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Problem-solving approach for salbutamol analysis by HPLC during pharmaceutical assay

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Abstract: When cationic basic compounds are chromatographed using hydro-organic mobile phase, the presence of anionic free silanols in the silica-based stationary phases results in broad and asymmetrical peaks. The addition of an ionic reagent to the mobile phase prevents analytes from accessing free silanols, improving peak shape. In this study, the chromatographic behavior of salbutamol sulfate as a basic compound was investigated under various conditions, including the use of different columns, mobile phases, and ion-pair reagents such as triethanolamine (TEA) and sodium heptane sulfonate (SHS). The retention and peak shape of chromatograms were both evaluated. The results show that pre-conditioning the column with TEA and including it in the mobile phase can prevent cationic analytes from accessing anionic silanols, resulting in improved peak shape. Furthermore, buffering the mobile phase is an important factor in keeping the pH constant throughout the process. The chosen method was validated in part. This study could be helpful for researchers and analyst to solve such problems with cationic basic components.

Key words: salbutamol, HPLC, silanol, triethanolamine

1. Introduction

High-pressure phase liquid chromatography (HPLC) is a well-established technique for analyzing a wide range of samples. However, even when using tried-and-true conditions, achieving a symmetrical peak can be challenging at times. Peak tailing and split peaks are common problems in silica-based HPLC, particularly in basic compounds where the organic component of the solvent is present in low concentration.[1,2] This phenomenon could be caused by the presence of anionic free silanol in silica-based stationary phases. Although peak symmetry has been significantly improved by reducing the number of residual silanols in high purity silica, peak distortion has not been eliminated satisfactorily, even products marketed as fully end capped columns commercially, are not 100 % end capped.[3] Free silanol groups are weakly acidic

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depending on the silica type.[1,4] Although the pKa is a fixed value in water, the amount of it can be vary in different composition of the mobile phase and cannot be easily predicted.[5] Thus anionic silanols introduce additional ion-exchange interactions in the chromatographic process at the typical working pH range of typical HPLC columns (pH 2-8).[1] Regardless of the strategy chosen, evaluation of silanol activity and the contribution of the various parameters that help to reduce it, is not simple due to the complexity of the interactions that can occur inside the column at the same time. Several strategies have been proposed to reduce or suppress silanol activity. It is critical to evaluate and comprehend the minimization of the silanol effect in order to improve the chromatographic behavior of basic compounds. Salbutamol sulfate (SS) is an anti-asthmatic and anti-COPD medication with two functional groups: a secondary amine and a phenolic-hydroxy group with pka values of 9.2 and 10.7, respectively. This means that a secondary amine functional group is cationic at pH lower than 8. While working in another project using SS, HPLC method adapted from the literature to analyzing pharmaceutical formulations; but the drug peak shape gradually became distorted, initial peak tailing changed to splited peaks. So, we have to study on different HPLC methods. In the case of SS, peak splitting could be due to the ionization of one functional group while the others are not (*Fig.* 1). In this study, various HPLC methods and conditions were tested. SS is a model drug to evaluate parameters such as:

1. Column type
2. Mobile phase composition
3. Ion pairs reagents
   a. Sodium 1-heptansulfonate (SHS)
   b. Triethanolamine (TEA)
4. Solvent matrix

To develop a suitable and validated HPLC method, the impact of these factors on the silanol activity of a C18 stationary phase under various experimental conditions was assessed by observing changes in retention and peak shape, the latter expressed qualitatively as tripled, doubled, tailed or single peak.

2. Experimental

2.1. Reagents

SS was kindly gifted by L.B. Bohle, Germany; purity 99 %, sodium heptane sulfonate, TEA, orthophosphoric acid (analytical reagent grade), acetonitrile and methanol (HPLC grade) were obtained from Merck, Germany. Fresh Milli-Q water prepared using equipment from Millipore Corporation (Milli-Q® Advantage A10, Merck, Germany) was collected and filtered through 0.45 μm membrane filter (Phenomenex, California, USA).

2.2. Apparatus and column

The Knauer HPLC system (Berlin, Germany) was made up of a Knauer controller quaternary pump and a

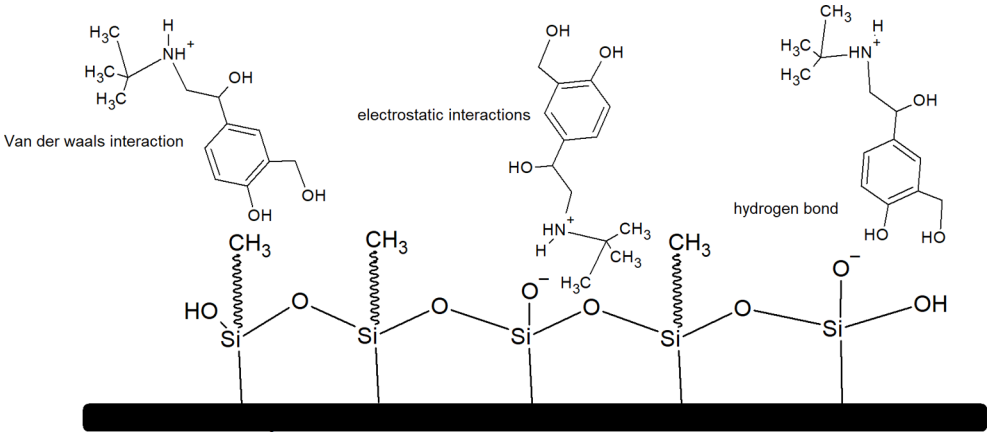

Fig. 1. Assumed interactions of cationic salbutamol in HPLC method.

UV detector (Knauer No. E 4310), and Agilent 1260 infinity liquid chromatography (Waldbronn, Germany) consisting of a temperature-controlling system (-5 to 80 °C), photodiode array (PDA) detector, low-pressure quaternary gradient pump, and degasser was used as HPLC instrument. The operation was controlled using EZ Chrome elite software and Agilent ChemStation software.

Brisa "LC²" column with the following characteristics was used: C18 Column, 150 mm × 4.6 mm i.d., 5 µm particle size and 120 Å pore size a fully "end capped" free silanol silica with a broad usable pH range (2-11). Another column was: a high-resolution C18 column (CLIPEUS C18 5 µm, 250 × 2.1mm, USA). All experiments have been done at room temperature.

3. Result and Discussion

3.1. Initial experiments

Salbutamol was analyzed for months using a C18 column (Brisa LC^2 C18), with the mobile phase consisting of orthophosphoric acid 0.5 % : acetonitrile 90 : 10, pH 2 ± 0.1[6], stock solution was prepared in water. The system was operated at a constant UV wavelength of 230 nm with a flow rate of 0.7 mL/min by 20 µL injection. Retention time was (RT) 4.4 min (*Fig.* 2(a)). But after about 150 injections the peaks gradually became distorted (*Figs.* 2(b) – 2(d)),

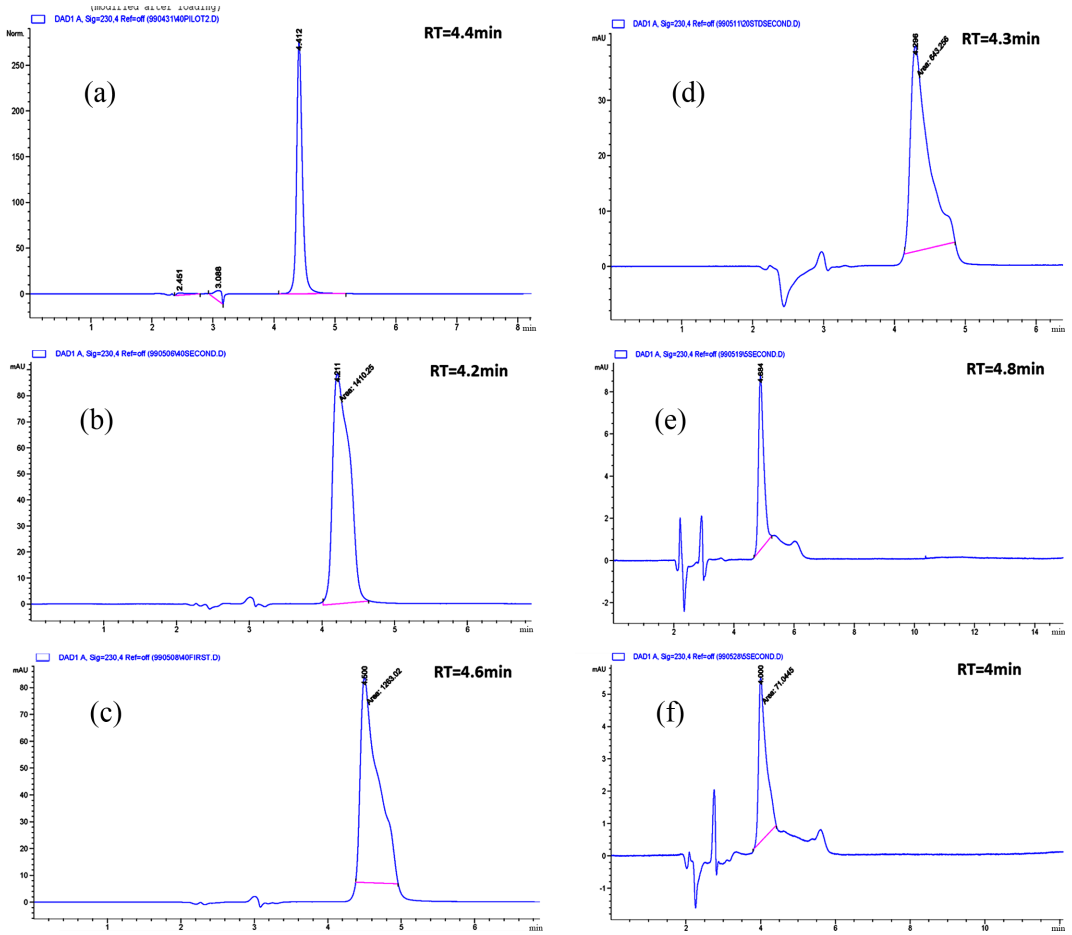

Fig. 2. SS peak shape after months altered and retention time does not change significantly; (a) salbutamol peak for months. (b)-(f): salbutamol peak distortion in different days. chromatography system characteristic: C18 column (Brisa LC^2 C18 Column 5 µm 15 × 0,46 cm), mobile phase consists orthophosphoric acid 0.5 %: acetonitrile 90: 10, pH 2 ± 0.1, stock solution was prepared in water.

initial peak tailing changed to splited peaks (*Figs.* 2(e) – 2(f)). The retention time does not change noticeably. To solve the problem, the pH of a fresh mobile phase was carefully adjusted, a new stock solution was prepared, lower sample concentrations were injected, deionized water sources were checked, and the system was washed for 2 hours with water at a low flow rate (0.5 mL/min). However, none of the strategies mentioned were able to solve the problem.

3.2. Check the column

Column was checked in order to evaluate any signs of collapse. The frit of the column was cleaned by sonication in a hydroalcoholic solution, the column was turned around and reverse flow with water. The peak shape improved, but peaks became tailed again after two days (data are presented in supplementary document, *Fig.* S1(a) and S1(b)). The frit was washed once more, but the peak has doubled (*Fig.* S1(c)).

3.3. Using another C18 column

To determine whether the problem was due to the column or not, a new column was tested under the same conditions after. All HPLC conditions remained constant. *Fig.* S2(a) – S2(c) shows that after a few days, the peak shape became distorted again, and the initial peak tailing changed to doubled peaks. As a result, we concluded that the problem was unrelated to the column.

3.4. The effect of the mobile phase

3.4.1. The addition of ion-pairing agents

3.4.1.1. Evaluation the effect of sodium 1-heptane-sulfonate (SHS)

2.78 mM SHS was added to the used mobile phase to evaluate the effect of SHS.[7] The retention time was increased (RT 10.5 min), but the peak was still doubled, as shown in *Fig.* 3(a). The hydrophobic interaction of SHS (*Fig.* 3(b)) with the alkyl chains bound to the stationary phase and the electrostatic interaction of anionic SHS with SS cationic amine are two possible hypotheses.[8] These events may lead to an increased retention of SS on the column (*Fig.* 3(b)) which is in agreement with Z.Y. Yang et al. findings[9] SS is a basic element that provides Ione-pair electrons. As a result, under acidic condition of the mobile phase, it will combine with H+ due to the attraction of the positive and negative charges, converting SS to the H+-conjugate. The H+-conjugate of SS then binds to the negative group of SHS in the mobile phase and is converted to a neutral molecule. As a result, when compared to the ionic form of SS, the neutral complex results in improved retention ability of SS on the C18 column, because the neutral complex has a weaker polarity than ionized SS, and compounds with similar polarities to the column are effectively retained or adsorbed. Furthermore, the nitrogen atom is masked by the H+-conjugate and result in a neutral complex. As a result, there is no

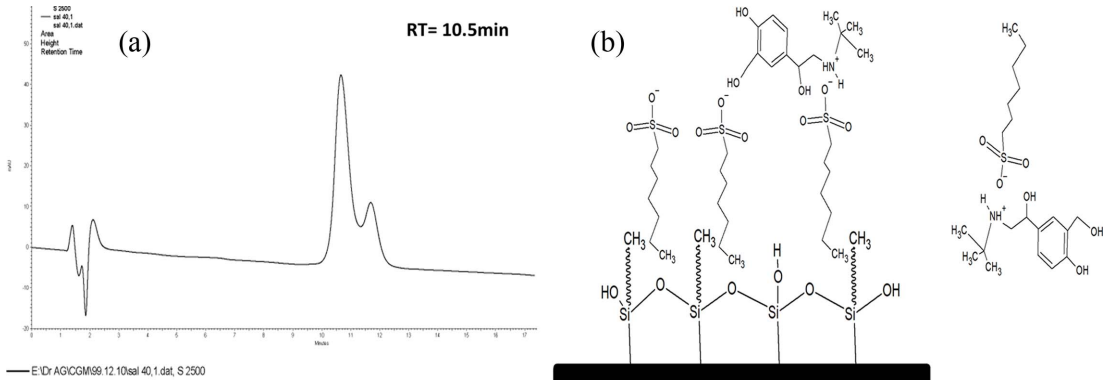

Fig. 3. Effect of SHS addition to the mobile phase. (a) salbutamol dissolved in water, chromatographic system characteristic: high-resolution C18 column (CLIPEUS C18 5 μm, 250 × 2.1mm, USA) and the mobile phase consist orthophosphoric acid 0.5 %: acetonitrile 90: 10, containing 2.78 mM SHS, pH 2 ± 0.1, (b) SHS interaction with silanol and SS.

nitrogen adsorption to the column, but it appears that the SHS concentration was insufficient to interact with all SS molecules. Because the formation of the neutral complex is dependent on the molar concentration of SHS in the aqueous phase.

3.4.1.2. Evaluation of the composition of the mobile phase

In this part of the experiment, we used the HPLC method developed by Nokhodchi *et al*.[10] In comparison to the previous method the differences are as follow; the organic phase was increased to 45 %, and methanol was used instead of acetonitrile. The concentrations of SHS and pH were increased to 6.8 mM and 7.2, respectively. Methanol: water was used as the sample matrix (45:55).

At this mobile phase pH amine functional group of SS (pKa 9.2) is supposed to be positively charged while the hydroxy phenolic group is neutral (10.2). Furthermore, the majority of free silanol groups have a negative charge (pka 4.5-7).

In comparison to the previous section, it is assumed that when SHS concentration increases, more van der Waals interaction of SHS with alkyl chains bound to the stationary phase occurs, allowing a negative layer of SHS to cover the surface of the column and increase SS retention. The availability of free silanol groups to interact SS was prevented by steric hindrance of SHS which can be another reason. Furthermore, as the SHS concentration increased, formation of the neutral complex (SHS-: SS+) increased. Finally, peaks were improved on the first day and appeared as a single peak, but after one day, peaks were doubled again (*Fig.* S3 in the Supplementary material).

3.4.1.3. Evaluation the effect of TEA

1) Pre conditioning of column

First, the column was flushed with gradient elution using solvent A: 100 mL DW (deionized water) containing 0.1 % TEA (95-50 %) and solvent B: ACN (5-50 %) delivered at a flow rate of 0.2 mL/min. This procedure was carried out to fill void spaces, remove hydrophilic and lipophilic contamination on frit, and to block free silanol groups with TEA. Then, various mobile phases were investigated.

2) Mobile phase1

The pre-conditioned column was eluted with a mobile phase containing ACN: DW 10:90 + 0.1 % TEA, pH was set at 9.5 by 100 µl of orthophosphoric acid 10 %. Triple peak emerged at about 15 min (*Fig.* S4 in the Supplementary material). In this pH some of hydroxy phenolic groups of SS and free silanol groups are negatively ionized. Whereas some of amines functional groups are positively ionized. The triple peak could be a result of different interaction of ions with column.

3) Mobile phase2

The pre-conditioned column was eluted with a mobile phase containing ACN: DW 10: 90 + 4.5 mM SHS. pH was decrease to 3.2 by 100 µL orthophosphoric acid 10 %. As shown in *Fig*. S5 in the supplementary material, peak was broadened. In this pH SS are positively ionized and interact with negatively ionized SHS however it is assumed that some of silanol groups are ionized but interact with TEA. Also, the hydrophobic region of SHS may interact with the alkyl bonded groups and ionized SHS interact with bonded TEA . All the mixed mechanisms could lead to peak broadening and increase RT.

4) Mobile phase 3

The pre-conditioned column was eluted with the same mobile phase composition as part 2.1 (ACN: phosphoric acid 0.5 % 10:90) the only difference was buffering the mobile phase by adding NaOH to reach to pH 2.5. *Fig.* 4(a) shows SS chromatogram dissolved in water and appeared as a single peak.

In this condition it is assumed that the amine group was positively charged, and TEA is bonded to the remained negatively charged free silanol groups. Other mechanism is that TEA bonded to alkyl chains of the stationary phase and positive charge of TEA covers the inner surface and repelled the cationic SS.[2] Also buffering the mobile phase maintain the pH constant during process. This method was tested in other days. When the same concentrations of SS were repeatedly injected the retention times are different (*Fig.* 4(b)). It may be caused by the elution of bonded TEA during the washing process. So, adding TEA 0.07 %[11] into the mobile phase was tested. The results are shown in *Fig.* 5. At the

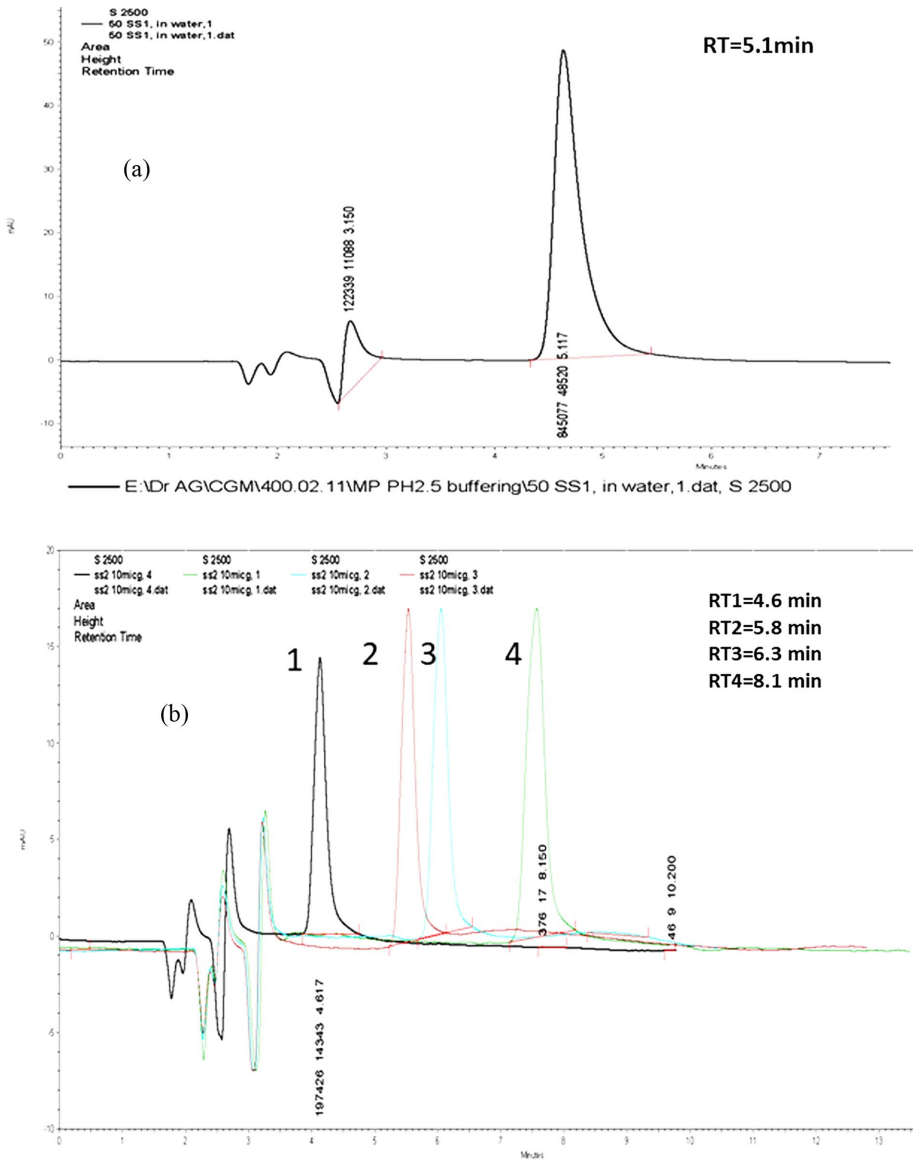

Fig. 4. Effect of column pre-conditioning and buffering the mobile phase. (a) 50 µg/mL SS in water, (b) consecutive injections of the 10 µg/mL SS in water. System characteristic: C18 column (Brisa LC2 C18 Column 5 µm 15 × 0,46 cm), mobile phase ACN: phosphoric acid 0.5 % 10:90, pH adjusted to 2.5 by NaOH, 230 nm.

beginning of an experimental day and at the end; the column was washed by DW containing 0.1 % TEA and 5 %ACN. It shows that in different days the SS peak appears as a single peak. This was set as the desired procedure. The validation parameters for this method are calculated according to ICH guideline.[12] and presented in *Table* 1. *Table* 2 presents the desired HPLC condition.

3.5. The effect of sample matrix

In tested methods when SS dissolved in methanol or mobile phase instead of water the peaks were not satisfactory and they became spilited (results not shown).

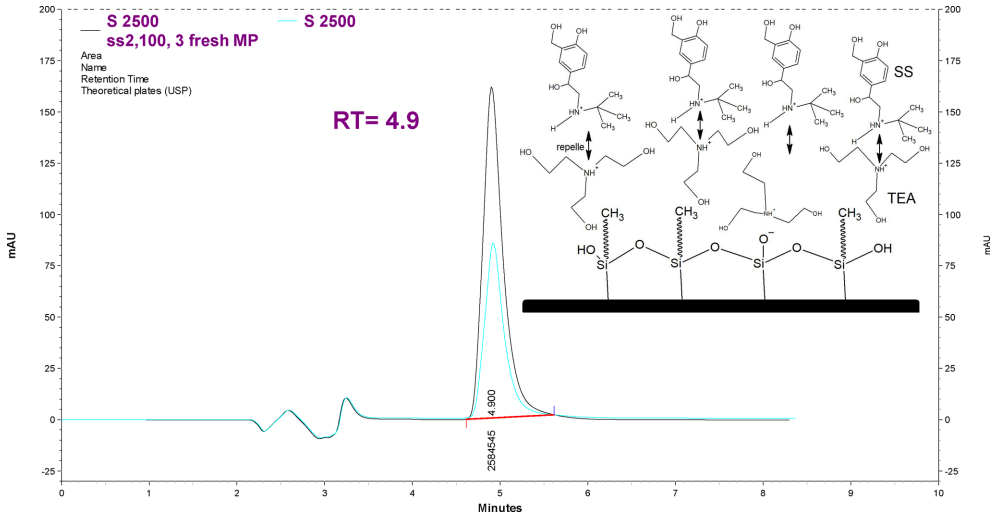

Fig. 5. Effect of adding 0.07 % TEA in mobile phase. Injections of the 50 µg/mL and 100 µg/mL SS in water. Chromatographic system characteristic: C18 column (Brisa LC2 C18 Column 5 µm 15 × 0,46 cm), mobile phase ACN: phosphoric acid 0.5 % 10:90 + TEA 5.74 mM, pH adjusted to 2.5 by NaOH, 230 nm.

Table 1. Validation parameters of developed method

Calibration equation	Regression coefficient (r)	LOD (µg/mL)	LOQ (µg/mL)	Concentration (µg/mL)	Accuracy ± SD %	Repeatability (RSD %)
y = 23809x + 15800	0.999	8.05	24.15	10	103.9 ± 2.88	2.77
				25	99.46 ± 3.23	3.24
				50	97.98 ± 1.1	1.12

Table 2. Desired HPLC method for SS analyzing

Column	Brisa LC^2, C18 Column, 150 mm × 4.6 mm i.d., 5 µm particle size and 120 Å pore size a fully "end capped" free silanol silica with a broad usable pH rang
Pre-wash condition	Column was flushed with gradient elution using solvent A: 100 mL DW (deionized water) containing 0.1 % TEA (95-50 %) and solvent B: ACN (5-50 %) delivered at a flow rate of 0.2 mL/min.
Mobile phase	ACN: phosphoric acid 0.5 %; 10:90 + 0.07 % TEA, adjust pH by NaOH to reach 2.5
Flow rate	0.7 mL/min
Detector	UV 230 nm

4. Conclusions

For decades, the HPLC technique was used for analyzing and determining active pharmaceutical ingredients. Despite a precise and sensitive method, sometimes finding a valid and robust method to achieve a suitable peak is not an easy approach. Basic compounds are known as a problematic molecule for analysing by HPLC, possibly due to their cationic nitrogen, which can interact with the anionic silanol groups of the stationary phase. In this study SS as a model drug has a basic nitrogen and was analyzed by HPLC. Although the column was end capped but after about 150 injection peak shape distorted. As stated in the literature the end capped columns were sometimes not able to solve this problem. Because of this we used different approaches to solve this problem, such as using different columns, mobile phases,

solvent matrices and adding ion pair agents.

Our results show that when column was pre-washed with TEA and also was added to the mobile phase, the peak shape improves over time. Several mechanisms were proposed: i) TEA interact with anionic free silanol groups due to its counter-charge ii) TEA bonding to an alkyl bonded stationary phase to form a positive layer that repels cationic SS molecules. All of these mechanisms act together to prevent the interaction of SS and free silanol groups and to improve peak shape.

This study could be beneficial for analyst and researchers how works in pharmaceutical industry or labs to solve such problems in analysis of cationic basic components.

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References

1. S. Carda-Broch, M. C. García-Alvarez-Coque and M. J. Ruiz-Angel, *J. Chromatogr A.*, **20**(1559), 112-117 (2018).
2. N. J. Eggers and C. M. Saint-Joly, *J. Liq. Chromatogr.*, **6**(11), 1955-1967 (1983).
3. J. Köhler, D. Chase, R. Farlee, A. Vega and J. Kirkland, *J. Chromatogr A*., **352**, 275-305 (1986).
4. A. Mendez, E. Bosch, M. Roses and U. Neue. *J. Chromatogr A.*, **986**(1), 33-44 (2003).
5. P. Rembischevski and A. L. Gemal, *J. Liq. Chromatogr. Relat. Technol.*, **22**(9), 1343-1353 (1999).
6. A. A. Eel-Zaher, M. A. Fouad and E. F. Eelkady, *Anal. Chem. Insights.*, **9**(9), 1-7 (2014).
7. United States Pharmacopeial Convention, *The United States Pharmacopeia*, USP 42 NF 37, United States (2019).
8. M. A. Mahrouse and N. T. Lamie, *Microchem. J.*, **147**, 691-706 (2019).
9. Z. Y. Yang, L. Wang and X. Tang, *J. Pharm. Biomed. Anal.*, **49**(3), 811-815 (2009).
10. W. Kaialy, G. P. Martin, M. D. Ticehurst, P. Royall, M. A. Mohammad, J. Murphy and A. Nokhodchi, *AAPS J.*, **13**(1), 30-43 (2011).
11. L. Mälkki and S. Tammilehto, *J. Pharm. Biomed. Anal.*, **11**(1), 79-84 (1993).
12. ICH Guideline, Q2(R1): Validation of Analytical Procedures: Text and Methodology Q2(R1) in ICH Harmonised Tripartite Guideline (2005).

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