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ORIGINAL ARTICLE

Niclosamide quantification in methyl-β-cyclodextrin after derivatization to aminoniclosamide

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Abstract A fluorescence derivatization method for the quantification of the anthelmintic drug niclosamide in organized media (methyl-β-cyclodextrin) is described. The derivatization reaction is based on a 3 h reduction reaction of the nitro group in niclosamide to an amino group by hydrogenation catalyzed by Pd/C in methanol:ethyl acetate (1:3). The aminoniclosamide exhibits a maximum of fluorescence at 431 nm (excitation at 280 nm) that is markedly enhanced when methyl-β-cyclodextrin is present. The methodology was assessed by the analysis of pure and dosage forms. The method showed a linear range between 0 and 2200 μg L^{-1} with a limit of detection of 45.7 nM and a precision (relative standard deviation) of 4.1% in niclosamide. The effect of various interferents, such as niclosamide, fructose and 2-cyano-6-metoxybenzothiazole, in several tolerance ratios, was investigated respected to aminoniclosamide.

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Introduction

Niclosamide (2′,5-Dichloro-4′-nitrosalicylanilide, Fig. 1) belongs to the family of medicines called anthelmintics drugs which are effective against most tapeworms, including the beef tapeworm, the dwarf tapeworm and the dog tapeworm [1]. Commercially known as bayluscide or niclocide in USA and Canada or clonitralid and Bayer 73 in Germany. Its mode of action against tapeworm species is to uncouple oxidative phosphorylation and it blocks the glucose uptake and inhibits respiration in cestodes and the anaerobic ATP production [2], widely used as a molluscide in tropical regions for control of freshwater snails [3] and also used as an intestinal taeniacide in humans [4].

In the literature there are references to several analytical techniques used in the quantification of niclosamide at trace levels. Methods for analysis of niclosamide in water include spectroscopy [5–8], liquid chromatography [9–12] and voltammetric determination [13, 14]. Residues of niclosamide have been analyzed by gas chromatography but this procedure requires indirect analysis of the hydrolysis product 2-chloro-4-nitroaniline [10, 15]. A derivatization method based on the reduction of the nitro group in niclosamide to the amino group using zinc powder in etanolic acid media, followed by a colour development reaction with p-benzoquinone has been proposed [6].

Niclosamide is not fluorescent but it can be analyzed by reduction to aminoniclosamide which exhibits a green fluorescence at 439 nm which can be enhanced using its ability to form inclusion complexes with cyclodextrins. Because of the organizing ability of cyclodextrin media,

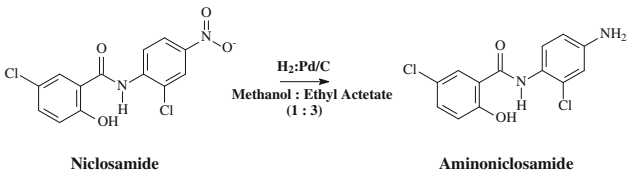

Fig. 1 Formation of aminoniclosamide

luminescent phenomena are enhanced, the molecules in the internal cavity are isolated from the surrounding environment and their excited states shielded from extinction processes. Cyclodextrins (CD) show selectivity depending on the relative size of the cavity and guest molecules [16–18]. The determination of a variety of organic compounds has been developed by exploiting the α-CD [19, 20], *β*-CD and methyl-*β*-CD [21–23]. Only a research work has been found to use cyclodextrins and dendrimers in combination to solve the problem of solubilisation of niclosamide in aqueous media, but no analytical determination was done [11].

In the present work a new procedure for the derivatization reduction reaction, based on a catalytic step for the quantitative transformation of niclosamide to aminoniclosamide, followed by the formation of an inclusion complex of aminoniclosamide with a cyclodextrin (CD) is described. Six CD derivatives were studied in order to obtain the one that provokes the highest fluorescence enhancement, which was methyl-*β*-cyclodextrin. The inclusion complex was analysed by fluorescence emission measurements.

Material and methods

Materials

Niclosamide (99%), α-cyclodextrin (α-CD), *β*-cyclodextrin (*β*-CD), methyl-*β*-cyclodextrin (with a degree of substitution of 1.6–2.0 per glucose units, M*β*-CD), hidroxypropyl-*β*-cyclodextrin (Hp-*β*-CD), sulphated-*β*-cyclodextrin (S*β*-CD), *γ*-cyclodextrin (*γ*-CD) and 2-cyano-6-metoxybenzothiazole (2Cy6M) were purchased from Sigma-Aldrich Química SA (Spain). Fructose was obtained from Panreac Química (Barcelona, Spain). All reagents were of high purity grade and used as received.

Stock solutions of aminoniclosamide (50 mg L^{-1}) were prepared in deionised water and diluted as required. Aminoniclosamide is light sensitive thus stock and standard [24] solutions were stored in light tight containers. Cyclodextrins solutions (10^{-2} M) were prepared in deionised water with resistivity higher than 4 MΩ/cm.

Derivatization reaction

Niclosamide, is one of the halogenated salicylanilides, which are highly hydrophobic having a very low aqueous solubility [25] and showing aromatic behaviour, do not fluoresce as a result of the strong electron-withdrawing nature of the nitro substituent, for that was planned its determination in aqueous media by reduction.

Aminoniclosamide (Fig. 1) were obtained by the selective catalytic reduction of niclosamide, under hydrogen atmosphere (4 bars) in methanol:ethyl acetate (1:3) solution, using Pd/C (10%) for 3 h at room temperature. Then, the yellowish solution obtained was filtered through a celite layer and the solvent was removed on rotary evaporator yielding the corresponding pure aminoniclosamide (99.9%) as yellowish solid. The synthesised aminoniclosamide were checked by ^{1}H-NMR and ^{13}C-NMR spectrometry, included distortionless enhancement by polarization transfer (DEPT) and heteronuclear single quantum correlation (HSQC) analysis (NMR Spectra are presented in the Supporting information). Mass spectrometry analysis of the product in a methanol solution resulted in a *m/z* peak at 296 g mol^{-1} which demonstrates the formation of aminoniclosamide.

Aminoniclosamide (2′,5-Dichloro-4′-aminosalicylanilide) NMR characterization

^{1}H-NMR (DMSO-d_6, 50 °C): *δ*:6.96 (m, 1H, H-5′), 7.04 (d, 1H, $J = 7.4$ Hz, H-3′), 7.36-7.39 (m, 1H, H-6′), 7.38 (d, 1H, $J = 8.8$ Hz, exc. D_2O, $N\underline{H_a}H_b$), 7.44 (m, 1H, H-4), 7.80 (d, 1H, $J = 8.8$ Hz, exc. D_2O, $NH_a\underline{H_b}$), 7.79-7.82 (m, 1H, H-3), 8.02 (dd, 1H, $J_1 = 7.9$ Hz, $J_2 = 1.6$ Hz, H-6), 10.52 (br.s, 1H, exc. D_2O, CONH), 11.55 (br.s, 1H, exc. D_2O, OH) ^{13}C-NMR (DMSO-d_6, 50 °C) *δ*:118.65 (C5′), 118.87 (C1), 120.37 (C3′), 123.33 (C3 + C1′), 124.41 (C6′ + C5), 130.00 (C2′), 130.58 (C6), 135.00 (C4), 138.65 (C4′), 159.84 (C2), 168.01 (C=O). *Supporting information contains the NMR spectra of aminoniclosamide and NMR spectra and data of niclosamide.*

Preparation and fluorescence measurements of the complex of aminoniclosamide with cyclodextrins

To aliquots of aminoniclosamide in deionised water were added increasing volumes of 10^{-2} M of the different CDs solutions and deionised water up to a final volume of 5 mL. These samples were sonicated for 10 min and their fluorescence spectra were recorded at 431 nm with excitation at 280 nm at room temperature. Calibration set of six samples was prepared by transferring appropriate aliquots of stock solution of pure aminoniclosamide, being the final concentration between 0 and 2000 μg L^{-1}.

Apparatus

Excitation between 199.4 and 672.8 nm and emission between 349.7 and 719.7 nm were obtained with a SPEX 3D luminescence spectrophotometer equipped with a Xenon pulse discharge lamp (75 W) as light source, and a CCD detector. After optimization, 5 nm slits and 0.1 s integration time were used. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance III spectrometer at 400 and 75.47 MHz, respectively, using TMS as an internal standard (chemical shifts in δ values and J in hertz). Mass spectra were recorded on a Finnigan LCQ DECA XP MAX (Finnigan, San Jose, CA) quadrupole IT equipped with API source, using ESI interface. The vaporizer and the capillary voltages were 5 and 4 V, respectively. The capillary temperature was set at 300 °C.

Data analysis

The interaction between aminoniclosamide and CD can be analysed using a Langmuir-type binding isotherm [26]. This model is described by the following equation:

$$\frac{C}{I} = \left(\frac{1}{BI_{\max}}\right) + \left(\frac{1}{I_{\max}}\right)C \quad (1)$$

where C is the CD concentration, I is the fluorescence intensity of the complex and B is the binding constant.

The quenching of inclusion complex fluorescence by interfering species can be described by a Stern–Volmer equation:

$$\frac{I_o}{I} = 1 + K_{sv}[\text{Interferent}] \quad (2)$$

where I_o is the fluorescence intensity without interferent, I is the fluorescence intensity observed in the presence interferent and K_{SV} is the Stern–Volmer constant [27]. The detection limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3.33 times (99.7% confidence degree) and 10 times the standard deviation of seven blanks divided by the slope of the calibration graph. The precision of the estimations was determined by analysing 10 samples containing 0.1 mg L^{-1} of aminoniclosamide and 6 mM CD.

Results and discussion

Fluorescence study

The increase of the relative fluorescence intensity of aminoniclosamide in the presence of different CD in water was used as a qualitative measure of the complexing stability of the inclusion complex. Experiments were conducted to analyse the interaction of aminoniclosamide with 10^{-2} M of α-CD, β-CD, Mβ-CD, Hβ-CD, Sβ-CD and γ-CD solutions. Figure 2 shows the fluorescence spectra of aqueous solution of aminoniclosamide in the presence of CDs.

The analysis of Fig. 2 shows a general fluorescence intensity enhancement when CD is presence compared when only in water but it is highest for Mβ-CD. The enhancement of fluorescence is observed because cyclodextrins offer a protective, more constrained microenvironment to an electronically excited fluorophore and so the resulting fluorescence is enhanced. The maximum fluorescence obtained for the inclusion complex formed between the fluorophore and a CD is a consequence of the aqueous medium surrounding it.

So, the relative higher enhancement with Mβ-CD probably results from a better fit of the aminoniclosamide molecule inside the cavity, helped for the methyl groups surrounding the host molecule. The fluorescence emission of aminoniclosamide in the presence of Mβ-CD is about twice that in its absence. Only the Sβ-CD provokes a bathochromic shift which is probably due to the stabilization effect of hydrogen bonding between the amino (–NH$_2$) group and water.

Quantification of aminoniclosamide in the presence of Methyl-β-CD

The influence of Mβ-CD concentration on the aminoniclosamide (0.1 mg L^{-1}) fluorescence was studied and the fluorescence intensity was enhanced when the Mβ-CD concentration increases (between 0 and 6 mM) at room

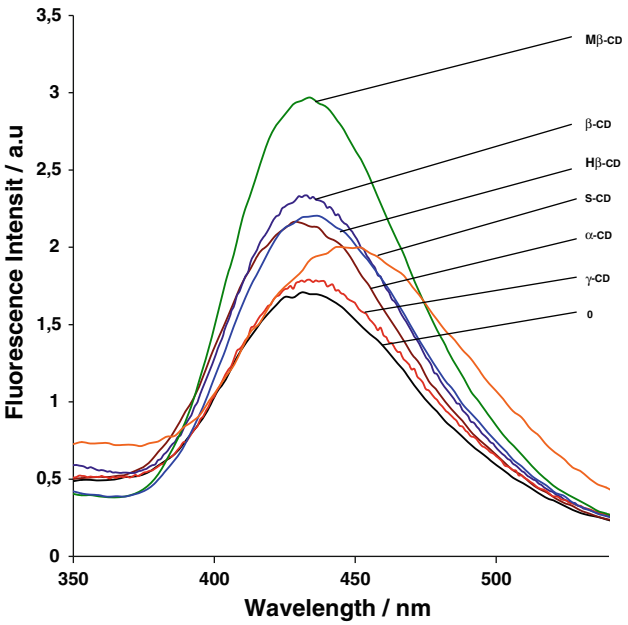

Fig. 2 Fluorescence spectra of aminoniclosamide: CDs

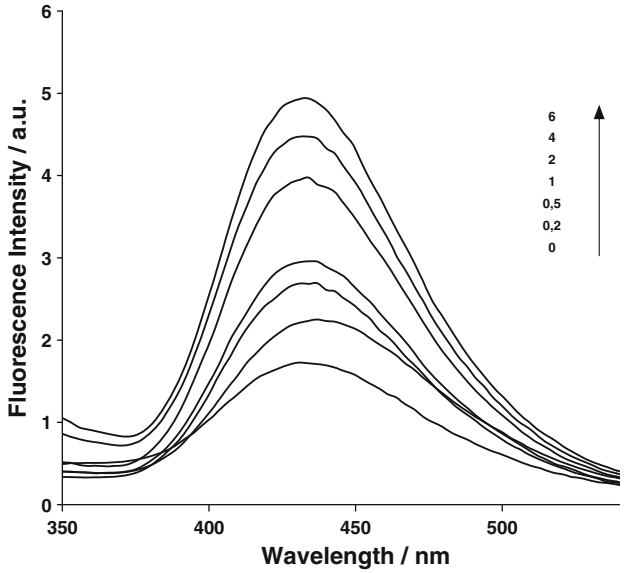

Fig. 3 Influence of [Mβ-CD] in the fluorescence intensity of aminoniclosamide

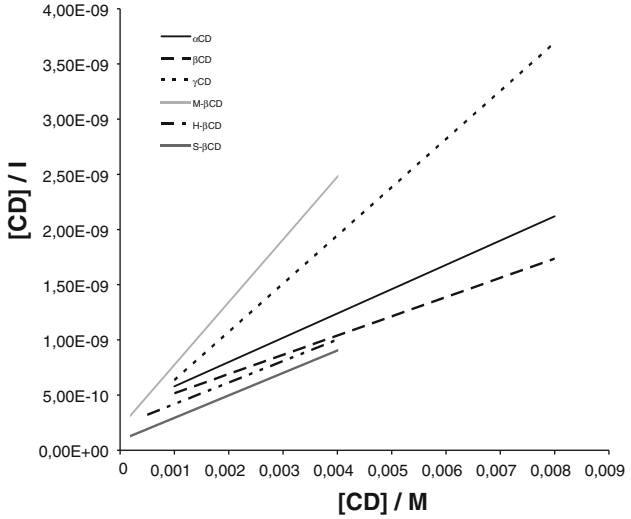

Fig. 4 Langmuir-binding isotherms of aminoniclosamide with the different CDs

temperature (Fig. 3). Because an increasing trend was always observed, the Mβ-CD concentration of 6 mM was chosen and maintained for maximum intensity of fluorescence.

Using the optimum values of the experimental factors the calibration graph obtained by the linear least-squares treatment (with a correlation coefficient of 0.999) was (concentration expressed as mM): $I_F = 917766$ [*Aminoniclosamide*] + 2×10^6. The calibration linear dynamic range was established between 0 and 2,200 μg L^{-1} respected to niclosamide. The LOD and LOQ were 45.7 and 137 nM, respectively for aminoniclosamide and correlated to niclosamide. The precision of the estimations was 4.1% (expressed as relative standard deviation).

Analysis of the CD binding capacity

For quantitative purposes the analysis of the binding capacity of the CD towards aminoniclosamide was studied using a *Langmuir-type binding isotherm* (Eq. 1) [26]. Figure 4 shows the plot of the experimental data and Table 1 resumes the data obtained by linear least-squares of the plots. The analysis of this Table shows that experimental data fits Eq. 2 quite well and a linear trend is observed throughout the entire tested concentration range. The binding constants (B) to all the studied CDs are summarized in Table 1.

As far as the analysis of aminoniclosamide is of concern we are interested in the formation of a stable inclusion complex, with a relatively high binding constant, but also that provokes a relatively high fluorescence intensity increase. The analysis of Table 1 shows that the CD that has the highest binding constant is Sβ-CD, with a $B = 3119 \pm 192$ M^{-1}, and the CD that provokes the highest fluorescence intensity increase is β-CD with $I_M = 57.3 \pm 0.2 \times 10^5$.

However, Sβ-CD provokes a relatively small fluorescence intensity increase $17.2 \pm 0.4 \times 10^5$ and β-CD has a relatively small binding constant ($B = 507 \pm 5$ M^{-1}). The CD that shows a relatively large binding constant and provokes relatively large fluorescence intensity increase is Mβ-CD with $B = 1879 \pm 29$ M^{-1} and $I_M = 52.2 \pm 0.8 \times 10^5$. Consequently the CD chosen to be used in the analytical method was Mβ-CD.

Interference studies

In order to assess the selectivity of the proposed fluorescence method, an interference investigation was performed using a solution containing 0.1 mg L^{-1} of aminoniclosamide to check the effects of concomitant molecules such as niclosamide, the raw material, and two other possible interferences, the excipient fructose and 2-cyano-6-metoxybenzothiazole.

Table 2 shows the results obtained using the method of tolerance ratios. For fructose the interfering effect quite small is and for 2-cyano-6-metoxybenzothiazole a 10% reduction in the recovery is observed for the 1:10 ration.

For niclosamide the effect is quite significative which shows that the reduction process should be quantitative and the full conversion of niclosamide to aminoniclosamide is required.

The interference of niclosamide is a consequence of the decrease of the fluorescence signal of the inclusion complex of Mβ-CD with aminoniclosamide in aqueous solution. In order to quantify the quenching provoked by

Table 1 Fitting results of data to a Langmuir-type binding isotherm

Cyclodextrin	I_{max} (a.u.)	*B*	*r*	*N*	Concentracion range (mM)
Alfa	4571963.8 ± 99800	600.8 ± 51.3	0.984	5	1–8
Beta	5739582.5 ± 21360	507.1 ± 4.8	0.984	5	1–8
Gamma	2290262.3 ± 56730	2253.6 ± 392.6	0.999	5	1–8
Methyl-Beta	5226607.7 ± 81660	1878.6 ± 29.0	0.996	6	0.2–6
Hydroxypropyl-Beta	5133133.5 ± 3822	872.9 ± 4.7	0.981	5	0.5–4
Sulphated-Beta	1726768.6 ± 37416	3119.1 ± 192.0	0.981	5	0.2–4

I_M maximum intensity, *B* binding constant, *r* correlation coefficient, *N* number of points

Table 2 Tolerable limits of interfering species

Interference	Aminoniclosamide:Interference	Recovery (%)
Niclosamide	1:1	96
	1:5	86
	1:10	80
Fructose	1:1	98
	1:5	98
	1:10	96
2-cyano-6-metoxybenzothiazole	1:1	96
	1:5	95
	1:10	90

niclosamide on the fluorescence of the inclusion complex a Stern–Volmer plot was done. Linear Stern–Volmer plots were observed with a $K_{SV} = 1.34 \pm 0.04 \times 10^5$ M^{-1}. The relatively high magnitude of the Stern–Volmer constant shows that niclosamide forms a stable complex with the fluorophore or, more likely, it competes with aminoniclosamide for Mβ-CD replacing it on the complex resulting on a non-fluorescent inclusion complex [27].

Conclusions

A reproducible and sensitive method for the determination of niclosamide in pharmaceutical formulations has been developed. The methodology is based on the selective catalytic reduction of niclosamide followed by the enhancement of the fluorescence when aminoniclosamide forms the inclusion complex with Mβ-CD. The derivatization reaction induces further selectivity and the utilization of an inclusion complex increases the sensitivity of the proposed methodology. This method can be used for analysis of pharmaceutical formulations or biological and environmental samples.

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