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Inclusion of Benzaldehyde Semicarbazone into β-Cyclodextrin Produces a Very Effective Anticonvulsant Formulation

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Abstract

Aryl semicarbazones can be considered a novel class of compounds presenting anticonvulsant activity. In order to improve the efficiency and bioavailability of benzaldehyde semicarbazone (BS) we used the host–guest strategy and β-cyclodextrin (β-CD) to prepare a BS/β-CD 1:1 inclusion compound, which was characterized by thermal analyses (TG, DTG, DSC), XRD powder pattern diffraction analyses, infrared data and ^{1}H, ^{13}C, 2D-ROESY NMR and ^{1}H relaxation times. The BS/β-CD inclusion compound was tested in rats using the maximum electroshock (MES) screen. The minimum dose necessary to produce anticonvulsant activity decreased from 100 mg/Kg (ip or vo) for the free semicarbazone to 25 mg/Kg/vo (75%) and 15 mg/Kg/ip (85%), indicating that the host–guest strategy that uses β-CD and BS is very effective and could be successfully employed in the preparation of pharmaceutical formulation of anticonvulsants.

Abbreviations: BS – benzaldehyde semicarbazone; CD – cyclodextrin; β-CD – β-cyclodextrin; MES – maximum electroshock screen; PM – physical mixture.

Introduction

Epilepsy is a leading neurological disorder, second only to stroke [1]. Current drug therapy for epilepsy suffers from the disadvantage that the convulsions of approximately 25% of the patients cannot be controlled by medication [2]. Moreover, the number of drugs useful for the treatment of epilepsy is remarkably small. A series of aryl semicarbazones have shown to possess excellent anticonvulsant activity in the maximal electroshock (MES) screen in rats [3–7], being more active than mephenytoin or phenobarbital, besides their low or absent neurotoxicity [4]. Considering a binding site hypothesis, it is likely that the semicarbazone moiety and the aryl ring align at complementary areas on a macromolecular complex with the receptor *in vivo*; these areas have been referred to as the hydrogen bonding domain and the hydrophobic unit [7].

Molecular encapsulation of drugs in cyclodextrins has been extensively studied recently with the aim of improving characteristics of pharmaceutical interest such as solubility in aqueous media, dissolution rate, stability and biovailability [8].

In a previous work we prepared a very effective anticonvulsant formulation using encapsulation of benzaldehyde semicarbazone (BS) in hydroxypropyl-β-cyclodextrin (HP-β-CD) [9]. In order to further improve the efficiency and bioavailability of semicarbazones we now used the host-guest strategy and β-CD to prepare a BS/β-CD 1:1 inclusion compound (Figure 1), which was characterized using FTIR, thermal analysis (TG/DTG, DSC), XRD pattern diffraction and ^{1}H, ^{13}C NMR, ^{1}H relaxation times and 2D-ROESY techniques. The advantages of β-CD are its lower cost and better suitability for oral administration [10]. The anticonvulsant activity of the BS/β-CD inclusion compound was tested in Wistar normal rats using the MES experimental model of epilepsy.

Experimental section

General

Infrared spectra were recorded on a IR-TF Galaxy 3000 Mattson spectrometer in the 4000–400 cm^{-1} range using KBr disks; NMR spectra were obtained with a Brucker DPX-200 Avance (200 MHz) or a Brucker DRX-400 Avance (400 MHz) spectrometer and deuteriated dimethyl sulfoxide (d^{6}-DMSO) as the solvent. Powder X-Ray diffraction patterns were recorded on a Rigaku Geiger-flex 2037 equipment using a copper tube and CuKα radiation (λ = 1.5405 nm). The TG/DTG curves were obtained with a Shimadzu TGA-50H balance under a nitrogen flow. TG curves were measured at 10 °C/min by heating the sample from 25 °C to 750 °C. DSC curves were obtained with a Shimadzu

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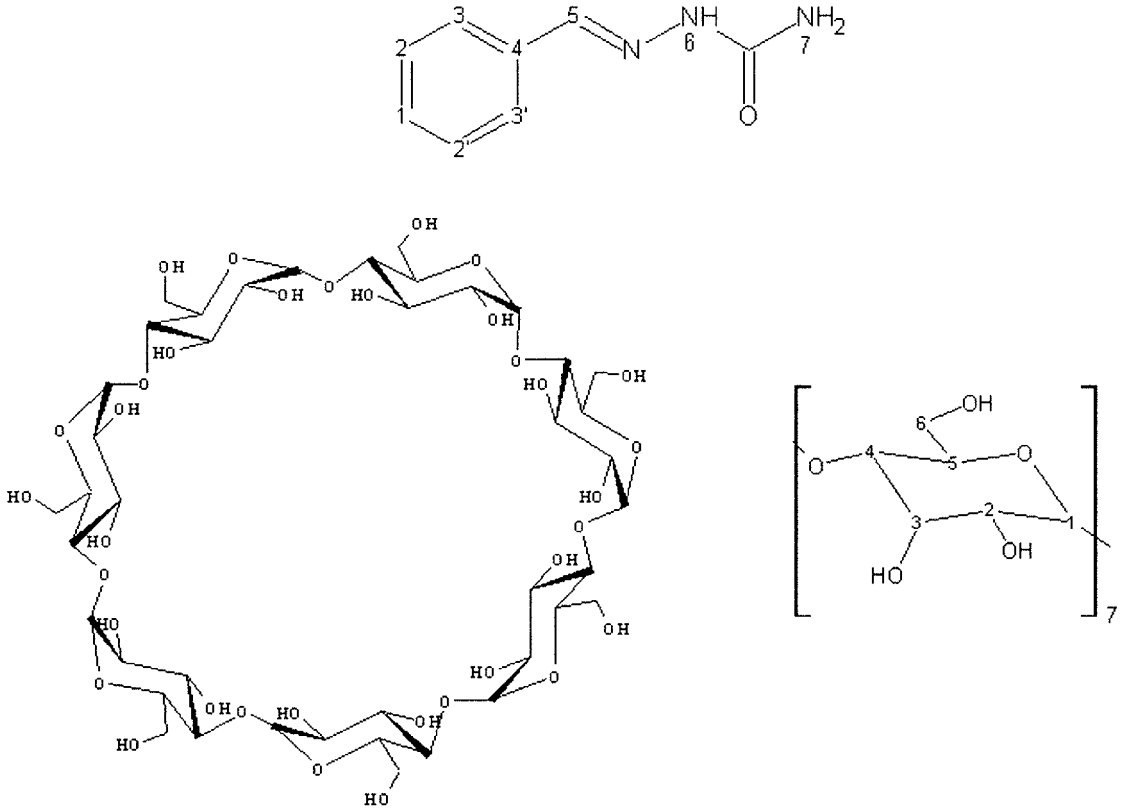

Figure 1. Structures of benzaldehyde semicarbazone (BS) and β-cyclodextrin (β-CD).

DSC-50 equipment using a 50mL/min nitrogen rate flow and 10 °C/min. Electronic spectra were recorded on a HP8453 diode array spectrometer.

Preparation of drug/CD solid complex

BS was obtained as described in the literature [3] and characterized by its melting point (216–217 °C), its infrared and ^{1}H, ^{13}C NMR spectra. The inclusion compound with β-cyclodextrin (β-CD) was prepared by mixing BS and β-CD in water in 1:1 molar ratio with stirring for 48 hours. The suspension was submitted to a freeze-drying process (Labconco Freezone model 177) during 72 hours. For comparison a 1:1 BS:β-CD physical mixture was obtained by gentle grinding of the components until a homogeneous powder was formed. All measurements were carried out immediately after the physical mixture preparation. The 1:1 BS:β-CD molar ratio in the inclusion compound was confirmed by the Higuchi and Connors method [11], measuring the BS absorbance at 281 nm in water with a 1 cm path length quartz cell.

Biological tests

Free BS and the BS/β-CD inclusion compound were administered by intraperitoneal route (ip) and by gavage (vo), in the MES experimental model of generalized tonic-clonic seizures. Doses are indicated in Results.

Animals

Wistar rats from the main breeding stock of the Institute of Biological Sciences, Federal University of Minas Gerais, Brazil, weighing 250–300 g were used in the MES tests. The animals were kept at 24 °C, in groups of 5 per cage receiving chow pellets and water *ad libitum*. The light/dark cycle was 12h:12h, with lights on at 7:00 am and lights off at 7:00 pm. Efforts were made in order to avoid any unnecessary distress to the animals, in accordance to the Guidelines for Animal Experimentation of Federal University of Minas Gerais, Brazil.

Induction and evaluation of maximum electroshock-induced seizures (MES)

Electroshock seizures were induced by electric stimulus, produced by an Elektroschockgerät apparatus (Karl Kolbe, Scientific Technical Supplies, Frankfurt, Germany) using a current of 70 mA, 60 Hz, during 1 second through a pair of ear clip electrodes.

The behavioral evaluation was carried out by measuring the tonic component in a four points scale as follows: **0** = no seizure; **1** = forelimb extension without hind limb extension; **2** = complete forelimb extension and partial hind limb extension; **3** = complete hind limb extension, which stays parallel to the tail. To evaluate the effect of decreasing on electroshock induced seizures severity, it was taken as criteria the blockade of complete fore- and hind limb extension (score $\leq$ 1).

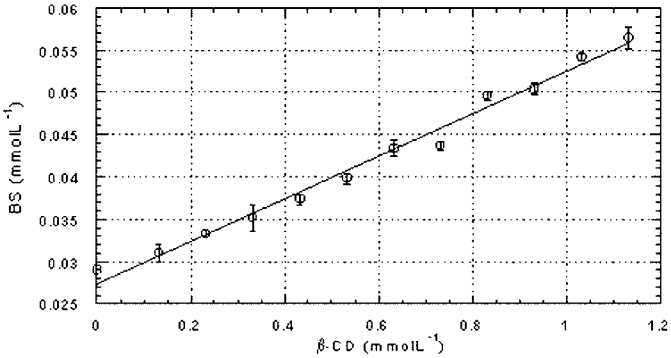

Figure 2. Solubility of BS as a function of β-CD concentration (Y = M_0 + M_1X; X = concentration of β-CD, M_0 = 0.0274 mmolL^{-1}, M_1 = 0.0250, R = 0.9935).

Statistical analysis

Scores of seizures were compared by Kruskal-Wallis non-parametric test for percentage comparison ($p < 0.05$) and considered statistically significant.

Results and discussion

Characterization of the inclusion compound between BS and β-CD

The 1:1 BS:β-CD stoichiometry was confirmed by the phase solubility method [11] which gave an A_L type diagram (see Figure 2).

The first evidence for host–guest interaction was obtained from the modification of the infrared absorptions of BS and β-CD upon inclusion. In the FTIR spectrum of β-CD the absorptions at 3400 cm^{-1}, 2925 cm^{-1}, 1640 cm^{-1} and 1025 cm^{-1} were attributed to ν (OH), ν (C–H), δ (O–H) and ν (C–O–C) respectively [12]. In the spectrum of BS the absorptions at 3463 cm^{-1}, 3395 cm^{-1} and 1600 cm^{-1} were attributed to ν (N–H), ν (NH_2) and ν (C=N) respectively. The ν (C–H) bands of BS were observed in the 2900-3100 cm^{-1} range. Two absorptions attributed to ν (C=O) were found at 1690 and 1650 cm^{-1} [13].

Comparison between the FTIR spectra of BS, the BS/β-CD inclusion compound and the physical mixture (Figure 3) reveal important changes upon inclusion. The BS ν (N–H) and ν (NH_2) bands at 3463 cm^{-1}, and 3395 cm^{-1} respectively were also observed in the spectrum of the physical mixture and in that of the inclusion compound. However, a narrowing of the β-CD absorptions at 3500–3400 cm^{-1} and 1100 cm^{-1} attributed ν (OH) and ν (C–O–C) was observed in the inclusion compound, probably due to the breaking of hydrogen bonds upon host–guest interaction and release of water molecules. Besides, the intensities of ν (C=O) at 1690 cm^{-1} and ν (C=N) at 1600 cm^{-1} of BS undergo a substantial decrease in the spectrum of the inclusion compound, which is not observed in the spectrum of the physical mixture, indicating molecular recognition of BS by the β-CD cavity. Crystal structure determinations of BS showed that the distance between the carbonyl carbon and the center

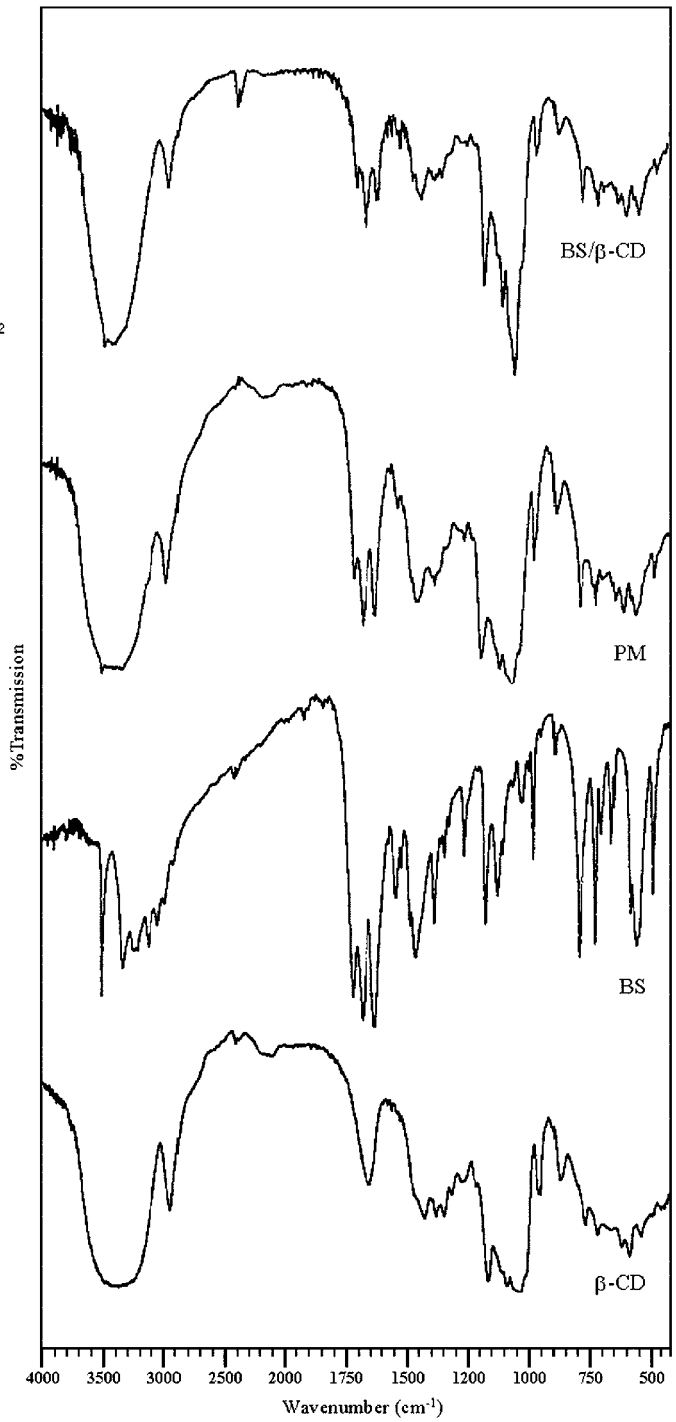

Figure 3. FTIR spectra of β-CD; BS; physical mixture (PM) and BS/β-CD inclusion compound.

of the aryl ring is 9.5 Å [14]. On the other hand it is well established that the length distance of β-CD is 7.9 Å [15], indicating that the cavity could accommodate the aromatic ring as well as part of the BS semicarbazone moiety.

The TG/DTG and DSC curves for β-CD and BS present thermal behaviors as related in the literature [11, 16].

The TG/DTG curves of the physical mixture exhibit thermal profiles associated to β-CD and BS (Figure 4A, B). The DSC curve shows four endothermic peaks at 70.6 °C, 214.7 °C, 306.3 °C and 326.3 °C, corresponding to β-CD

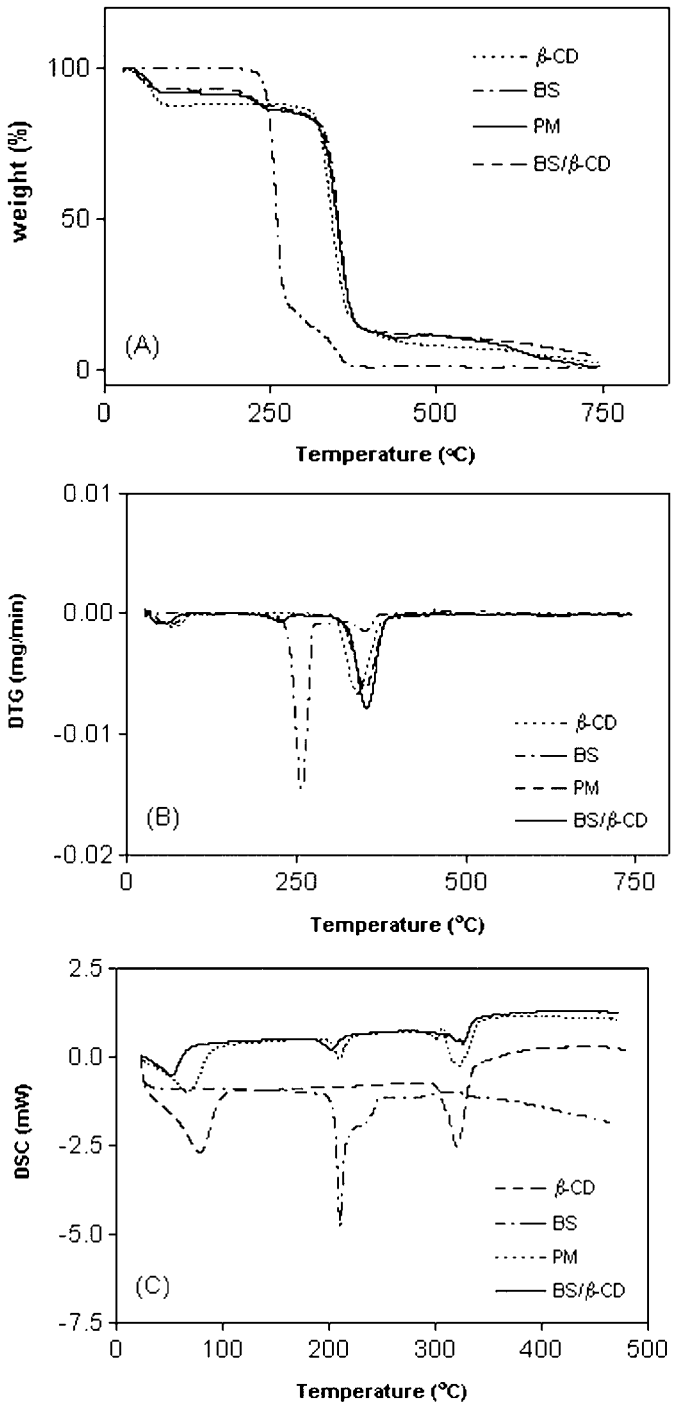

Figure 4. (A) TG curves of β-CD, BS, physical mixture (PM) and BS/β-CD inclusion compound; (B) DTG curves of β-CD, BS, physical mixture (PM) and BS/β-CD inclusion compound; (C) DSC curves of β-CD, BS, physical mixture (PM) and BS/β-CD inclusion compound.

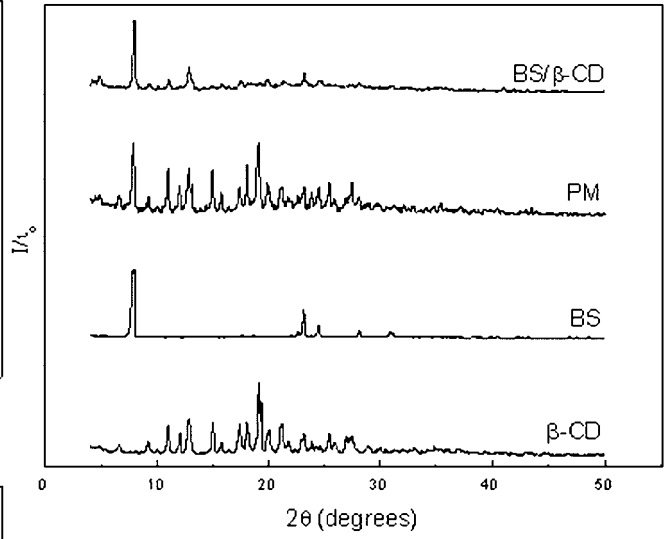

Figure 5. XRD patterns of β-CD; BS; physical mixture (PM); BS/β-CD inclusion compound.

and BS thermal phenomena. The last two peaks, attributed to melting and caramelization of β-CD are observed separately, in contrast to the DSC curve of β-CD, which shows only one thermal event (Figure 4C).

The thermal behavior of the BS/β-CD inclusion compound is entirely different. Its TG curve (Figure 4A) presents a weight loss in the 30–80 °C range attributed to the release of water molecules followed by a second loss in the 190–250 °C range, corresponding to partial BS decomposition, possibly due to the release of the semicarbazone moiety, as suggested by the percentage weight loss (6-7%). Further decomposition occurs at 360 °C, as evidenced by the DTG curve (Figure 4B). The DSC curve of the BS/β-CD inclusion compound exhibits one endothermic event at 58.7 °C, but the strong peak at 78.3 °C and 70.6 °C originally observable in the β-CD and in the physical mixture curves respectively is now absent, indicating the release of water molecules upon inclusion. In addition, the broad peak at 208.8 °C corresponds to the BS melting with decomposition, in accordance with the BS melting point determination. This peak occurs at a lower temperature in the inclusion compound in comparison to free BS, as a consequence of the host–guest interaction, which results in lower thermal stability. Finally the peak at 332.8 °C can be associated to melting and caramelization of β-CD, which occurs at higher temperature in the inclusion compound.

The XRD powder pattern diffraction analyses gave further support for the formation of a supramolecular compound between BS and β-CD. Figure 5 shows the X-ray diffraction patterns of BS, β-CD, the physical mixture and the BS/β-CD inclusion compound. The XRD powder diffraction patterns of BS and β-CD exhibit sharp peaks, characteristic of crystalline compounds. The XRD pattern of the physical mixture shows peaks characteristic of BS and β-CD. In contrast, the BS/β-CD inclusion compound presents a pattern that suggests a loss of crystallinity with formation of a less organized system upon inclusion.

Table 1 reports the ^{13}C NMR signals of BS and of BS in the BS/β-CD inclusion compound as well as the variation in chemical shifts (Δ) upon inclusion. The signals in the spectra of BS and β-CD were in agreement with data obtained previously by us [17] or reported in the literature [16, 18, 19].

Upon host–guest interaction, all hydrogen signals of BS shift to lower frequencies (data not shown) and the carbon signals to higher frequencies. Interestingly, the C1, CH and C=O signals exhibit the most significant shifts upon interac-

Table 1. ^{13}C NMR spectra of free benzaldehyde semicarbazone (BS) and of BS in the BS/β-CD inclusion compound (d^6-DMSO)

	BS	BS/β-CD	Δ
C=O	156.868	157.276	0.408
CH	139.356	140.239	0.883
C1	129.049	129.465	0.416
C2, C2′	128.598	128.891	0.293
C3, C3′	126.567	126.833	0.266
C4	134.793	134.851	0.058

Table 2. ^{1}H NMR relaxation times (τ_1, s^{-1}) of free benzaldehyde semicarbazone (BS), BS in the BS/β-CD inclusion compound, BS in the physical mixture (d^6-DMSO) and relaxation time ratios

	BS	BS/β-CD	$\tau_{1_{complex}}/\tau_{1BS}$	PM*	τ_{1PM}/τ_{1BS}
NH_2	0.230 ± 0.002	0.157 ± 0.001	0.68	0.198	0.86
H3, H3′	1.209 ± 0.001	0.749 ± 0.001	0.62	1.059	0.86
H2, H2′	1.233 ± 0.001	0.771 ± 0.001	0.63	1.024	0.85
CH	0.646 ± 0.007	0.451 ± 0.005	0.70	0.559	0.87
NH	0.660 ± 0.006	0.367 ± 0.002	0.56	0.510	0.77

τ_1 values for BS and BS/β-CD are average of three experiments.
* Physical mixture obtained in d^6-DMSO solution with 1:1 BS:β-CD molar ratio.

tion, suggesting inclusion of the BS molecule from the aryl ring to the carbonyl oxygen of the semicarbazone moiety into the β-CD cavity, as ascertained by infrared data.

Table 2 lists ^{1}H relaxation times (τ_1) for free BS, BS in a physical mixture prepared in d^6-DMSO and BS in the BS/β-CD inclusion compound, as well as the $\tau_{1_{complex}}/\tau_{1BS}$ and $\tau_{1_{physical\,mixture}}/\tau_{1BS}$ ratios. The ^{1}H relaxation times (τ_1) of BS are slightly different from those previously reported [9] because the experiments were performed in different NMR equipments.

Changes were observed in all relaxation times upon inclusion. The relaxation time ratios indicate a much stronger interaction in the inclusion compound than in the physical mixture. To further support BS inclusion a 2D-ROESY NMR experiment of the BS/β-CD complex was performed in d^6-DMSO (due to the BS insolubility in water and D_2O). The cross peaks revealed interaction of CH and all aromatic hydrogens of BS with H3 and H5 of β-CD. In addition, H3 and H3′ of BS interact with OH2, OH3 and with H2, H4 of β-CD, and H1, H2, H2′ of BS with H1 of β-CD, giving more stringent evidence for formation of the inclusion compound.

Biological test: Comparison of the anticonvulsant effect of free benzaldehyde semicarbazone (BS) and the BS/β-CD inclusion compound in the maximum electroshock (MES) screen

In the MES model, thirty minutes after injection BS blocked the hind limb extension in about 90% of the animals (n = 5) at 100 mg/Kg/ip as observed in the literature [3]. The BS/β-CD inclusion compound blocked completely the hind limb extension at 25 mg/Kg/ip in 100% of the animals, at 15 mg/Kg/ip in 60% of the animals and at 25 mg/Kg/vo in

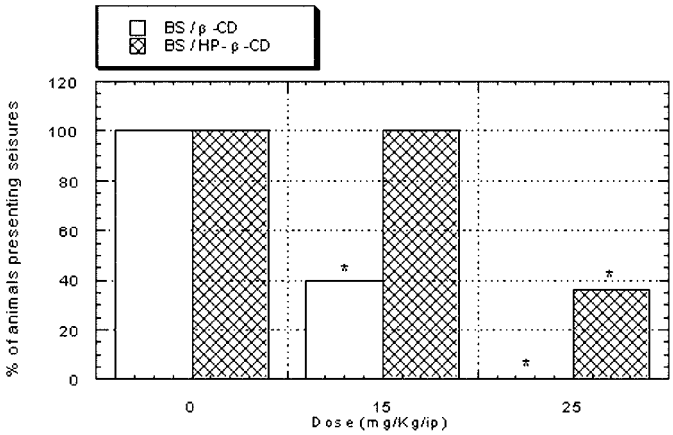

Figure 6. Percentage of animals presenting seizures per dose of the BS/β-CD and BS/HP-β- CD inclusion compounds (n = 5, $p < 0.05$).

60% of the animals (Figure 6). In the control group all animals (n = 3) presented complete hind limb extension (score 3).

In the MES model of epilepsy the minimum dose necessary to produce anticonvulsant activity decreased from 100 mg/Kg (ip or vo) for the free semicarbazone to 25 mg/Kg/vo (75%) and 15 mg/Kg/ip (85%) for the BS/β-CD inclusion compound. Comparison with the results obtained previously by us for the BS/HP-β-CD inclusion compound, which allowed dose reduction of 75% ip (see Figure 6) and 65% vo [9] reveals that the host–guest strategy that uses β-CD is even more effective. The reasons for this difference could be either the lower water solubility of the BS/β-CD inclusion compound as compared to the BS/HP-β-CD analogue, or the β-CD greater adhesion to the mucous wall [20], which would allow a more sustained release. Further studies are underway in order to get a better understanding of those differences.

In conclusion, taking into consideration that currently used drugs cause significant side effects, which may limit their maximal usefulness, the new strategy could be successfully employed in the preparation of pharmaceutical formulations of anticonvulsants.

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