte peak areas. Regression parameters, specifically the slope, intercept and regression coefficient (r), were calculated

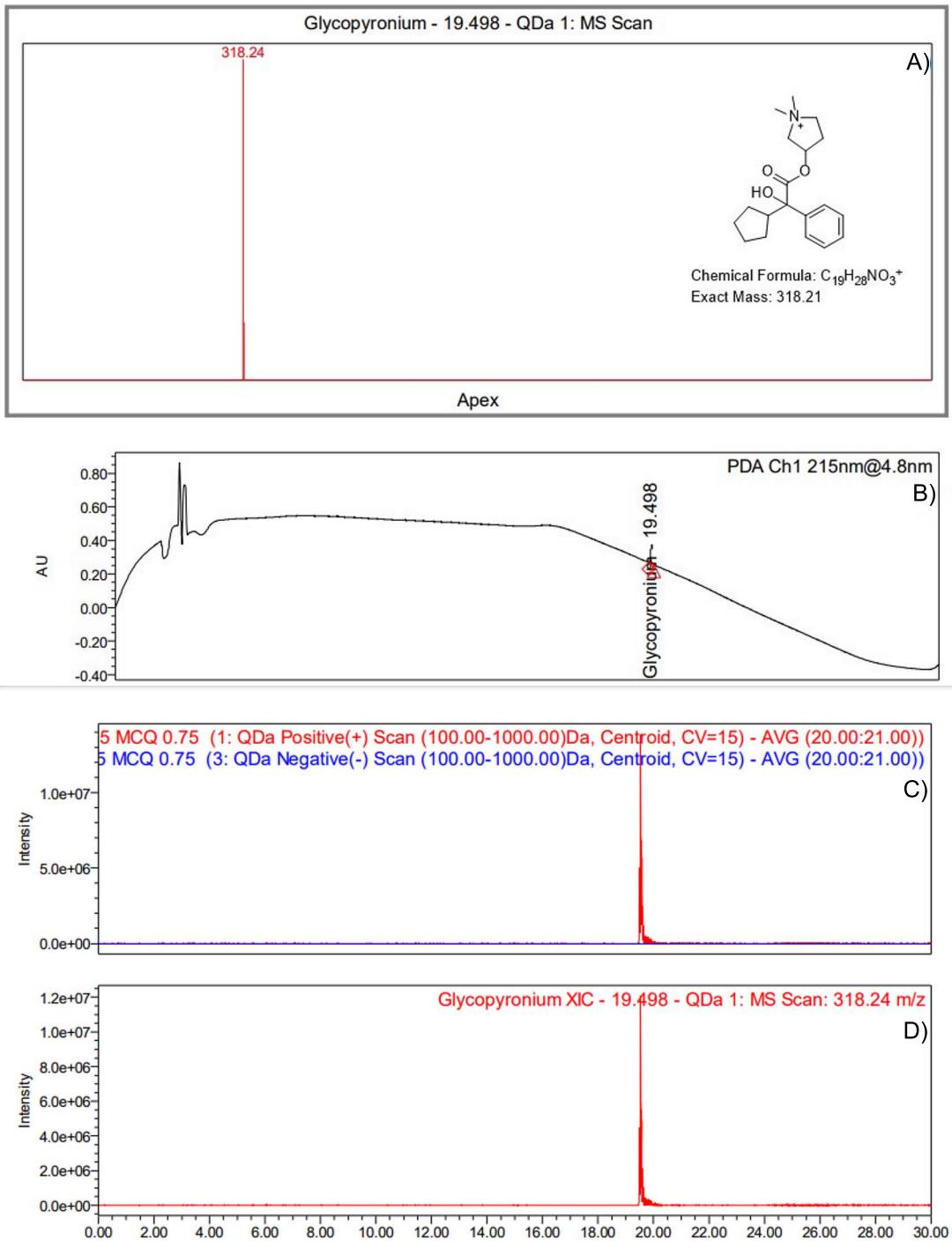


Figure 2 GB standard solution (180 μg/mL, 0.5% formic acid in acetonitrile); (A) mass spectrum in the main ion scan, (B) PDA chromatogram, (C) scan chromatogram, (D) XIC.

using the least squares technique. The minimum limit for detecting a substance was calculated using the lowest peak concentration with a signal-to-noise ratio of at least 10:1 and a RSD of 20%. The lowest concentration peak was also used to calculate the LOD with a signal-to-noise ratio of at least 3:1.

We established the order of concentration for GB, FFD and their related elements to be linear. See Tables IV and V for values of the regression parameters. All values comply with the linearity standards. The method's sensitivity for quantitative analysis was established by defining the LOD and LOQ. We determined these impurity values experimentally using the signal-to-noise (s/n) approach. Tables IV and V show that a signal-to-noise ratio of 3:1 and 10:1 is acceptable for LOD and LOQ, respectively.

LOD and LOQ. It was calculated using the following equations:

(σ): The standard deviation in the lowest concentration.

Table IV Results of method validation parameters for GB and impurities.

	GB	B	C	D	F	G	H	I	J	K	L	M	N	O
LOD (ng/mL)	–	1.29	5.36	0.83	3.31	1.67	0.61	2.54	0.91	1.05	0.43	1.34	0.78	0.56
LOQ (ng/mL)	–	3.91	14.9	2.53	10.0	5.06	1.86	7.13	1.85	3.77	4.14	4.01	2.50	3.45
Linearity														
Concentration range (ng/mL)	900–3600	48.1–1922.7	50.3–2010.0	49.9–1997.7	48.1–1923.4	50.0–2001.6	48.9–1956.1	48.8–1950.0	49.9–1994.0	49.9–1995.7	49.8–1990.2	49.8–1991.9	45.9–1837.8	50.0–2000.8
Slope	3615.6	27.9	71.3	52.3	151.1	272.0	368.3	197.2	260.2	290.5	258.8	149.9	121.5	489.5
y-intercept	−20243	1036	20.4	−180.3	−3061	−950	−1087	−28.2	−284.1	3520.8	−141.4	398.6	75.6	3747.1
R^2	0.99991	0.99541	0.99998	0.99999	0.99854	0.99980	0.99993	0.99932	0.99996	0.9998	0.9995	0.9994	0.9999	0.9997
Accuracy														
Level 1 (ng/mL)	901.5	0.96	1.01	1.00	0.96	0.98	0.98	0.98	1.00	1.00	1.00	1.00	0.92	1.00
Recovery 1 (%)	99.3	91.9	94.2	90.0	92.9	94.1	95.3	96.1	92.9	92.9	100.0	95.9	96.1	98.0
Level 2 (ng/mL)	1803.0	19.23	20.10	19.98	19.23	19.51	19.56	19.50	19.96	19.96	19.94	19.92	18.38	20.01
Recovery 2	101.8	101.0	99.0	99.0	99.6	97.6	99.4	98.8	99.6	99.6	98.6	99.4	98.3	97.6
Level 3 (ng/mL)	3606.0	76.9	80.40	79.91	76.94	78.04	78.24	78.00	79.76	79.83	79.76	79.68	73.51	80.03
Recovery 3 (%)	100.4	100.2	99.3	98.3	100.1	99.0	100.4	101.6	100.1	100.1	98.9	100.4	102.1	99.9
Precision														
Results (%)	101.8	1.1	1.0	1.0	1.0	1.1	1.2	1.0	1.0	1.0	1.0	1.0	1.0	1.1
RSD%	1.69	4.27	6.26	4.44	5.94	5.38	8.34	7.97	9.87	6.47	5.94	4.58	7.57	5.59
Solution stability (hour)	48	48	12	12	48	48	48	48	48	36	48	12	48	48

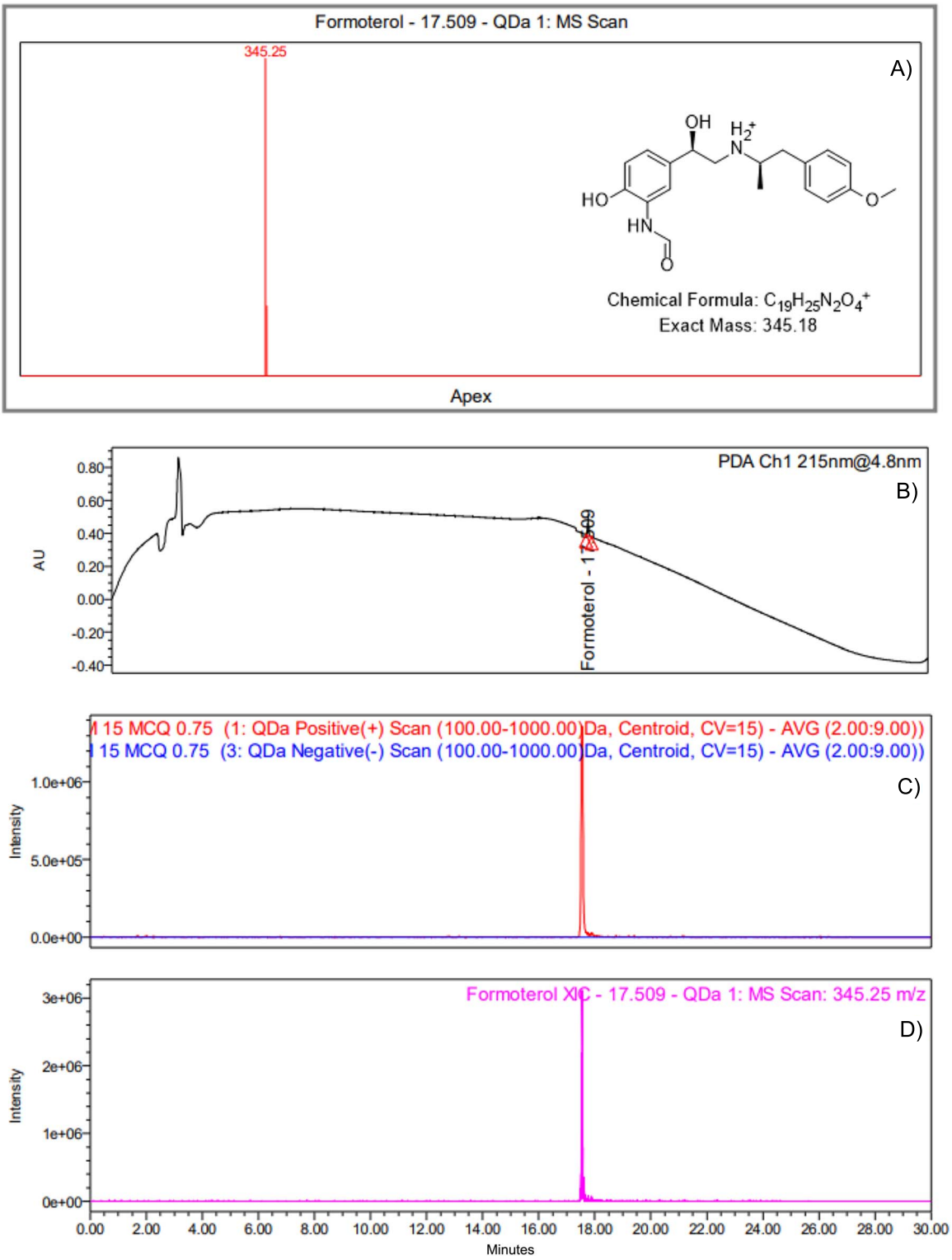


Figure 3 FFD standard (100 μg/mL, 0.5% formic acid in acetonitrile); (A) mass spectrum in the main ion scan, (B) PDA chromatogram, (C) scan chromatogram, (D) XIC.

(S): The slope of the calibration curve.

$$LOD = \frac{3.3\sigma}{S}$$

$$LOQ = \frac{10\sigma}{S}$$

Precision

For the evaluation of method's precision, the values of RSD% were calculated: GB (0.07%), GB impurity B (0.41%), GB impurity C (0.33%), GB impurity D (0.13%), GB impurity F (0.76%), GB impurity G (0.53%), GB impurity H (0.57%), GB impurity I (0.42%), GB impurity J (0.61%), GB impurity

Table V Results of method validation parameters for FFD and impurities.

	FFD	A	B	C	D	E	G	H	I
LOD (ng/mL)	–	0.23	0.22	0.19	0.18	0.13	0.31	0.21	0.32
LOQ (ng/mL)	–	0.47	0.47	0.48	0.48	0.48	0.50	0.47	0.50
Linearity									
Concentration range (ng/mL)	500–2000	0.5–18.7	0.5–18.9	0.5–19.1	0.5–19.3	0.5–19.3	0.5–19.8	0.5–18.9	0.5–19.9
Slope	4460.2	4606.4	4499.9	4326.6	4238.8	3238.5	25601.4	3243.5	4275.8
y-intercept	−1863.6	−156.07	−385.89	288.51	758.01	−238.29	−6976.8	86.64	916.58
R^2	0.99985	0.99999	0.99978	0.99977	0.99957	0.99999	0.99941	0.90604	0.99966
Accuracy									
Level 1 (ng/mL)	501.0	0.43	0.45	0.44	0.43	0.46	0.47	0.46	0.48
Recovery 1 (%)	99.9	91.9	94.2	90.0	92.9	94.1	95.3	96.1	97.5
Level 2 (ng/mL)	1002.0	9.40	9.36	9.44	9.65	9.51	9.67	9.27	9.80
Recovery 2	99.2	101.0	99.0	99.0	99.2	98.3	99.4	98.8	99.2
Level 3 (ng/mL)	2004.0	37.31	36.95	37.08	38.27	38.05	39.78	37.81	40.46
Recovery 3 (%)	99.3	100.2	99.3	98.3	101.6	99.4	100.4	101.6	102.3
Precision									
Results (%)	99.9	1.1	1.0	1.0	1.0	1.1	1.2	1.0	1.0
RSD%	0.84	2.99	6.26	4.35	7.65	6.29	8.34	7.97	9.87
Solution stability (hour)	48	48	12	48	48	36	48	36	48

Table VI Results of LOD, LOQ and s/n datas for GB and impurities.

	LOD (ng/mL)	s/n	LOQ (ng/mL)	s/n	%RSD
GB impurity B	1.29	6	3.91	14	1.8
GB impurity C	5.36	4	14.9	16	5.4
GB impurity D	0.83	4	2.53	12	2.4
GB impurity F	3.31	3	10.0	9	1.7
GB impurity G	1.67	3	5.06	18	3.9
GB impurity H	0.61	5	1.86	16	1.0
GB impurity I	0.61	5	1.86	16	2.0
GB impurity J	0.61	3	1.86	16	3.0
GB impurity K	0.61	5	1.86	16	1.2
GB impurity L	0.61	3	1.86	16	1.5
GB impurity M	0.61	5	1.86	16	1.0
GB impurity N	0.61	6	1.86	16	1.0
GB impurity O	0.61	5	1.86	16	4.2

Table VII Results of LOD, LOQ and s/n datas for FFD and impurities.

	LOD (ng/mL)	s/n	LOQ (ng/mL)	s/n	%RSD
FFD impurity A	0.23	3	0.47	16	3.9
FFD impurity B	0.22	5	0.47	14	3.6
FFD impurity C	0.19	4	0.48	13	2.0
FFD impurity D	0.18	3	0.48	19	3.6
FFD impurity E	0.13	5	0.48	14	2.4
FFD impurity G	0.31	4	0.50	19	3.0
FFD impurity H	0.21	5	0.47	16	4.92
FFD impurity I	0.32	3	0.50	16	5.7

K (0.30%), GB impurity L (0.96%), GB impurity M (1.42%), GB impurity N (0.32%), GB impurity O (0.21%), FFD (0.01%), FFD impurity A (0.74%), FFD impurity B (0.15%), FFD impurity C (0.16%), FFD impurity D (1.31%), FFD impurity E (0.39%), FFD impurity G (0.15%), FFD impurity H (1.75%) and FFD impurity I (0.57%). The obtained values fulfilled the required criteria (RSD 2% for active ingredients and 10% for impurities).

The RSD% should not be >2.0% in the results of the active substances assay. The RSD% between the peak areas of the respective compounds should be <10.0% for precision study.

Accuracy and recovery

Accuracy for active substances was determined by analyzing samples prepared in triplicate at 50, 100 and 200% of the

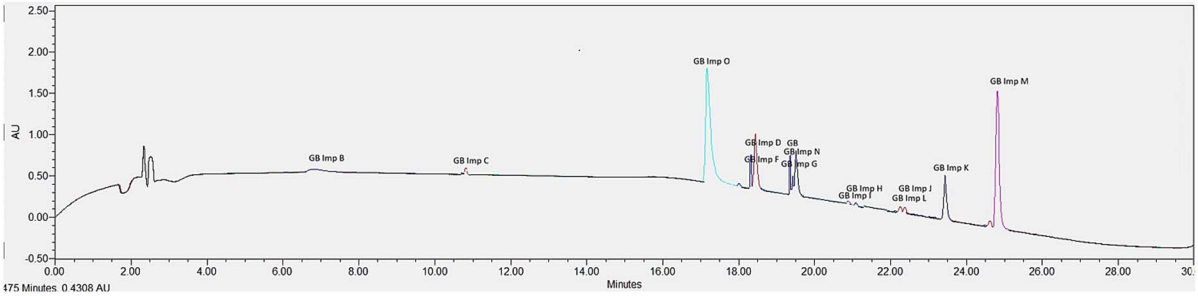


Figure 4 Blend chromatogram of GB and impurities.

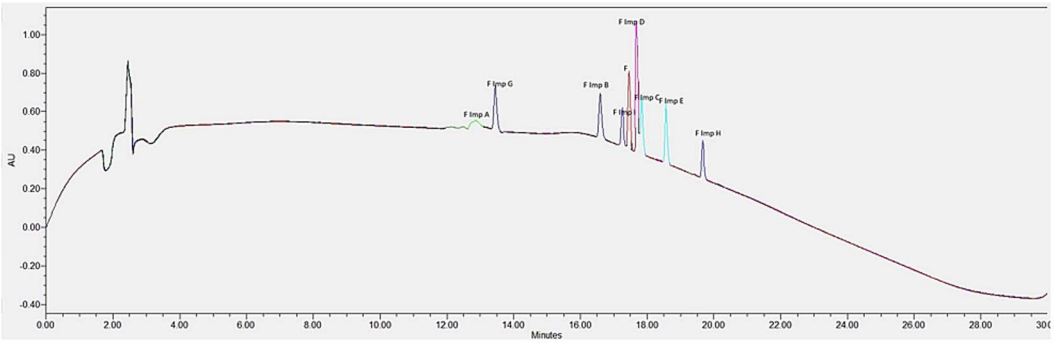


Figure 5 Blend chromatogram of FF and impurities.

respective target assay concentrations. Accuracy for impurity quantification was determined by analyzing samples prepared in triplicate at the respective LOQ, 100% and 400% of the target quantification concentration. Accuracy was calculated as recovery (Rec): Rec (%) = experimental concentration/theoretical concentration × 100. Acceptance criteria were 98.0–102.0% of the normal value for recovery of active substances in assay analysis. The acceptance criteria for impurities; 80.0–120.0% for 400% and 100% levels, 70.0–130.0% for LOQ level.

To evaluate the accuracy of the proposed method, the recovery values were calculated for sample solutions spiked with GB and FFD at 50, 100 and 200% of the limit concentrations. To evaluate the accuracy of the proposed method for impurities, the recoveries were calculated for sample solutions spiked with their impurities at LOQ, 100% and 400% of the limit concentrations. They are all within the acceptance criteria (recoveries: 98.0–102.0% for drug, 70.0–130.0% for impurities at LOQ concentration, 80.0–120.0% for impurities at 100% and 200% and 400% concentration) and solution stability was 12 hours for all samples are presented in Tables IV and V. The precision results show that the drug and impurities are successfully recovered in the excipient and diluent matrix.

Conclusion

MS is a method utilized to identify and measure drugs and metabolites. It is applied widely in research and development, and quality control (QC) laboratories, and includes measuring drug doses and related compounds. The pharmaceutical industry necessitates extremely strict limits for associated substances in drugs and drug products. Major genotoxic impurities, leachable compounds or unknown impurities can be extensively employed to measure the quantity of active substance and impurities, particularly as mass detectors become increasingly prevalent and accessible. In this respect, our work entails designing and validating a UPLC MS technique for monitoring GB, FFD and their related pharmacopoeial compounds. This technique has successfully identified and measured impurities at an exceptionally low level that would otherwise be undetectable by other available detectors and technologies. A rapid, replicable and sensitive UPLC MS (QDa) method has been developed to measure GB, FFD and 21 corresponding impurities (consisting of GB impurities B, C, D, F, G, H, I, J, K, L, M, N, O and FFD impurities A, B, C, D, E, G, H, I) in the drug product. Figure 4 shows the blend chromatogram of GB and impurities, and Figure 5 shows the blend chromatogram of FF and impurities. This approach has been validated against the existing ICH guidelines. The newly developed method exhibits sensitivity, specificity, linearity, precision, stability and accuracy. The conducted tests included quantification and impurity assessment, which were carried out using a straightforward and swift sample preparation technique. The method was effectively employed in QC release and market samples.

Author contributions

Serdar Seckın (Writing—review & editing) and Serap Saglik Aslan (Project administration)

Supplementary data

Supplementary data are available at *Journal of Chromatographic Science* online. Supplementary Figures S1 through S23 contain MS chromatograms for all impurities.

Conflicts of interest

All authors declared that they have no conflicts of interest with regard to the studies described in this manuscript.

Funding

There was no funding obtained to help with the development of this manuscript.

References

1. World Health Organization. Chronic Obstructive Pulmonary Disease (COPD). Fact Sheet, No. 315. Geneva: World Health Organization, 2022.
2. Mathers, C.D., Loncar, D.; Projections of global mortality and burden of disease from 2002 to 2030; *PLoS Medicine*, (2006); **3**(11): e442. https://doi.org/10.1371/journal.pmed.0030442.
3. Lomborg, Bjørn, ed. *Global Problems, Local Solutions: Costs and Benefits*. Cambridge: Cambridge University Press, 2013. https://doi.org/10.1017/CBO9781139600484.
4. Vestbo, J., Hurd, S.S., Agustí, A.G., Jones, P.W., Vogelmeier C., Anzueto A., *et al.*; Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease: GOLD Executive Summary. *American Journal of Respiratory and Critical Care Medicine* (2013);**187**(4): 347–365. https://doi.org/10.5005/jp/books/12063_1.
5. Kumar, V., Abbas, A.K., Aster, J.C.; *Robbins and Cotran pathologic basis of disease*, 9th ed. Elsevier Saunders, Philadelphia, (2015).
6. Reilly, J. J., E. K. Silverman, and S. D. Shapiro.; Chronic Obstructive Pulmonary Disease. In Dan, L.L., Anthony, S.F., Dennis, L.K., Stephen, L.H., J., L.J., Joseph, L., (eds.). Harrison's Principles of Internal Medicine, 18th ed. McGraw-Hill, Toronto (2011).
7. Decramer, M., Janssens, W., Miravitlles, M.; Chronic obstructive pulmonary disease; *Lancet*, (2012); **379**(9823): 1341–1351. https://doi.org/10.1016/S0140-6736(11)60968-9.
8. Mason, R., Broaddus, V.C., Martin, T.; *Murray and Nadel's textbook of respiratory medicine*, 5th ed. Saunders, Philadelphia, (2010).
9. Rabe, K.F., Hurd, S., Anzueto, A., Barnes, P.J., Buist, S.A., Calverley, P., *et al.*; Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary; *American Journal of Respiratory and Critical Care Medicine*, (2007); **176**(6): 532–555. https://doi.org/10.1164/rccm.200703-456SO.
10. Nathell, L., Nathell, M., Malmberg, P., Larsson, K.; COPD diagnosis related to different guidelines and spirometry techniques; *Respiratory Research*, (2007); **8**(1): 89–95. https://doi.org/10.1186/1465-9921-8-89.
11. AstraZeneca. Bevespi Aerosphere (Glycopyrrolate and Formoterol Fumarate) Inhalation Aerosol Prescribing Information. Wilmington, DE: AstraZeneca Pharmaceuticals LP, 2023. https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/208294s000lbl.pdf.
12. European Pharmacopoeia Commission.; Glycopyrronium Bromide (Monograph 1783), In *European Pharmacopoeia*, 9th ed. European Directorate for the Quality of Medicines & HealthCare (EDQM), Strasbourg 2016.
13. European Pharmacopoeia Commission.; "Formoterol Fumarate Dihydrate (Monograph 1724/1783)." In *European Pharmacopoeia*, 9th ed. Strasbourg: European Directorate for the Quality of Medicines & HealthCare (EDQM), 2018.
14. Suzuki, É.Y., Simon, A., da Silva, A.L., Amaro, M.I., de Almeida, G.S., Agra L.C., *et al.*; Effects of a Novel Roflumilast and Formoterol Fumarate Dry Powder Inhaler Formulation in Experimental Allergic Asthma, *International Journal of Pharmaceutics* (2020); **588**: 119771.
15. Akapo, S.O., Asif, M.; Validation of a RP-HPLC method for assay of formoterol and its related substances in formoterol fumarate dihydrate drug substance; *Journal of Pharmaceutical and Biomedical Analysis*, (2003); **33**(5): 935–945. https://doi.org/10.1016/S0731-7085(03)00425-4.
16. Merey, H.A., El-Mosallamy, S.S., Hassan, N.Y., El-Zeany, B.A.; Validated chromatographic methods for the simultaneous determination of mometasone furoate and formoterol fumarate dihydrate in a combined dosage form; *Bulletin of Faculty of Pharmacy, Cairo University.*, (2016); **54**(1): 99–106. https://doi.org/10.1016/j.bfopcu.2016.02.001.
17. El-Bagary, R.I., Fouad, M.A., El-Shal, M.A., Tolba, E.H.; Forced Degradation of Mometasone Furoate and Development of Two RP-HPLC Methods for Its Determination with Formoterol Fumarate or Salicylic Acid, *Arabian Journal of Chemistry* (2016); **9**: 493–505. https://doi.org/10.1016/j.arabjc.2015.05.005.
18. F.L., Wilson, I.A., Maziarz, M., Rainville, P.D.; Ultra High-Performance Liquid Chromatography Method Development for Separation of Formoterol, Budesonide, and Related Substances Using an Analytical Quality by Design Approach. *Journal of Pharmaceutical and Biomedical Analysis* (2021); **193**: 113729. https://doi.org/10.1016/j.jpba.2020.113729.
19. Trivedi, R.K., Chendake, D.S., Patel, M.C.; A Rapid, Stability-Indicating RP-HPLC Method for the Simultaneous Determination of Formoterol Fumarate, Tiotropium Bromide, and Ciclesonide in a Pulmonary Drug Product, *Scientia Pharmaceutica* (2012); **80**: 591–603. https://doi.org/10.3797/scipharm.1204-06.
20. Gujarati, P.Z., Thula, K.C., Maheshwari, D.G.; Stability indicating HPLC method for simultaneous estimation of mometasone furoate and formoterol fumarate in combined dosage form; *Pharmacophore*, (2014); 5(2): 219–230.
21. Salem, Y., Hammouda, M.E., Saleh, N., El-Enin, M.A., El-Ashry, S.M.; Validated Ion Pair Chromatographic Method for Simultaneous Determination and In Vitro Dissolution Studies of Indacaterol and Glycopyrronium from Inhaled Capsule Dosage Form, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* (2018); **204**: 464–474. https://doi.org/10.1016/j.saa.2018.06.078.
22. Rumpler, M.J., Sams, R.A., Colahan, P.; Regulatory Control of Glycopyrrolate in Performance Horses Using Validated UHPLC/MS-MS Methods, *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* (2012); **889–890**: 130–137. https://doi.org/10.1016/j.jchromb.2012.02.008.
23. Storme, M.L., Kindt, W., Goeteyn, K., Reyntjens, J.F., Bocxlaer, V.; Quantitative determination of glycopyrrolate in human plasma by liquid chromatography–electrospray ionization mass spectrometry: the use of a volatile ion-pairing agent during both liquid–liquid extraction and liquid chromatography; *Journal of Chromatography B*, (2008); **876**(1): 24–30. https://doi.org/10.1016/j.jchromb.2008.10.013.
24. Erukulla, K.K., Rengitham, S.S.; Synchronous estimation of glycopyrrolate and formoterol in bulk and pharmaceutical dosage form by RP-HPLC method; *Indian Journal of Pharmaceutical Education and Research*., (2018); **52**(4): s47–s55. https://doi.org/10.5530/ijper.52.4s.75.
25. Seckin. S.; Analysis of Glycopyrronium Bromide, Formoterol Fumarate Dihydrate and Related Substances, *PhD diss.*, *İstanbul University, Institute of Health Sciences, Department of Analytical Chemistry*, 2023. Supervised by S. Saglik Aslan.
26. International Conference on Harmonisation (ICH). ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology Q2(R1). Geneva: ICH Steering Committee, 2005.

© The Author(s) 2026. Published by Oxford University Press. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (https://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact reprints@oup.com for reprints and translation rights for reprints. All other permissions can be obtained through our RightsLink service via the Permissions link on the article page on our site—for further information please contact journals.permissions@oup.com.
Journal of Chromatographic Science, 2026, 64(9), bmag030
https://doi.org/10.1093/chromsci/bmag030
Article