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The potential of Corona Charged Aerosol Detector for investigation of telmisartan - β-cyclodextrin inclusion complexes

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Summary

Cyclodextrins (CDs) are widely used in pharmaceutical analysis due to its biodegradability and eco-friendly character. The particular structure of CDs, characterized by hydrophobic cavity, enables the formation of inclusion complexes with variety of organic compounds. As structures lacking chromophores, CDs could not be detected by Photodiode Array (PDA) detector and Corona Charged Aerosol Detector (CAD) was introduced as the most appropriate detector for the formed complexes. The aim of the study was to investigate the degree of complexation between telmisartan, used as a model substance, and β-CD. Moreover, the effect of different β-CD concentrations (5-15 mM) and pH of the aqueous part of mobile phase (3-7) on the degree of complexation was also assessed. The intensity of the formed complex appeared to be the highest when 15 mM β-CD was used for the complexation. Also, it was noticed that the increase in peak areas with an increasing β-CD concentration was more evident at pH 7 in comparison with the same trend at lower pH values. The reproducibility of the measurement was confirmed by low relative standard deviation (RSD) of peak areas within five measurements. These findings support the use of HPLC-CAD methods for studying the process of inclusion complexes formation.

Keywords: β-Cyclodextrin; Inclusion complex; Corona Charged Aerosol Detector; Telmisartan;

1. Introduction

Liquid chromatography is one of the most frequently used techniques in drug analysis, during research and development, as well as in routine control of pharmaceutical substances and pharmaceutical products (1). To achieve the desired chromatographic efficiency, it is favorable to use organic solvents, in the first place acetonitrile. Most organic solvents used in HPLC are toxic and could harmfully impact the environment and human health as well (2-4). To avoid the negative consequences of large amounts of acetonitrile used during the analysis, different "greening" strategies were applied (4). Nowadays, development of green liquid chromatography methods is a recognized trend in pharmaceutical analysis (5, 6). In that respect, CDs are applied as mobile phase additives allowing an increase of water and simultaneous decrease of organic solvent content, resulting in the consequent reduction of analyte's retention times (7, 8). CDs are well-known cyclic oligomers derived from starch. Their particular structure, characterized by a hydrophilic outer surface and an internal relatively hydrophobic cavity, is responsible for the good water solubility, as well as ability to fully or partially encapsulate hydrophobic molecules of suitable size within their cavity (9-11).

In this study, telmisartan (Figure 1), as a representative of the group of sartans, was selected as a model substance. Physicochemical characteristics of telmisartan made it a suitable candidate to be incorporated in CD cavity. Telmisartan is amphoteric compound, weakly dissolved in water, but moderately to highly dissolved in methanol or acetonitrile (12).

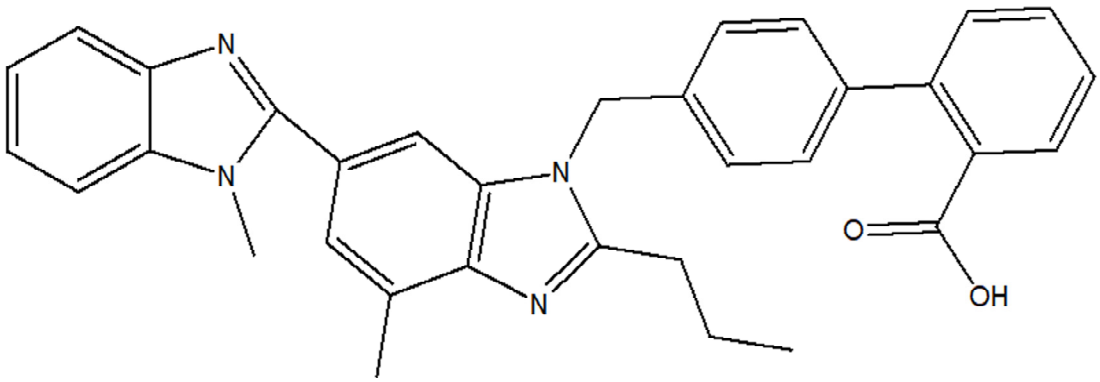

Figure 1: Chemical structure of telmisartan

The ability to form inclusion complexes with structurally diverse drugs served as a base from which numerous applications of CDs were made in the pharmaceutical industry (6, 13). CDs are used to modify the unpleasant smell or taste of the pharmaceuticals, their physicochemical instability, low solubility in water and poor bioavailability. For this reason CDs are profoundly useful in pharmaceutical industry and are incorporated in a number of pharmacopeia sources (14). Moreover, regarding the prominent solubilizing potency, as well as, unfavorable permeability through hydrophobic bio-membranes, CDs are used very successfully in formulations intended for oral, ocular, nasal and, even, parenteral route of drug application (15). Having in mind the great attention CDs have attracted, it would be of high importance to characterize the formed complexes in more detail, and define the parameters which significantly influence the complexation procedure.

Regarding the detection of the complex, there are certain limitations originating from the lack of chromophores in the structure of CDs. Several methods for evaluation of inclusion complexes can be found in literature, applying mass spectrometry (MS) or nuclear magnetic resonance (NMR) (11, 16, 17). However, these techniques are rather expensive, so would not be the first choice for routine analysis. Therefore, a strong need for detector generating sensitive and universal response, independent of spectral or physicochemical qualities of the investigated compounds, arises. Having in mind the aforementioned, the purpose of the study was to investigate the potential of CAD in determining the degree of complexation. CAD, as a detector based on aerosol charging, generates a universal response over a broad dynamic range regardless of the analyte's structure. Among all so far constructed aerosol based detectors, it stands out as an easy-to-use and highly sensitive mass dependent detector (18, 19). Thus, suitability of CAD to investigate the complexation process occurring between telmisartan and β-CD was tested for the first time. We assessed the influence of certain parameters such as β-CD concentration and pH of the sample on the intensity of the complex.

1.1 Theory

During the past years, scientists have dealt with finding the appropriate technique to characterize the complexes formed between CDs and various organic hydrophobic compounds (17) . ESI-MS was recognized as soft ionization technique able to study the stoichiometry of the formed complexes (16), however the information about the degree of complexation was not provided. NMR was also successfully used for stoichiometry determination, as well as assessing the complex stability (20, 21), but the same limitation as in case of ESI-MS occurred. Furthermore, CAD, as a universal detector producing a response independent on the analyte's structure, was the detector of choice (22, 23). CAD's principle of functioning is reflected in nebulization of analytes together with mobile phase, leading to droplets formation. Analyte particles are produced while

droplets are dried to remove the mobile phase. Further, generated aerosol is reaching the reaction chamber where it is collided with a stream of positively ionized nitrogen that has primarily passed over high voltage corona wire. The positive charge is then transferred to the opposing stream of analyte particles and the transferred charge is generating a response directly proportional to the amount of the analyte investigated (23). The intensity of the signal is dependent on the amount of charge the analyte surface can receive, so the detector's response would mostly depend on the mass of investigated analyte (22). Implied phenomenon encompass that larger particles carry more charge than smaller ones. As a particle detector, it is believed that CAD produces the equal amount of particles if the same amount of different materials is entering detector under the stable conditions. However, there is variability in CAD response which originates from the influence of different factors affecting the process of particle formation. Taking into account the evaporation process, mobile phase components have to meet the certain volatility criteria, similar to MS mobile phase requirements (24). In the same course, higher content of organic solvent in the mobile phase is beneficial. Additionally, mobile phase with higher percentage of organic modifier regulates production of larger number of substance particles via lower viscosity. Furthermore, the decreased mobile phase flow rate also contributes to enhanced detector response, as in the case of HPLC-MS. Apart from being a detector of choice for analytes lacking chromophores, CAD's additional advantage is its ease of use reflected in small number of parameters to be optimized (22, 23).

2. Experimental

2.1 Chemicals and reagents

Reference standard substance of telmisartan (2-[4-[[4-methyl-6-(1-methylbenzimidazol-2-yl)-2-propylbenzimidazol-1-yl]methyl]phenyl]benzoic acid) was purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). β-CD was obtained from Acros organics (Geel, Belgium). For mobile phase preparation, deionized water obtained from a Simplicity 185 purification system, Millipore (Billerica, MA, USA), was used. HPLC-grade acetonitrile was also purchased from Sigma Aldrich, while ammonium hydroxide and acetic acid used for pH adjustment were obtained from Centrohem d.o.o. (Stara Pazova, Serbia) and Merck (Darmstad, Germany), respectively. pH of the aqueous phase and working samples was adjusted on PHM210 Standard pH-meter (Radiometer Analytical SAS, Lyon, France) equipped with glass electrode. Before use, mobile phase and working samples were filtered through 0.45 μm nylon membranes (Agilent Technologies, Santa Clara, CA, USA).

2.2 Sample preparation

Stock solution of telmisartan was prepared by dissolving the appropriate amount of the reference standard substance in the mixture of water and acetonitrile (50:50, v/v) to obtain the concentration of 0.5 mg mL-1. β-CD stock solutions were prepared by dissolving an appropriate amount of β-CD in deionized water to obtain the following concentrations: 5 mmol L-1, 10 mmol L-1 and 15 mmol L-1. pH of the working solutions was adjusted by addition of ammonium hydroxide or acetic acid. pH was set to 3, 5 or 7 and five working solutions were prepared at each pH level. The first working solution was consisted of 100 μL of stock solution of telmisartan, 760 μL of deionized water with adjusted pH and 140 μL of acetonitrile. The second working solution consisted of 760 μL of the stock solution of β-CD in 10 mM concentration, 50 μL of deionized water with pH set to a certain level and 190 μL of acetonitrile. The remaining three working samples contained mixture of stock solution of telmisartan (100 μL), stock solution of β-CD in 5 mM, 10 mM and 15 mM concentration, respectively (760 μL) and acetonitrile (140 μL). The complexation was assisted by vortexing the samples for 1 h before the analyses.

2.3 Instrumentation

Experiments were carried out on Dionex Ultimate 3000 HPLC system, equipped with Diode Array Detector (DAD) and Corona Veo Charged Aerosol Detector (Thermo Fisher Scientific, Germering, Germany), serially connected. Separation was achieved on HypersilGOLD-C4 (150 x 4.6mm, 5μm) column (Thermo Fisher Scientific, Germering, Germany). The temperature of the column was set to 25 ˚C. Gradient elution with mobile phase consisted of mixture of water and acetonitrile was applied according to the program presented in Table I. pH of the aqueous part of the mobile phase was set to 3, 5 or 7 with addition of acetic acid or ammonium hydroxide. Detection on DAD was performed at 295 nm, while CAD signal intensity was examined by adjusting the parameters presented in Table II. Injection volume was 10 μL.

Table I Gradient elution program

Time (min)	Mobile phase flow (mL min^{-1})	Content of acetonitrile (%)	Content of aqueous phase (%)
0	0.7	10	90
14.0	0.7	90	10
14.1	0.7	10	90
15	0.7	10	90

Table II Parameters of CAD

Parameter	Optimized values
Evaporation temperature	50 ˚C
Filter constant	5 s
Gain range	10 pA
Power function	1
Data rate	10 Hz

The stoichiometry of the formed complexes was confirmed by injecting the samples into a mass spectrometer with electrospray ionization (ESI-MS). Experiments were performed on TSQ Quantum Access Max (Thermo Fisher Scientific Inc.).

2.4 Docking study

Molecular docking studies were performed applying an on-line available software Autodock v4.2. (The Scripps Research Institute, CA, U.S.A.) (25). β-CD was labeled as host compound and its x-ray crystal structure necessary for the study was retrieved from the Brookhaven protein data bank. Calculation of pKa and selection of dominant forms of telmisartan at different pH values were performed using Marvin Sketch 4.1.13 ChemAxon Ltd (Budapest, Hungary) (26). Conformations of telmisartan with minimal energy content across the examined pH range were obtained by the MOPAC/AM1 method of Chem 3D Ultra 7.0.0 (Surrey, UK) (27). They were further used as ligands to β-CD for docking study. Auto Dock Tools (ADT) was used for preparation, running and analyzing the docking simulation. The Lamarckian Genetic Algorithm (LGA) was chosen to search for the best conformers (28). During the docking process, a maximum of 100 conformers was considered for each compound. A grid of 40 points in each direction (x, y, and z) with grid spacing of 0.375 Å was built centered on the center of ligand molecule. The maximum number of energy evaluation was set to 2 500 000.

3. Results and discussion

The stability of the complex formed between CDs and various organic hydrophobic compounds mostly depends on size complementarity between drug molecule and CD cavity. For that reason, β-CD was chosen as the appropriate one, since there is evidence that large cavity of β-CD is a better host for a phenyl group (29, 30). This group is also present in telmisartan, which theoretically gives the advantage to β-CD in comparison with α-CD possessing smaller cavity. Formed inclusion complex is additionally stabilized by numerous hydrogen bonds and dipole-dipole interactions between certain groups in the structure of telmisartan and primary and secondary

hydroxyl groups present inside and outside the β-CD cavity (31), which was confirmed with docking study explained in more detail in section 3.1.

To get insight into the whole complexation procedure and to determine the degree of complexation and influential parameters, it is necessary to determine the stoichiometry between drug and β-CD, and propose the most probable type of binding. Complex stoichiometry was proven to be 1:1. Molecular ions of 515.6 m/z, 1135.9 m/z and 1650.6 m/z corresponding to telmisartan, β-CD and telmisartan – β-CD complex, respectively, were detected in ESI + mode. To preclude the possibility of complexes formed in 1:2 or 2:1 ratio, broader m/z range was investigated. Across the range from 100 – 3000 m/z, no 2785.6 m/z or 2165.2 m/z corresponding to 1:2 or 2:1 complexes, could be detected. Moreover, employed ESI-MS confirmed that free drug and formed complex are eluting at the same retention times. Knowing the stoichiometry, docking study was performed to predict the complex structures.

3.1 In – silico prediction

Docking study was performed to predict the type of binding occurring between telmisartan and β-CD at different pH values. The results of the performed study gave an impression of the orientation of the ligand molecule towards the cavity of β-CD. When pH of the sample and aqueous mobile phase is set to 3, telmisartan is predominantly present in its dicationic form (81.15 %) with both amino groups protonated. Benzimidazole part of the structure of telmisartan is incorporated in β-CD cavity (Figure 2a). Benzoic acid part in its non-ionized form remains free for interactions with silanol groups of stationary phase. On the other hand, in less acidic conditions (pH 5), number of protonated amino groups is equal to the number of deprotonated carboxyl groups for 56.59 % of the molecule, so electroneutral state contributes to the overall lipophilicity of telmisartan and enables the insertion of benzimidazole part of the structure into the cavity of CD, leading to inclusion complex formation (Figure 2b). On the other hand, for 30.01 % of the molecule both amino groups of benzimidazole part of the structure are protonated at pH 5. Deprotonated carboxyl groups are available for interactions with stationary phase. Finally, at pH 7 the structure with only carboxyl group deprotonated is dominant (88.10 %). However, the pathway of the complex formation remained the same as in previous two cases. Benzimidazole part of the structure of telmisartan is entering β-CD cavity (Figure 2c). These findings indicate that the type of binding is the same at all examined pHs, leading to the conclusion that ionization of carboxylic group and amino groups of benzimidazole makes the difference. At pH 3, interactions between free benzoic acid and stationary phase outreach the interactions leading to complex formation, unlike the other investigated acidity conditions.

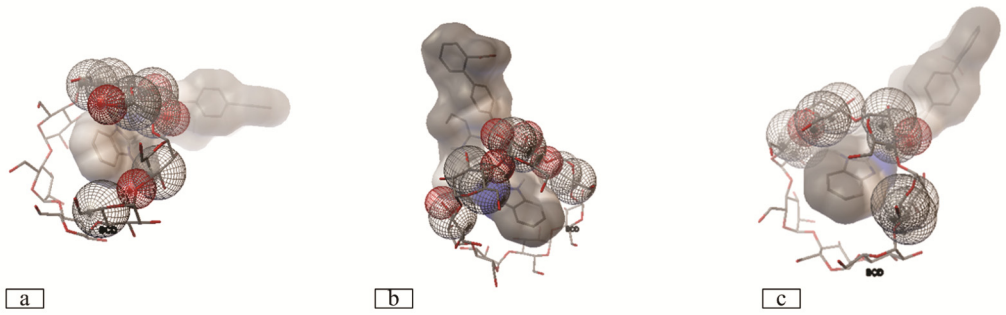

Figure 2: Docking structures
2a Telmisartan - β-CD complex formed at pH 3
2b Telmisartan - β-CD complex formed at pH 5
2c Telmisartan - β-CD complex formed at pH 7

3.2 Investigation of the influence of β-CD concentration and pH on the complexation

CAD was also employed to determine the degree of complexation and investigate its dependence on pH and β-CD concentration. The concentration of telmisartan was kept constant in all samples, while β-CD concentration was varied from 5-15 mM, as well as pH of the samples and aqueous part of the mobile phase. The influence of pH and β-CD concentration on the degree of complexation was investigated by analyzing the samples containing telmisartan only, β-CD only and their mixture. As previously mentioned, DAD was not the best choice for detection of complexes due to its inability to detect compounds without chromophores in their structure. The reason for employing DAD was to determine the retention time of free telmisartan. Furthermore, its role was to confirm that concentration of telmisartan was equal in all of the investigated samples (Figure 3).

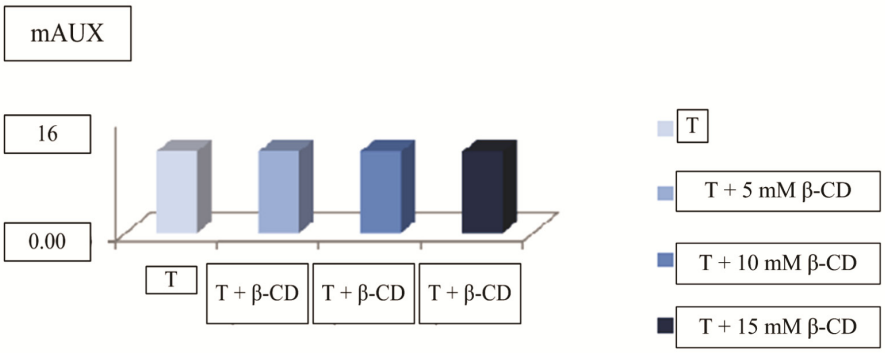

Figure 3: Peak areas originating from free and complexed telmisartan with increasing β-CD concentrations detected by DAD

On the other hand, CAD was capable to detect free β-CD as well as the complex formed between telmisartan and β-CD. When analyzing the mixture of telmisartan and β-CD with HPLC-DAD the obtained chromatogram contained only one peak. LC – ESI – MS showed that retention time of the observed peak was the same as retention time of free telmisartan leading to a conclusion that free telmisartan and formed inclusion complex are eluting at the same retention time. On the other hand, when analyzing the same mixture with HPLC-CAD, two peaks could be observed (Figure 4).

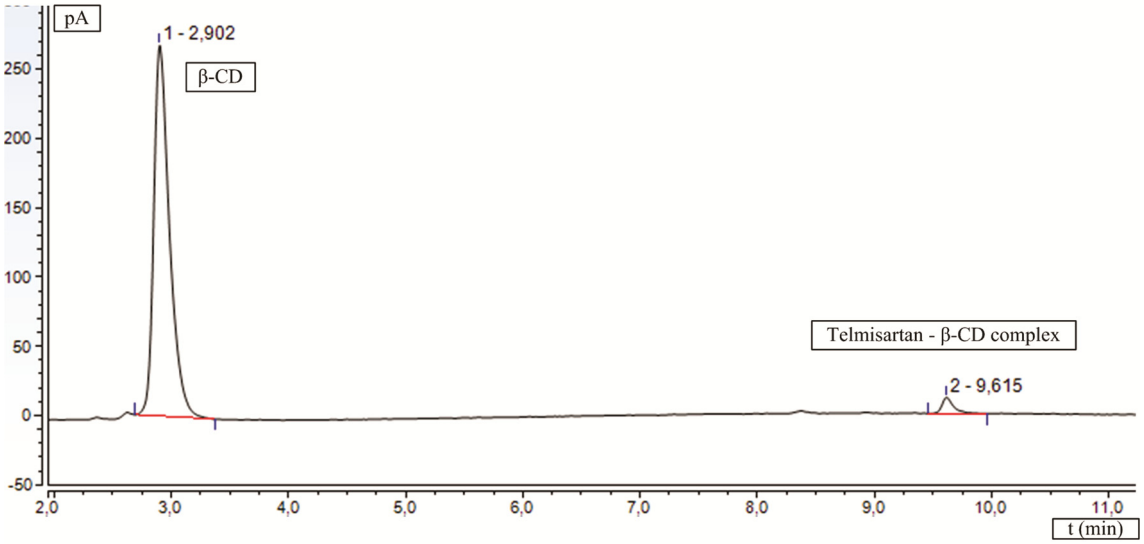

Figure 4: Chromatogram of β-CD and complex between telmisartan and β-CD obtained from HPLC-CAD

The retention time (tr) of the first peaks is equal to retention time of free β-CD (tr = 2.90 min) previously analyzed, confirming that the first peak originates from free uncomplexed β-CD. Retention time of the second peak present was the same as retention time of free telmisartan (tr = 9.61 min). Consequently, the second peak represents telmisartan - β-CD complex. The influence of varying β-CD concentrations on complexation was investigated under constant concentration of telmisartan in the mixture. In the same manner the influence of different pH on the complexation procedure was also assessed. The results are presented in Table III.

Table III Signal intensities of the analyzed samples from DAD and CAD at pH = 3, pH = 5 and pH = 7

	pH = 3			pH = 5			pH = 7		
Sample	DAD	CAD		DAD	CAD		DAD	CAD	
		β-CD	T + β-CD		β-CD	T + β-CD		β-CD	T + β-CD
T	A = 21.6703 t_r = 9.48	-	A = 1.0945 t_r = 9.55	A = 16.8259 t_r = 10.48	-	A = 1.1129 t_r = 10.54	A = 18.9936 t_r = 11.38	-	A = 0.4787 t_r = 11.46
β-CD (10 mM)	-	A = 45.4792 t_r = 2.77	-	-	A = 32.3598 t_r = 2.79	-	-	A = 30.4554 t_r = 2.78	-
T + β-CD (5 mM)	A = 21.6744 t_r = 9.47	-	A = 1.1362 t_r = 9.54	A = 21.7340 t_r = 10.59	-	A = 1.4154 t_r = 10.65	A = 19.5229 t_r = 11.30	-	A = 0.9638 t_r = 11.37
T + β-CD (10 mM)	A = 21.6059 t_r = 9.47	A = 42.0940 t_r = 2.78	A = 1.1737 t_r = 9.53	A = 22.1996 t_r = 10.61	A = 30.1101 t_r = 2.79	A = 1.5213 t_r = 10.68	A = 19.2248 t_r = 11.06	A = 28.9039 t_r = 2.78	A = 1.2102 t_r = 11.13
T + β-CD (15 mM)	A = 21.5967 t_r = 9.47	-	A = 1.2197 t_r = 9.53	A = 22.7271 t_r = 10.63	-	A = 1.5286 t_r = 10.70	A = 19.8777 t_r = 10.80	-	A = 1.4993 t_r = 10.89

T – Telmisartan; A – Peak area (mAUX for DAD, pA for CAD); t_r – retention time

When analyzing free β-CD and β-CD in mixture with telmisartan, in the same concentration, the difference in the peak area of β-CD was evident (Figure 5).

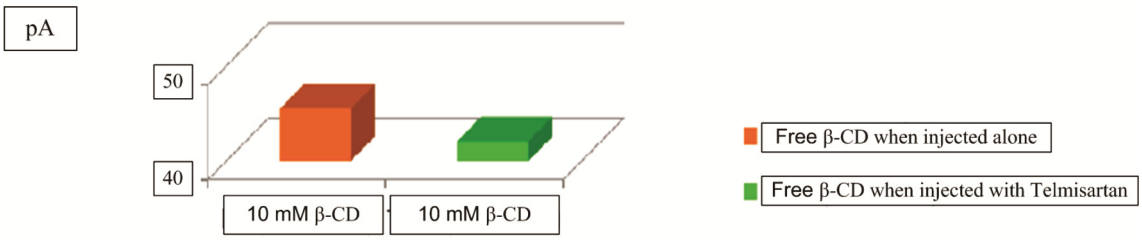

Figure 5: Difference in peak area of β-CD in the same concentration when analyzed free and in mixture with telmisartan obtained in HPLC-CAD

Moreover, peak areas of telmisartan–β-CD complex were increased with increasing β-CD concentrations from 5-15 mM. The difference in peak area of free β-CD when it was analyzed separately and in mixture with telmisartan also confirmed the hypothesis that the degree of complexation is higher with increasing concentrations of β-CD (Figure 6). The simultaneous detection on DAD showed that the signal of telmisartan was constant (Figure 3), confirming the hypothesis that the signal intensity

on CAD increases only due to the degree of complexation. Furthermore, the degree of complexation was growing with an increasing concentration of β-CD in the sample across the investigated pH range (Figure 6 and 7).

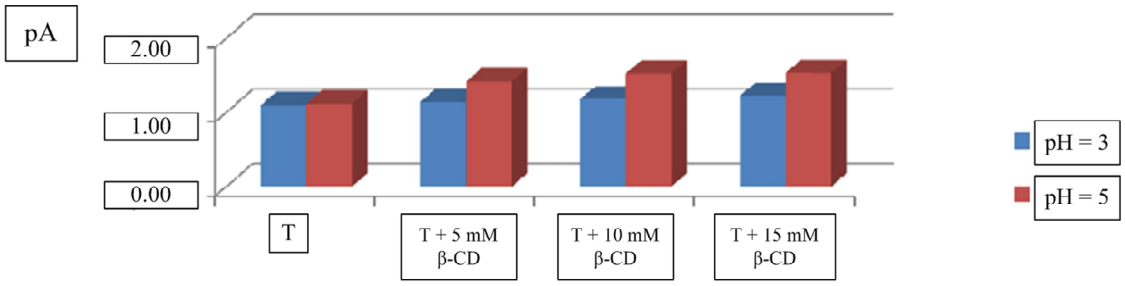

Figure 6: Comparison of degree of complexation with increasing β-CD concentrations at pH 3 and pH 5

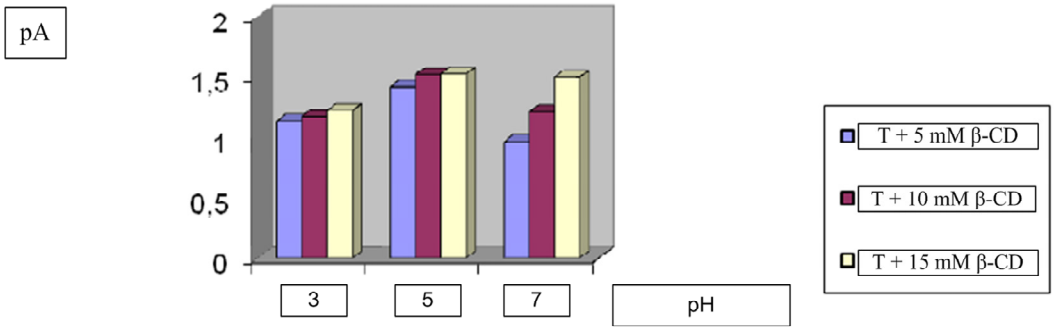

Figure 7: Comparison of degree of complexation with increasing β-CD concentrations at pH 3, pH 5 and pH 7

3.3 Reproducibility of the measurement

The reliability of the presented results was examined through the reproducibility test. Low variances between replicates confirmed reproducibility of the complexation. Furthermore, significantly lower variances between replicates compared to the variances between the samples with different concentration of complexation agent excluded the

possibility that the variances between samples with different concentration of complexation agent originate from the imprecision of the sample preparation (complexation) or instrument. To assess the reproducibility of the measurement, five identical samples were prepared and analyzed respectively. Five samples containing mixture of telmisartan with 5 mM β-CD, five samples containing telmisartan in the same concentration with 10 mM β-CD and five samples containing the same concentration of telmisartan with 15 mM β-CD were prepared and analyzed. As a measure of reproducibility, relative standard deviation (RSD) was calculated, for both HPLC-PDA and HPLC-CAD analysis. All of the obtained RSD values were less than 5% indicating good reproducibility. Results are presented in Table IV.

Table IV Assay of reproducibility of the measurement

Measurement number	T + 5 mM β-CD		T + 10 mM β-CD		T + 15 mM β-CD	
	DAD	CAD	DAD	CAD	DAD	CAD
1	20.2146	1.4491	21.6330	1.4764	16.9340	1.1187
2	20.0409	1.4381	21.7395	1.5373	16.4978	1.1876
3	19.9300	1.4224	21.6763	1.4921	16.2257	1.1111
4	19.7824	1.4567	21.5925	1.5602	15.8532	1.1474
5	19.7072	1.4410	21.5302	1.6443	15.6645	1.0357
RSD (%)	1.02	0.89	0.37	4.37	3.12	4.99

4. Conclusion

This study highlighted the potential ofto use HPLC-CAD in complexation procedure elucidation. The repeated measurements confirmed that CAD response is reproducible. Therefore, the reliability of the obtained results was also proven. An increase in β-CD concentration affected the intensity of the complex in a positive manner. Effect of pH on the complexation was also shown. At pH 7 the difference in peak areas of the complex with increasing β-CD concentrations was larger in comparison with the observed differences at pH 3 or 5. Finally, HPLC-CAD could be selected as one of the methods for studying complexes with β-CD. Moreover, due to its performances and simplicity, it could accompany the other techniques in analytical research and development.

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5. Literature

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