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IMPROVED DELIVERY THROUGH BIOLOGICAL MEMBRANES: PREPARATION AND INVESTIGATION OF STABLE DOPAMINE CHEMICAL DELIVERY SYSTEM SAMPLES

Nicholas Bodor, Hassan H. Farag[$], and Farghaly A. Omar[$*]

Center for Drug Discovery, College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, Florida 32610

ABSTRACT: *Crystalline dopamine chemical delivery system and its cyclodextrin complex could be prepared. The crystalline compound showed better stability at different storage conditions. The iv injection of aqueous solutions of the complex showed higher brain delivery than the DMSO solution of the pure compound. The ip Injection of the aqueous solution of the complex delivered good concentration of the deacylated dopamine quaternary (dopamine prodrug-moiety) to the brain.*

INTRODUCTION

Parkinsonism, a striatal dopamine deficiency syndrome, is a disability condition which may afflict at least one of every thousand population[1]. Drugs which restore the level of dopamine in the striatum and the hypothalamus proved to affect the management of this ailment[2]. Levodopa is considered the drug of choice and has been used for more than 25 years as a palliative treatment of the condition. In spite of the advantages of the use of the drug, it is not free from undesirable side effects which are mainly systemic[3,4].

The authors reported the preparation of a dopamine chemical delivery system (DA-CDS) (4) using the dihydropyridine/ pyridinium salt redox chemical delivery system[5-7] for the delivery of dopamine preferentially to the brian. The iv administration to rats gave 15-fold enhancement of delivery of the parent pro-dopamine quaternary (5) to the brian than that to the blood at 40 min post dosing[5].

One mg/Kg iv injection of DA-CDS to rats resulted in potent dopaminergic activity illustrated by 80% decrease in prolactin secretion[8]. Levels of dopamine and its major metabolites have been shown to increase in striatum and hypothalamus in rats treated with the CDS[9]. Hence DA-CDS could be considered a good candidate for the management of Parkinsonism with expected longer duration and less systemic side effects.

The prepared compound suffered from two disadvantages. The first is the instability of the solid samples prepared following the reported procedure[5] and the second is its poor water solubility. These two disadvantages could limit the practical use of the compound as drug. For this reason the investigation of the possibility of preparing a solid polymorph of DA-CDS which could possess reasonable shelf stability was considered. Also the ability and extent of the compound to form inclusion complex with 2-hydroxypropyl-ß-cyclodextrin was investigated. Meanwhile the effect of the complexation on the delivery of the parent pro-dopamine quaternary (5) was studied following iv administration of the complexed compound.

EXPERIMENTAL

All melting points were carried out in open capillary heated in mineral oil bath and are uncorrected. Elemental analyses were performed at Atlantic Microlab, Inc., Norcross, GA. NMR

$*) To whom correspondence should be addressed.

$) Present address, Dept. of Med. Pharm. Chemistry, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

spectra were recorded on a Varian EM390 instrument. All chemical shifts reported are in δ units (parts per million) relative to tetramethylsilane. Ultraviolet Spectra were determined using Cary Model 219 spectrophotometer. High performance liquid chromatography investigations were carried out using a Water 6000A solvent delivery system, Water U6K injector, Water 440 dual channel absorbance (with 254 nm filter) and a dedicated SP4290 integrator.

N-[ß-[3,4-bis(pivaloyloxy)phenyl]ethyl]carbamoylpyridine (2):

To an ice cold suspension of finely powdered 5.16 g (0.02 mol) of N-nicotinoyldopamine (1) in 100 ml of chloroform, 6.6 ml of triethylamine were added. To the mixture 8.43 g (0.07 mol) of trimethylacetyl chloride were added dropwise while stirring. The mixture was then refluxed while stirring for 24 hours. Chloroform was distilled under reduced pressure and the viscous oily residue was dissolved in the least amount of boiling ether and rapidly filtered through folded filter paper. The ether solution was then brought to boiling and drops of petroleum ether were added until the first turbidity attained. The mixture was then kept in the refrigerator overnight. The solid was filtered and crystallized from ether/pet. ether (60-80°C) to give 7.3 g (86% yield) of mp 112-114°C.

^{1}H-NMR ($CDCl_3$) in δ ppm: 1.33 (18H,s,2(CH_3)$_3$), 2.83 (2H,t, J= 7Hz, C_6H_3-C$\underline{H}_2$), 3.58 (2H,t, J=7Hz ,N-C$\underline{H}_2$), 6.68-6.87 (1H,broad diffused triplet, CONH), 6.9-7.1(3H,m,C_6H_3-), 7.26 (1H,dd, $J_{5,4}$= 8Hz, $J_{5,6}$= 5Hz; C-5 pyridine proton),8.00 (1H,2 triplets, $J_{4,2}$= 2Hz, $J_{4,5}$; $J_{4,6}$= 8Hz; C-4 pyridine proton), 8.58 (1H, dd, $J_{6,5}$= 5Hz, $J_{6,4}$= 2Hz; C-6 pyridine proton) 8.86 (1H,d, $J_{2,4}$= 2Hz; C-2 pyridine proton). Shaking the sample for 3h with D_2O at 35°C results in disappearance of the amide peak at 6.68-6.87 and simplification of the quartet at 3.58 to triplet at 3.8, J= 7Hz.

1-Methyl-3-{N-[ß-[3,4-bis(pivaloyloxy)phenyl]-ethyl]carbamoyl}pyridinium methosulfate (3):

To a solution of 5.0 g of compound 2 in 20 ml of acetone, 20 ml of ether and 2 ml of dimethyl sulfate were added. The mixture was kept under mild reflux while stirring overnight. The white fine crystalline solid was separated by filtration, washed by dry ether and dried in a desiccator. Its purity was tested by HPLC, and any non quaternized impurity was removed by either stirring the solid under reflux with ether several times or adding 1 ml of dimethyl sulfate to its suspension in 10 ml of ether and reflux while stirring overnight. yield 78%, mp 153-155°C.

^{1}H-NMR(d_6-DMSO), δ ppm: 1.27 (18H, s, 2(CH_3)$_3$), 2.87 (2H, t, J= 7Hz, C_6H_3-C$\underline{H}_2$), 3.44-3.67 (2H, m, N-CH_2), 4.37 (3H, s, N-CH_3), 7.0-7.24 (3H, m, C_6H_3), 8.07-8.24 (1H,m, C-5 pyridine proton), 8.68 (1H, d, J= 7Hz, C-4 pyridine proton), 8.97-9.10 (2H, m, C-6 pyridine proton + CON$\underline{H}$, D_2O exchange results in simplification of the multiplet to a doublet integrated to one proton assigned to C-6 pyridine proton), 9.24 (1H, s, C-2 pyridine proton).

1-Methyl-3-{N-[ß-[3,4-bis(pivaloyloxy)phenyl]ethyl] carbamoyl} -1,4-dihydropyridine (4):

To an ice cold solution of 10.42 g (0.02 mol) of pure compound 3 in 100 ml of dearated water, was added 200 ml of ethyl acetate. To the stirred mixture 5.43 g of anhydrous $NaHCO_3$ were added followed immediately by portion-wise addition of 12.5 g of $Na_2S_2O_4$. Stirring was continued at 0°C under nitrogen atmosphere and protected from light for 3 hours. At the end of the reaction time the mixture acquired a lemon yellow color. The organic layer was separated and the aqueous layer was extracted with 50 ml of cold ethyl acetate. The cold combined organic extract was washed with 50 ml of ice cold 5% Na_2CO_3 solution, separated and dried over anhydrous sodium sulfate. All the extraction and washing processes were carried out under nitrogen atmosphere. Ethyl acetate was then distilled off under reduced pressure at 25°C and the residue was flushed with dry nitrogen gas for at least 15 minutes. The yellow viscous residue was protected from light by wrapping the flask with aluminum foil and dried under high vacuum for at least 12 hours.

Crystallization of the resulted yellow froth was then achieved by adding 10 ml of peroxide-free, sodium-dried ether and scratching the solution until a fine lemon yellow crystalline solid was separated. The solid product was then filtered off, washed with peroxide-free sodium-dried ether and dried in a vacuum desiccator under nitrogen. The yield of the pure crystalline CDS (mp 103-105°C) was about 60%.

^{1}H-NMR ($CDCl_3$), δ ppm: 1.38 (18H, s, $2(CH_3)_3$), 2.8 (2H, t, J= 7Hz, C_6H_3-$C\underline{H}_2$), 2.9 (3H, s, N-CH_3), 3.0 (2H, broad doublet, C-4 dihydropyridine protons), 3.53 (2H, q, J= 7Hz, N-CH_2), 4.53-4.73 (1H, m, C-5 dihydropyridine proton), 5.3 (1H, broad triplet, CONH), 5.65 (1H, dd, J= 7&2Hz, C-6 dihydropyridine proton), 6.9-9.1 (3H, m, C_6H_3). D_2O exchange experiment resulted in disappearance of the broad triplet at 5.3 and the quartet at 3.35 changed to triplet centered at 3.7 (2H, J= 7Hz).

Preparation of DA-CDS/HPCD complex:

To a solution of 5.0 g of hydroxy-propyl-ß-cyclodextrin (HPCD) in 5 ml of deionized and dearated water, 0.3 g of finely powdered crystalline DA-CDS was added. The mixture was sonicated in an ice bath under nitrogen for 1 hour and left to equilibrate at room temperature and under nitrogen for further 45 min. The mixture was then filtered through medium porosity centered-glass funnel. The clear pale yellow solution obtained was lyophilized giving a pale yellowish white fine solid, which was then analyzed by HPLC for its contents of DA-CDS.

Analytical methods

Improved high pressure liquid chromatography (HPLC) methods were developed for this study. The column used for chemical oxidations, solubility and stability studies was Water's Nova-pack phenyl cartridge (8 mm ID) assembled in Water's RCM 8X10 cartridge holder. The solvent system consisted of 60% acetonitrile, 40% 0.05 M ammonium hydrogen phosphate and 0.0025 M tetrabutylammonium phosphate. At flow rate of 2 ml/min. the retention times were: 2.4 min for dipivaloyl quaternary compound (3); 6.0 min. for 1,6-dihydro-isomer and 7.0 min for 1,4-dihydro-isomer. The column used for the distribution studies was assembled Water's Nova-pack C18 cartridge (8 mm ID). The solvent system consisted of 65% acetonitrile and 35% 0.008 M KH_2PO_4. At a flow rate of 1 ml/min. the parent deacylated quaternary (5) had a retention time of 4.1 min.

Chemical Oxidation:

a) Alcoholic silver nitrate:

To 1 ml of 1% solution of crystalline compound (4) in methanol, 5 ml of saturated alcoholic silver nitrate solution were added, the mixture was left for 5 min. and then centrifuged. The supernatant was then analyzed by HPLC.

b) Potassium ferricyanide :

To 1 ml of 0.01 M potassium ferricyanide solution in 20% aqueous acetonitrile, 1 ml of a $1.69X10^{-3}$ M solution of DA-CDS in acetonitrile was added, mixed well and kept at 21±1°C. The mixture was analyzed by HPLC at different time intervals.

Solubility in hydroxypropyl-ß-cyclodextrin (HPCD) solution:

An excess of the finely powdered crystalline DA-CDS was added to each of the appropriate concentration of HPCD in deionized water (0,5,10,15 and 20% w/v solutions). The resulting suspensions were sonicated in an ice bath under nitrogen for 30 min. and then kept at 32±1°C under nitrogen atmosphere for 45 min. to equilibrate. The mixtures were then filtered through 0.45 μm membrane filter and analyzed by HPLC.

Stability of DA-CDS on storage:

Both the crystalline sample and the HPCD complex of DA-CDS were tested for stability at different storage conditions. The samples were well dried first and then stored in dark brown bottles under the following conditions:

A) Under nitrogen and dry conditions in the refrigerator (about 4°C).
B) Under nitrogen and dry conditions at room temperature (about 21°C).
C) Under air in opened bottles in the refrigerator.

D) Under air in opened bottles at room temperature.

The samples were analyzed each month for their content of CDS by HPLC. The CDS content was calculated using the non quaternized compound (2) as an internal standard. The sum of the peak areas of the 1,6- and 1,4- isomers was used to quantitate the sample content of the CDS.

Distribution after ip injection:

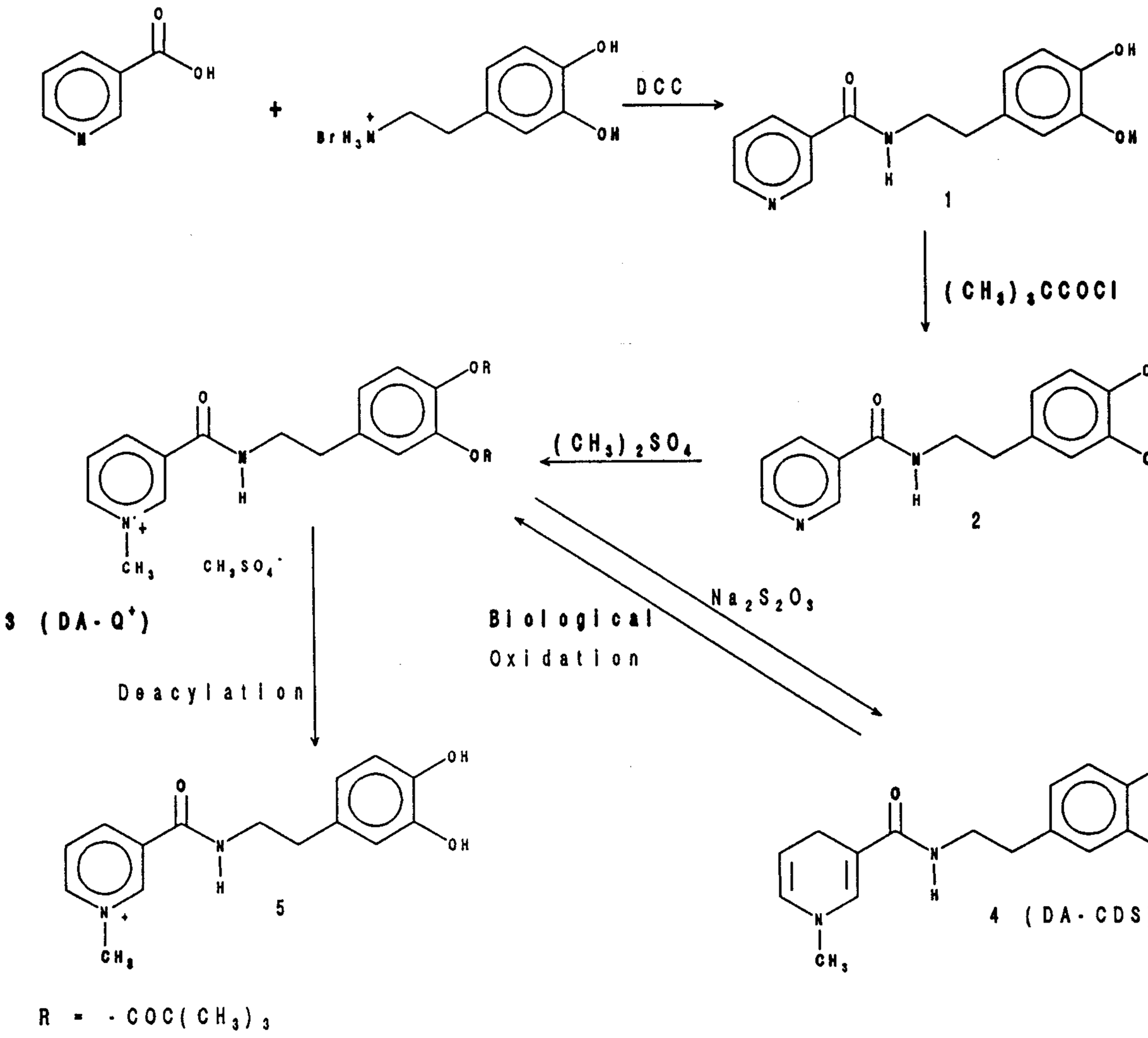
Conscious male sprague-Dawley rats of average weight 175±15 g, were used. DA-CDS HPCD-complex was given, in a dose equivalent to 40 mg DA-CDS per Kg rat body weight, by ip injection as a 50% w/v solution in deionized water. At appropriate time periods the animal was sacrificed by decapitation and trunk blood, brain, liver, kidney, lung and heart were collected and cooled on dry ice. About 1 ml of the thawed blood was added to a tared and cooled test tube containing 3 ml of 5% DMSO in acetonitrile. The tubes were afterwards weighed to determine the weights of blood in each sample. The thawed complete organ or about one g organ tissue was homogenized with 0.5 ml of water, 3 ml of 5% of DMSO in acetonitrile was then added and the mixture sonicated for 5 minutes in ice cold bath. The mixture was centrifuged, filtered and the filtrate was analyzed by HPLC for compound 2. A standard calibration curve for each organ was done by adding the appropriate volume of a stock solution of compound 2 in DMSO to a homogenized organ that was obtained from a control animal. The homogenate was then extracted as above and the extract was used to construct the corresponding organ calibration curve.

RESULTS AND DISCUSSION

DA-CDS (4) was prepared following the reported method[5] (scheme 1) with slight modification which results in higher yield and eventually gave crystalline stable final compound. In the dipivalylation of nicotinoyldopamine step, chloroform was used as the solvent and triethylamine as the acid scavenger. The product did not need any chromatographic purification, only crystallization from ether/pet. ether (60-80°C) to give 86% yield of the pure compound 2 (reported 73%).

Quaternization with methyl iodide was compared to that with dimethyl sulphate, the latter was found better even though the yield of the quaternary iodide is higher. The produced methosulphate salt (3) showed higher degree of purity and more water solubility than the corresponding iodide salt. Water solubility of the quaternary salt can represent a limiting factor in its reduction to the dihydro derivative. Higher solubility has shorten the reaction time and consequently decreased the amount of degradation impurities. The best solvent for quaternization was found to be a 1:1 mixture of boiling acetone-ether. Use of higher boiling point solvents resulted in degradation and a discolored product was obtained. Sometimes quaternization was not complete even after 24 hours reflux time. The non quaternized impurities were removed prior to reduction by extraction with boiling ether or through addition of excess dimethyl sulphate and further reflux of the reaction mixture.

Several conditions were tried for obtaining pure crystalline DA-CDS through reduction of the corresponding quaternary derivative e.g. aqueous medium in presence or absence of immiscible solvent or different concentrations of methanol, different molar proportions of sodium bicarbonate and sodium dithionite. Modification of the reported method[5], by increasing the reaction time to three hours and keeping the mixture stirred in ice bath under nitrogen atmosphere gave best results. Separation, extraction and other processes should be also carried out under nitrogen. Addition of peroxide-free, sodium-dried ether to the absolutely dry solid froth (the dihydro compound) resulted in a lemon yellow crystalline solid leaving red to pale brown mother liquor. The obtained solid compound could then be crystallized from boiling ether/pet. ether under nitrogen atmosphere to afford a pure crystalline product with melting point 103-105°C (reported for the solid froth 45-48°C)[5].



Scheme 1

The HPLC analysis of the crystalline DA-CDS (4) and the solid froth showed both to contain different combinations of two isomers, a minor slightly more polar, suggested; according to reports on similar reactions[10], to be the 1,6-dihydro-isomer and a major 1,4-dihydro-isomer. The ratio of the peak areas in case of the solid froth samples vary considerably, e.g., 5-30% depending on the conditions of the synthesis and isolation procedure. The ratio in the solid crystalline samples varied within a small range (about $15 \pm 1\%$).

Effect of Aging on Solid Froth Samples:

The solid froth samples were difficult to get and to keep absolutely dry (hygroscopic). The best dried sample submitted for elemental microanalysis was found to contain 6.1% of water. The solid content of the dihydropyridine was found to decrease quickly if not kept in a refrigerator and under nitrogen. At room temperature or at temperature higher than 4°C the main degradation products were found to be the 5,6-water addition product and the quaternary derivative (3). Since the rate of hydration of the 1,4-isomer is faster than that of the 1,6-analogue[10] the ratio of the 1,6-isomer to that of the 1,4-was found to increase by aging indicating that the hydration of dihydropyridines in solid froth samples is the main degradation process.

Effect of Aging on Crystalline Samples:

The solid crystalline compound was easier to dry and resisted hydration upon storage. At room temperature in a desiccator, or even in an opened container, oxidation was found to be the main degradation process. The average overall loss of dihydro-derivative in open air and at room temperature in a two months period was 21±1%. The 1,6-dihydro-isomer decreased by about 35% while the 1,4-analogue decreased by about 18% during the same period.

Chemical Oxidation:

A methanolic solution of the crystalline DA-CDS was found to be oxidized with alcoholic silver nitrate solution exclusively to the corresponding quaternary derivative. Monitoring by HPLC revealed that, both the 1,4- and 1,6-dihydro-isomer peaks disappeared and the only peak detected was that of the quaternary compound. With 0.01 M potassium ferricyanide solution both isomers were oxidized exclusively to the quaternary derivative but at different rates (in 1 min. 85% of the 1,6- isomer and 11% of the 1,4- isomer) . The apparent $t_{1/2}$ of the oxidation was found to be 22 and 354 seconds for the 1,6- and the 1,4- dihydro-isomers respectively. The 1,6-isomer appeared to be oxidized about 16 times faster than the 1,4-isomer under these conditions.

Solubility in Cyclodextrin:

Figure 1 shows the phase solubility of DA-CDS in aqueous 2-hydroxypropyl-β-cyclo-dextrin (HPCD). As expected, the solubility increased as a linear function of HPCD concentration, i.e. first order dependance. The concentrations used in this study ranging from 0-20% (w/v). The solubility increased dramatically from 0.02 mg/ml at 0% HPCD concentration to 6.12 mg/ml at 20% HPCD, corresponding to 30.5 mg DA-CDS per g HPCD. However solid inclusion complex samples of DA-CDS with HPCD could be prepared up to 52 mg DA-CDS per one gram complex. This could be accomplished by using 50% w/w aqueous HPCD solution and finely owdered crystalline DA-CDS. The mixture was sonicated in an ice cold bath under nitrogen gas

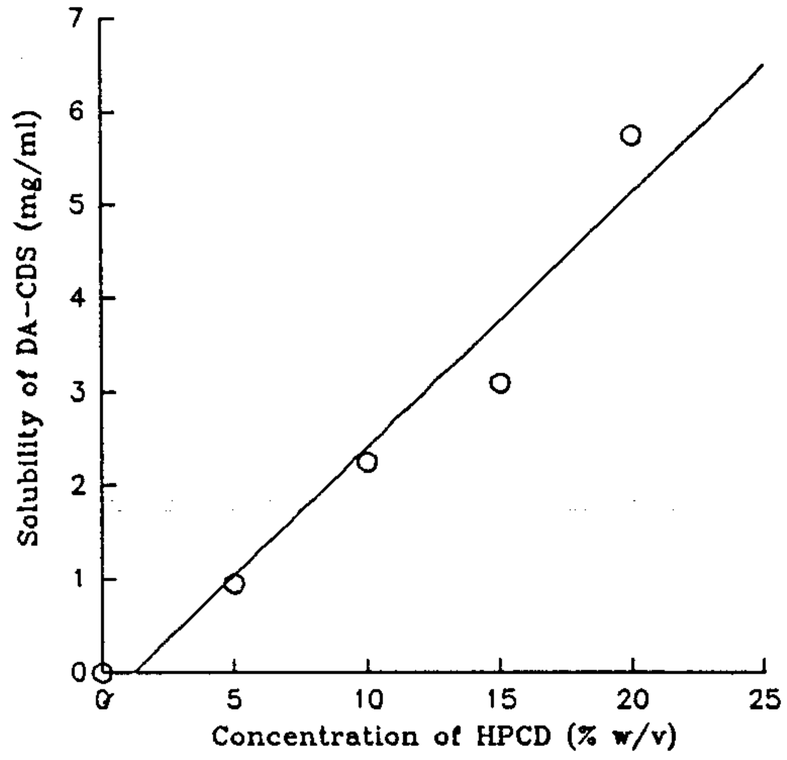


Fig. 1: Solubility of DA-CDS (4) in Aqueous Solutions of HPCD

for 45 min. prior to equilibration, filtration and freeze drying.

Stability of the Crystalline and Complex Samples on Storage:

The shelf stability of the solid crystalline DA-CDS and its HPCD inclusion complex was determined at room temperature (21±2°C) and at 4°C; under nitrogen and in air either in dry conditions or at the usual uncontrolled conditions of the laboratory. The samples were kept under these conditions for 6 months and were then monitored by HPLC for their total contents of the dihydropyridine derivatives (1,6- and 1,4-isomers). The crystalline samples proved to be more stable than the cyclodextrin complex at any storage conditions (Fig. 2,3). This could be explained by the fact that, the cyclodextrin complex, due to its method of preparation and being amorphous would have higher residual water contents even after the regular drying conditions. The amorphous solid could enable more water to be taken relative to its crystalline form[11,12]. This residual water by virtue of enhancing the molecular mobility within the amorphous solid[13] will not only increase the rate of hydration of the dihydropyridine moiety but also may increase the rate of oxidation with molecular oxygen. This also can explain the instability of the froth DA-CDS samples at room temperature. The best storage condition found

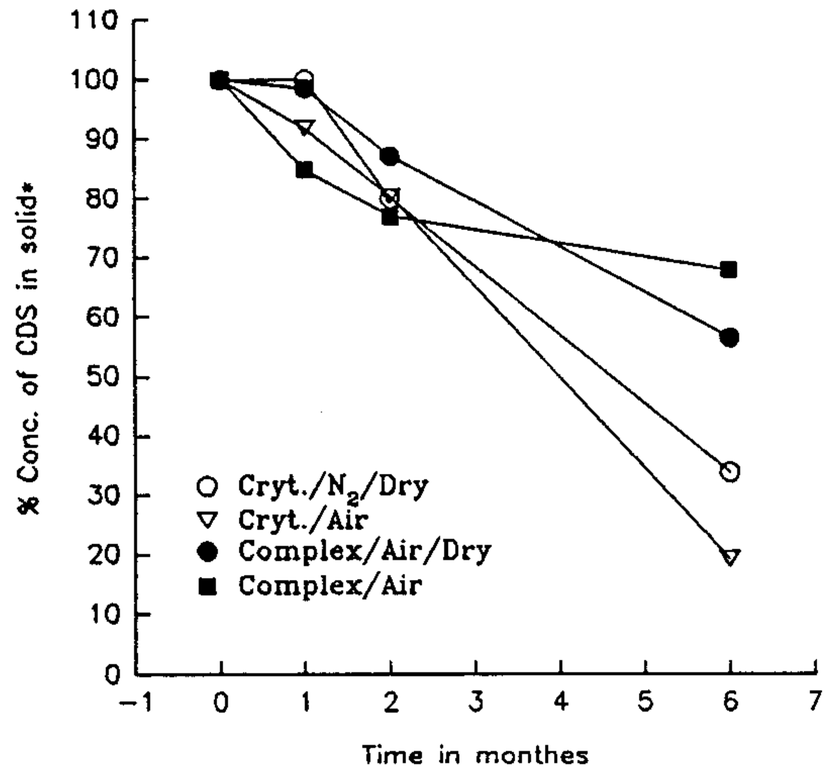


Fig.2: Shelf stability of crystalline DA–CDS (4) and its HPCD complex at room temperature.

* % calculated with respect to the labelled amount in both the crystalline and the complex.

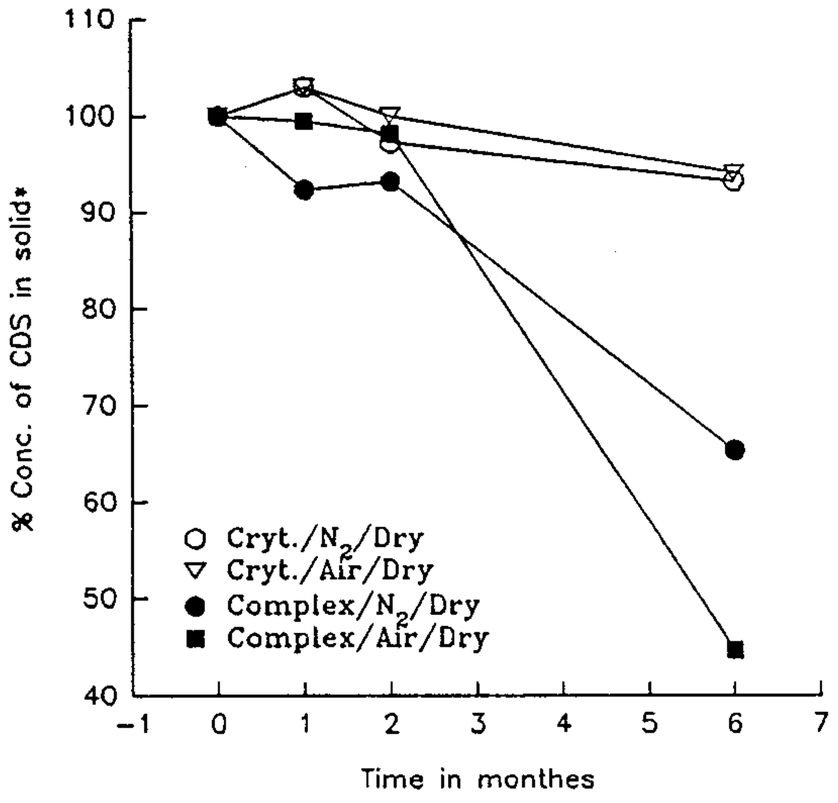


Fig.3: Shelf stability of crystalline DA–CDS (4) and its HPCD complex at 4°C..

* % calculated with respect to the labelled amount in both the crystalline and the complex.

was to keep the well dried crystalline compound in a tightly closed and sealed dark bottle under nitrogen at/or below 4°C.

Distribution after IP injection:

Compound 4 was given by ip route to conscious male Sprague-Dawley rats in the form of 50% aqueous solution of the corresponding HPCD complex and at a dose equivalent to 40 mg DA-CDS per Kg rat body weight. Blood and selected organs were analyzed by HPLC for their contents of the CDS and/or its metabolites.

No DA-CDS could be detected in any of the organs or blood at any time point. This should not be unusual, since this highly lipophilic compound would have great distribution and at the same time it would be biologically labile through deacylation and/or oxidation. The only metabolite which could be detected in all organs and blood, under the used conditions, was the fully deacylated quaternary 5 which could be considered the ultimate pro-dopamine compound. Figure 4 illustrates the results of the analysis, which proved that the DA-CDS would be stable enough to be absorbed from the intrapretonium cavity. The only organ which showed higher concentrations than the brain was the heart which may indicate a specific receptor binding interaction.

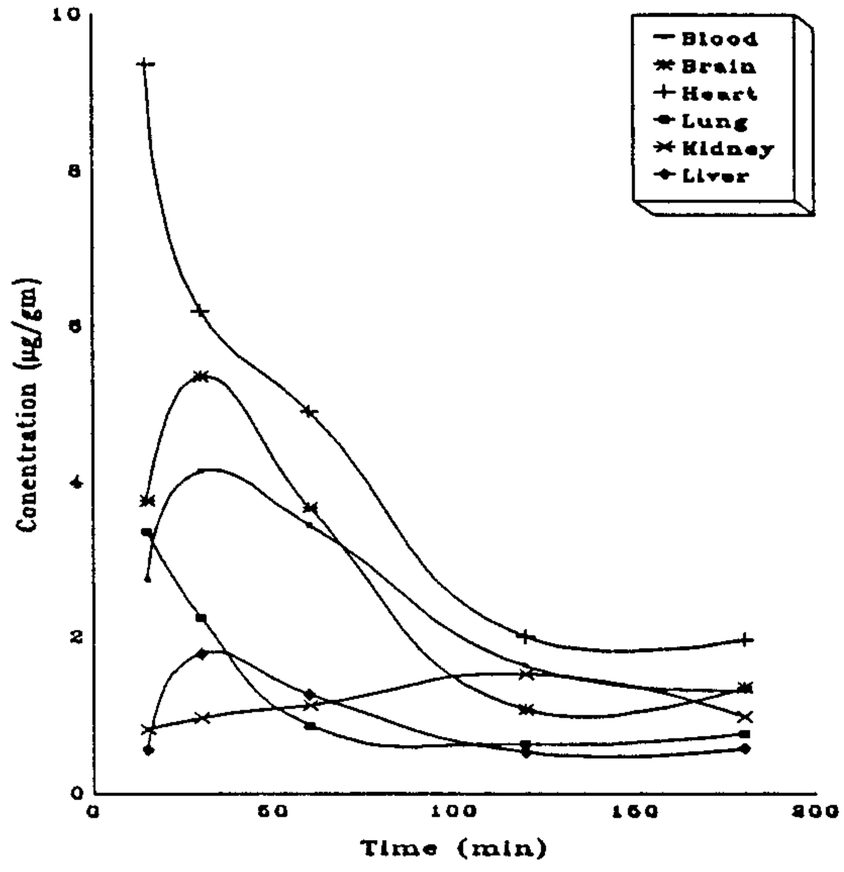


Fig. 4: Concentration of parent quaternary (5) after ip injection of HPCD complex of DA-CDS (40 mg/kg).

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التوجيه الجيد خلال الأغشية البيولوجية: دراسة تحضير نظام كيميائى ثابت لتوجيه الدوبامين الى المخ

نيكولاس بودور - حسن حسن فرج - فرغلى عبد الحميد عمر
مركز استكشاف الأدوية - كلية الصيدلة - جامعة فلوريدا
الولايات المتحدة الأمريكية
قسم الكيمياء الصيدلية الطبية - كلية الصيدلة - جامعة اسيوط

أمكن فى هذا البحث تحضير مشتقات كيميائية لمادة الدوبامين فى صورة مبلورة ثابتة وكذلك فى صورة متراكب لهذه المشتقات مع سيكلودكسترين بهدف زيادة الإتاحة البيولوجية لاكبر كمية ممكنة من جرعة الدوبامين فى مراكز التأثير بالجهاز العصبى المركزى.

وقد تميز كل من المشتق فى صورته البلورية وكذلك فى صورة المتراكب مع سيكلودكسترين بدرجة ثبات عالية ضد الأكسدة والحلمأه تحت ظروف التخزين.

كما لوحظ أن المتراكب مع سيكلودكسترين يزيد من درجة ذوبان هذه المشتقات فى الماء بحيث يتيح الحقن الوريدى بالمحلول المائى بدلا من المحلول فى داى ميثيل سلفوكسيد وقد أدى الحقن عن طريق الغشاء البريتونى للمحلول المائى الى وصول مشتق الدوبامين بدرجة تركيز عالية الى المخ.

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