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Study of the lorazepam: cyclodextrin inclusion complexes using electrospray ionization mass spectrometry

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Abstract—As an example of drug–cyclodextrin interactions in aqueous media, the cyclodextrin complexation of lorazepam was studied by electrospray ionization mass spectroscopy (ES-MS). It was concluded that highly concentrated aqueous hydroxypropyl-cyclodextrins are more suited for the drug complexation and that the inclusion complex includes one molecule of the drug with two and three cyclodextrin molecules as well as two molecules of the drug with three molecules of hydroxypropylcyclodextrins. It was postulated that in the hydroxycyclodextrin cavity the drug molecule decomposes via the elimination of one molecule of formaldehyde. © 2001 Elsevier Science Ltd. All rights reserved.

An important part of chemical research is to determine the existence as well as alter the physico-chemical properties of various drugs in aqueous media. If the drug molecule has aromatic groups that are complementary in size to the cyclodextrin cavity, then cyclodextrin is the molecule of choice to achieve these goals.¹ One of the most commonly used sedatives (particularly for anxiety and insomnia)² that has aromatic moieties to which cyclodextrins can bind are benzodiazepines.³ Therefore, it seems appropriate to explore their physical properties in aqueous cyclodextrins.

The objective of this work is to explore the possibility of using the ES-MS technique to better understand the nature of the benzodiazepine-cyclodextrin inclusion complex. Lorazepam (L) as one important member of the benzodiazepine family was selected to study the capabilities of cyclodextrin (CD) to form detectable benzodiazepine-cyclodextrin inclusion complexes (Scheme 1). The most widely used cyclodextrin in chemistry is β -cyclodextrin (β -CD). Naturally, its capability to form inclusion complexes with these drugs is of great importance, but it is also significant to explore the influence of the cyclodextrin cavity size on the inclusion complex formation. This can be accomplished by broadening the complexation study to cover α - and γ -cyclodextrins (α -CD and γ -CD). On the other hand, β-cyclodextrin has relatively low solubility in water,⁴ therefore to explore the influence of the concentration on the formation of the cyclodextrin complexes, hydroxypropyl derivatives of α -, β -, and γ -cyclodextrins were also included in this study.⁵ These cyclodextrins (hydroxypropyl-\alpha-cyclodextrin, HP-\alpha-CD; hydroxypropyl-β-cyclodextrin, HP-β-CD; hydroxypropyl-γcyclodextrin, HP- γ -CD) have both higher solubilities and binding capabilities than normal cyclodextrins.⁶ To explore the influence of polyhydroxyl molecules already present in natural fluids on the cyclodextrin drug complexation, the study also includes such additives as glycerol, PEG, and propylene glycol.⁷





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The formation of the inclusion complexes between benzodiazepines and cyclodextrins is known,⁸ but the inclusion complex 'behavior' in aqueous solution is still not completely understood. The earlier inclusion complexation studies were based on solubility studies using UV-Vis, CD, IR, and DSC techniques.⁹ Recent complexation studies were performed in mixed or organic solvents¹⁰ rather than in water since most of the benzodiazepines have very low solubility in water. In many instances additives such as glycerol help to dissolve the drugs, although water is the media in which cyclodextrin application is utilized the most.¹¹ In this paper only the results obtained from electrospray ionization mass spectroscopy (ES-MS) will be presented¹² although in some instances our extensive NMR studies of the benzodiazepines will be used for comparison.

Contrary to a traditional spectroscopic approach, such as is the case with NMR¹³ spectroscopic study, ES-MS spectroscopy allows us to detect and explore all kinds of different molecular associates between the guest and the host molecules.¹⁴ 'Soft' ionization methods have been employed to study the non-covalent interactions in the gas-phase successfully.¹⁵ Recently, Bartlett¹⁶ and others¹⁷ showed that ES-MS is a very useful soft technique for the study of molecular complexes.

We were challenged in our studies by the fact that our guest lorazepam (and the majority of the other benzodiazepines as well) has very poor water solubility (~0.1 mM). Fortunately, our NMR studies of the lorazepamcyclodextrin inclusion complexes reveal that to observe a substantial change in the chemical shift of the aromatic portion of the spectra, the required molar ratio L:CD is around 1:100. The concentration ratio used in the experiments are also determined by the fact that signals of the inclusion complexes should be sufficiently strong to be clearly separated from the spectra baseline.¹⁸ For practical purposes, the concentration of cyclodextrins was as high as 100 mM,¹⁹ while the lorazepam concentration was kept at a constant 0.1 mM.

In all our ES-MS studies with HP-CD 1:2²⁰ and 2:3²¹ lorazepam-cyclodextrin inclusion complexes were observed as demonstrated on the examples of lorazepam (L) inclusion complexes with the HP- α -CD (Fig. 1). Although the concentration of cyclodextrin is 1000 times higher than the drug concentration, the instrument detector sensitivity is tuned to lorazepam and in this way a better noise to signal ratio for inclusion complexes was observed. The free guest molecule signal (L+Na=344) is very small in comparison with the HP- α -CD inclusion complexes, indicating that practically the entire drug is complexed by the cvclodextrin. It appears that the complex with two HP- α -CD and one L is the most dominant inclusion complex, although there are signals of drug complexed three HP- α -CDs (Fig. 1).

In the ES-MS spectra of lorazepam inclusion complexes with HP-CDs (HP- α -CD, HP- β -CD, and HP- γ -CD) the observed m/z is 30 mass units below the expected molecular mass. This is clearly not the case when α -CD or γ -CD as host molecules were used (Table 1). The influence of the hydroxypropyl moiety on the cyclodextrin ring does not only increase the solubility in water and the cyclodextrin binding capability, but it also alters the chemical stability of the drug. It is obvious that the presence of the hydroxypropyl group catalyzed the elimination of the molecular unit with m/z 30. The first postulate is that lorazepam and HP-α-CD form acetal A through a reaction of the hemiacetal hydroxy group of HP-CD with the hydroxyl group of lorazepam (Scheme 2). In this way a molecule of water is eliminated (m/z=18). According to our ES-MS data even if this acetal is formed it decomposes and its signals cannot be observed.

Further evidence that the acetal **A** is an unlikely product formed in aqueous **L** and HP- α -CD comes from our chromatographic study. Both reverse phase column and reverse phase TLC chromatographic separations of the water solution made from lorazepam (0.1 mM) and HP- α -CD (100 mM) were performed. Only



Figure 1. The ES-MS spectra for the aqueous lorazepam (0.1 mM) and HP- α -CD (100 mM).

Table 1. Major peaks and host–guest ratio between lorazepam (L) as guest and cyclodextrins (α -CD, γ -CD, HP- α -CD, HP- β -CD, and HP- γ -CD)²² as host molecules obtained in water

Cyclodextrin (host)	Host:guest -ratio	Major peaks observed, m/z
α-CD	1:1	1319 $[\alpha$ -CD+L+Na] ⁺
	2:1	1157 $[2\alpha$ -CD+L+2Na] ²⁺
γ-CD	2:1	2937 $[2\gamma-CD+L+Na]^+$
		1480 $[2\gamma-CD+L+2Na]^{2+}$
	3:1	2130 $[3\gamma-CD+L+2Na]^{2+}$
HP-α-CD	2:1	2260-2898, cluster of 12 peaks 58
		units apart
		$[2HP-\alpha-CD+L-H_2CO+Na]^+$
	3:2	1773 (1802) to 2092, cluster of 12
		peaks 29 units apart
		$[3HP-\alpha-CD+2L-2H_2O+2Na]^{2+}$
HP-β-CD	2:1	2580-3226, cluster of 12 peaks 58
		units apart
		$[2HP-\beta-CD+L-H_2CO+Na]^+$
	3:2	2015–2379, cluster of 12 peaks 29 units apart
		$[3HP_BCD+2L_2H_CO+2N_2]^+$
HP-γ-CD	2.1	2852-3491 cluster of 12 peaks 58
	2.1	unite apart
		$[2HP_{2}CD \perp I_{2}H CO \perp N_{2}]^{+}$
	3.2	2257-2581 cluster of 12 peaks 29
	5.4	units apart
		$[2 H P \propto C D + 2 L = 2 H C O + 2 N a^{12} +$
		$[5117-\gamma-CD+2L-2\Pi_2CO+2Na]$

UV-detectable molecules in the solution do not have attached cyclodextrin moieties.²³ In two separate experiments, aqueous solutions of HP- α -CD and lorazepam were stirred at room temperature (first experiment) or refluxed overnight (second experiment). In both cases we were not able to detect the presence of lorazepam, instead we detected two dominant products. At room temperature the dominant product seems to be heterocycle I (Scheme 2) and at higher temperatures ketone **P**.²⁴

The influence of the concentration of cyclodextrin on the formation of a higher order of inclusion complexes, as well as a relatively low affinity of benzodiazepines to form cyclodextrin inclusion complexes is perfectly demonstrated in Fig. 2. From our ¹H NMR studies we know that there is practically no change in the chemical shift for aromatic signals of 0.1 mM lorazepam if the cyclodextrin concentration is below 10 mM. Furthermore, disproportion of the guest and host proton signals in the NMR spectra of this aqueous solution renders the NMR method a difficult experimental technique. When HP- α -CD is used in only 10-fold excess (Fig. 2) there is no ES-MS detectable cyclodextrin complexation of lorazepam. Only the free drug, the free HP- α -CD, and the self-complexed HP- α -CD signals were observed (Fig. 2).

In all highly concentrated aqueous cyclodextrins, the ES-MS signals that indicate double complexation of the drug was observed (Table 1).²⁵ The drug tends to be complexed with at least two hydroxypropylcyclodextrins, while the formation of the complex with three hydroxypropyl cyclodextrins and two lorazepam molecules is also strong. The size of the cavity seems to not have an influence on the intensity of the 3:2 complex. This can be explained by the fact that the hydroxypropyl group can stabilize the cyclodextrin inclusion complex in two ways. One is by shielding a large portion of the drug's benzene moiety from the bulk of water because it cannot fully fit into the HP-α-CD cavity. Another is by surrounding the drug's benzene moiety to make it fit better into large HP- γ -CD cavity. This, of course, cannot be accomplished in α -CD, β -CD, and γ -CD; therefore, it is reasonable to expect that the ES-MS intensity of signals for drug complexation with these cyclodextrins is not as strong.

One can argue that polyhydroxyl molecules can actually help the formation of cyclodextrin complexed lorazepam in a similar manner as covalently attached hydroxypropyl groups do in HP-CDs. According to our ¹H NMR spectroscopic studies, the presence of polyhy-



Scheme 2. Possible pathways for decomposition of lorazepam in aqueous HP-CDs.



Figure 2. The ES-MS spectra for the aqueous lorazepam (0.1 mM) and HP- α -CD (1 mM).

droxyl molecules such as glycerol increase the solubility of lorazepam in water, but at the same time decrease the solubility of the cyclodextrins. It also appears that there is no substantial difference on the cyclodextrin lorazepam binding constant, which is estimated to be between 10^{-4} and 10^{-3} mol⁻¹ L. However, 100 mM concentration of the cyclodextrin with glycerol is hard to maintain homogeneous. The ES-MS spectroscopic studies fully agree with the ¹H NMR finding. There is some difference of the ion distribution. For instance, no double charged CD species can be observed when glycerol is present. Strong signals for the sodium cation with glycerol are present in the ES-MS spectra (m/z 115 and 190).²⁶

In summary, noncovalent lorazepam–cyclodextrin inclusion complexes could be studied using 'soft' ES-MS techniques. The formation of cyclodextrin inclusion complexes occurs and can be easily detected with the ES-MS technique in highly concentrated aqueous cyclodextrins. To achieve that, a host–guest molar ratio of 1000:1 in 100 mM aqueous cyclodextrin is required. Although quite different, the ES-MS and NMR studies complement each other quite well. We also confirmed the advantage of hydroxypropylcyclodextrins to form detectable inclusion complexes with lorazepam and eliminate the formaldehyde molecule from lorazepam. The typical complexation ratio is 2:1 and 3:2 for hydroxypropylcyclodextrin–lorazepam inclusion complexes.

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- 12. The ES-MS spectra were acquired with a sector instrument with a mass of charge (m/z) range of 5000. A Micromass Autospec M mass spectrometer with an electrospray source was used. The ES-MS parameters (i.e. pressure, temp., dielectric capillary distance and the voltage on the needle, etc.) were kept constant in each series of solutions. A flow rate of 10 μ L/min was applied using volumes of 100 μ L of sample solutions with the constant concentration of lorazepam being 10^{-4} M and cyclodextrin concentration variations from 10^{-1} to 10^{-4} M.
- 13. On the NMR time scale the recorded NMR chemical shifts for guest molecule represent an average chemical shift resulting from fast equilibrium that involve free guest molecules as well as guest molecules involved in various cyclodextrin inclusion complexes.
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- 18. When the lorazepam–cyclodextrin concentration ratio is 1:100 or 1:1000 practically all lorazepam is in the inclusion complex. Therefore, it is reasonable to propose that the concentration of the inclusion complex present in the solution is equal to initial concentrations of lorazepam (0.1 mM). Furthermore, the hydroxypropylcyclodextrins used are a mixture of variously substituted cyclodextrins. For instance, HP- α -CD is a mixture of mono-, di-, three-,

four-, penta-, and hexahydroxypropyl- α -cyclodextrins. Except for mono- and hexahydroxypropyl- α -cyclodextrin all the others can have many isomers are a result of attaching hydroxypropyl to the different primary hydroxyl groups of the glucose unit of the cyclodextrin ring. For instance, due to cyclodextrin asymmetry there are six dihydroxypropyl- α -cyclodextrin (1,2-, 1,3-, 1,4-, 1,5, and 1,6-. Pairs 1,2- and 1,6- as well as 1,3- and 1,5are not equivalent. In the ES-MS all six dihydroxypropyl- α -cyclodextrin isomers generate one set of signals). The same is applicable for other hydroxypropylcyclodextrins. Therefore, in the case of HP- α -CD six ES-MS 1:1, 36 1:2, and 108 1:3 ES-MS detectable inclusion complexes will be formed in the solution. Of course the most intense will also be the one that has the most isomers. To generate reasonably strong signals of these inclusion complexes the lorazepam-cyclodextrin ratio is required to be as high as possible.

- 19. Considering our reasoning mentioned above (variety of HP-CD inclusion complexes with lorazepam) it would be optimal to prepare a 0.1 mM concentration of lorazepam in 1000 mM (1 M) concentration of HP-CD. Although these cyclodextrins will allow us to prepare this highly concentrated solution the cyclodextrin crystallizes from the solution after several hours. Further problems appear with carrying out the ES-MS experiment. Crystallization of the cyclodextrin occurs in the injection needle and injection chamber must be cleaned after every ES-MS recording even when the concentration of the cyclodextrin is 0.1 mM. With these difficulties associated with experimental studies, high concentration cyclodextrin solutions such as 1000 mM (1 M) were not possible and a majority of our studies were performed with 0.01 M cvclodextrin concentration (1:100 guest-host ratio), but to obtain the better spectra presented in this paper the concentration of cyclodextrin was 0.1 M.
- 20. The signal values for the L:HP-CD 1:2 complex are observed with the elimination of formaldehyde. This reaction also occurs under simulated laboratory conditions and a paper of synthetic formaldehyde elimination from diazepams will be published separately. For examples of elimination of formaldehyde from β -oxo alcohols, see: Olsen, S.; Aalrust, E.; Blom, H. Chem. Ber. 1957, 90, 1389-1398; Olsen, S.; Aalrust, E.; Blom, H. Chem. Ber. 1957, 90, 765-771. The reaction is very similar to the elimination of carbon monoxide from barbituric acids. For this reaction, see: Wulff, G.; Clarkson, G. Carbohydr. Res. 1994, 257, 81-95; Jursic, B. S. Tetrahedron Lett. 2000, 41, 5325-5328. The basic ES-MS signal at m/z = 2260 corresponds to $[2\alpha$ -CD+L-HCHO+Na]⁺ = $[2\times$ 973+321-30+23]. After these ES-MS signals 10 addition signals for various HP- α -CD isomers in the complex separated by 58 units follow the 2α -CD+1L complex signal.
- 21. The values for this complex start with $[3\alpha$ -CD+2L-2H₂CO+2Na]²⁺ = $[3\times973+2\times321-2\times30+2\times23]^{2+}=3547$. This amounts to m/z = 1803.5, which corresponds to our first signal at 1773. This signal is followed by 12 signals separated by m/z = 29, a half molecular weight of one hydroxypropyl unit.
- 22. We were not able to detect the β -CD inclusion complex with lorazepam due to fact that β -CD has relatively low water solubility.

- 23. Chromatographic study was performed with Merck TLC plates with C18-silica gel (5×20 cm) with a fluorescent indicator. Methanol was used as the solvent with slow increases of water (gradient chromatography). A similar solvent was used for reverse phase column chromatography. We were not able to detect nor isolate any UV-absorbing compound from the solution of L in aqueous HP- α -CD except lorazepam. Therefore, we believe that formation of acetal A under these conditions is unlikely.
- 24. In the first experiment lorazepam (3.2 mg; 0.01 mol) and hydroxypropyl- α -cyclodextrin (140 mg; ~0.1 mmol) in 50 mL were stirred and sonicated at room temperature overnight. The aqueous solution was extracted with chloroform, and the chloroform solution was dried over anhydrous sodium sulfate and evaporated. The TLC analysis in chloroform shows the formation of one dominant product with $R_{\rm f}$ 0.26. Our ¹H NMR is consistent with the monochloro derivative of I for which experimental data are available (Pharmazie 1988, 43, 362). In the second experiment the same aqueous solution with the same composition of lorazepam and hydroxypropyl-acyclodextrin was refluxed for 45 minutes. In the beginning white crystals of lorazepam were on the surface of the solution and the walls of the round-bottomed flask. After several minutes the solution became clear. The cooled reaction mixture was extracted with chloroform (3×15 mL), the chloroform solution dried over anhydrous sodium sulfate and evaporated to dryness. Considering

both TLC chromatography in chloroform and ¹H NMR spectra there are no traces of lorazepam in the residue. There are three products detected by the TLC. The major product has R_f values of 0.22, while the lorepazam value should be 0.04 (thin-layer chromatography was performed using plastic-based 0.25 mm thick silica gel 60 F-254 plates with chloroform as solvent). In the ¹H NMR (CDCl₃) spectra signals that corresponds to lorazepam such as two doublets at 5.048 (J=9 Hz) and 4.541 (J=9 Hz) for the CHOH unit are not present and aromatic signals are shifted to higher ppm values (~0.6 ppm). This is all in agreement with the assumption that product **P** is formed in the course of the reaction.

- 25. Due to the low solubility of β -cyclodextrin in water the ES-MS of aqueous 100 mM β -CD was not possible to record. On the other hand, results from the 10 mM aqueous β -CD ES-MS studies were not reproducible.
- 26. Adding glycerol to 100 mmol aqueous α and γ -cyclodextrin causes the precipitation of the cyclodextrin. This is not the case with freshly prepared aqueous HP- α -CD, HP- β -CD, and HP- γ -CD. Nevertheless, the instrument injection needles become quickly clogged and precipitation is formed in the injection chamber which makes spray inconsistent and obtained results hard to reproduce. Therefore, our studies with glycerol as an additive were carried out with 10 mM aqueous hydroxypropylcy-clodextrins where the formation of the lorazepam cyclodextrin inclusion complexes are not as strong.