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Macromolecular prodrugs. II. Esters of L-dopa and α-methyldopa

B. Zorc [a], M. Ljubić [a], S. Antolić [a], J. Filipović-Grčić [a], D. Maysinger [b], T. Alebić-Kolbah [b] and I. Jalšenjak [a]

[a] *Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb (Croatia) and*
[b] *Department of Pharmacology and Therapeutics, McGill University, Montreal (Canada)*

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Summary

L-Dopa and α-methyldopa were attached by an ester linkage to α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA), a hydrophilic polymer, previously proposed as a drug carrier. Ester bonding was achieved by means of 1-benzotriazolylcarbonyl (Btc) group as both an N-protecting and C-activating group in the starting amino acids. In the same way several simple esters of L-dopa and α-methyldopa were prepared. Release of active substances based on hydrolysis of PHEA adducts was studied in vitro, and the following (pseudo) first order release rate constants for L-dopa and α-methyldopa were obtained, 1.06×10^{-3} and 6.91×10^{-4} min^{-1}, respectively. In addition, characterization of the PHEA-L-dopa adduct was carried out in vivo using an intracerebral microdialysis technique in order to evaluate the prolonged release eventually achieved.

Introduction

The preparation of polymeric drug adducts, in which active substances are covalently linked to polymeric matrixes, is presently suggested as an effective way to prolong the pharmacological activity and minimize unfavorable side effects. Covalent bonds of limited stability in biological media are the most suitable for attaching an active substance to a macromolecular backbone, when a slow and gradual release of the active substance in the body is desired. Ester bonds should be considered first, when the structure of the drug molecule allows such a type of binding (Duncan and Kopeček, 1984).

N-substituted poly(aspartamides), so-called proteinoids, are readily and inexpensively prepared polymers with various applications. α,β-Poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) (1) has been studied as a blood plasma expander (Neri et al., 1973; Antoni et al., 1974, 1979) or as drug carrier (Giammona et al., 1987, 1989, 1991). Poly[*N*-(3-aminopropyl)aspartamide] shows fairly selective antibacterial, antifungal, and antitumor activity (Kovacs et al., 1967). Some polyaspartamides have been used as models in studies of protein-immobilization techniques on insoluble carriers (Drobnik et al., 1979a) and the distribution of synthetic polymers in vivo (Rypaček et al.,

Correspondence to: B. Zorc, Faculty of Pharmacy and Biochemistry, University of Zagreb, 41000 Zagreb, A. Kovačića 1, Croatia.

1980). Poly(*N*-hydroxyethyl)-DL-aspartamide-silica, a new packing for hydrophilic-interaction chromatography, was prepared and used for the separation of peptides, nucleic acids and other polar compounds (Alpert, 1990).

PHEA is an especially interesting and promising drug carrier since it is water soluble, nontoxic, nonantigenic, and biodegradable when exposed to a complex set of enzymes (Drobnik et al., 1979b). Hydroxyl groups of PHEA permit the attachment of selected carboxylic acids to the polymer. Several pharmacologically active agents such as benzoic acid, acetylsalicylic acid, 4-acetamidobenzoic acid, naproxen, 4-biphenylacetic acid, ketoprofen, ibuprofen, and alclofenac have been covalently linked by esters bonds to PHEA (Giammona et al., 1987, 1989, 1991).

In this paper we describe the attachment of two well known drugs L-dopa and α-methyldopa to PHEA.

L-dopa (3-hydroxy-L-tyrosine; 3,4-dihydroxy-L-phenylalanine) the immediate biological precursor of dopamine, is the most commonly used drug in the treatment of Parkinson's disease. Long-term therapy with L-dopa is, however, associated with a number of therapeutic problems (Bianchine et al., 1976). The main disadvantages of L-dopa is low water solubility, its sensitivity to chemical oxidation and peripheral decarboxylation. Only 1% of the administered dose reaches the brain after oral administration. To overcome these problems several delivery systems have been developed such as brain implant and microspheres including neurotransmitter dopamine or its precursor L-dopa (McRae-Degueurce et al., 1988; During et al., 1989; Maysinger et al., 1992).

α-Methyldopa [3-(3,4-dihydroxyphenyl)-2-methyl-L-alanine] is an antihypertensive agent which is used in the treatment of moderate to severe hypertension. Since α-methyldopa has a catechol nucleus like L-dopa itself, it is quite prone to oxidation.

Various esters and other derivatives of L-dopa and α-methyldopa have been synthesized, systematically protecting one or more of the main sites of metabolism in the molecules, improving stability and bioavailability of these drugs (Hajos and Sohar, 1966; Bodor et al., 1977). By linking L-dopa and α-methyldopa to a hydrosoluble polymer, prodrugs of interest can be obtained.

In our previous investigation 1-benzotriazolylcarbonyl (Btc) group was introduced to amino acids (Butula et al., 1981) and it was successfully used in preparation of amino acids esters (Matijević-Sosa et al., 1985) and in peptide synthesis (Butula et al., 1983; Zorc et al., 1990). Therefore, we have chosen the 'benzotriazole method' for binding L-dopa and α-methyldopa to PHEA and for preparation of their esters.

Materials and Methods

Materials

All melting points are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 457 spectrophotometer and UV spectra on a Pharmacia LKB Ultrospec Plus and Pye Unicam SP-100 spectrophotometers. A HPLC system with UV absorbance detection (ABI Applied Biosystem 783 Programmable Absorbance Detector) was used. Specific rotation data were taken on an Opton polarimeter. Viscosity measurements were carried out at 25°C using an Ostwald viscosimeter, with a flow time for water from 100 to 200 s. Polymer solutions were dialyzed against several changes of deionized water using Visking Dialysis Tubing (18/22 inch; Serva) with a molecular weight cut-off of 12000–14000. For thin-layer chromatography, silica gel sheets, Kieselgel 60 F_{254} (Merck) were used. Solvent systems were chloroform/methanol 9:1 and dioxane/water 9:1. Column chromatography was performed on silica gel 0.063–0.200 mm. L-Aspartic acid was purchased from Kemika (Zagreb), L-dopa from Merck (Darmstadt) and α-methyldopa from Lek (Ljubljana). All solvents were of analytical grade quality and were dried and distilled prior to use. All solutions for HPLC were prepared with HPLC-grade solvents (Fisher, Montreal).

Chemistry

α,β-Poly(N-hydroxyethyl)-DL-aspartamide (PHEA) (1) PHEA was prepared by aminolysis of polysuccinimide [PSI; poly(2,5-dioxo-1,3-pyrrolidinediyl)] with ethanolamine. PSI was prepared by thermal polycondensation of L-aspartic

acid (L-Asp) in the presence of phosphoric acid under reduced pressure, at 160°C. The method described by Neri et al. (1973) was followed but a few modifications were introduced (L-aspartic acid instead of DL-aspartic acid was used and the reaction was run at lower temperature). According to Kokufuta et al. (1978) the optical purity of PSI decreases rapidly with rise in temperature from 160 to 180°C. Running the polycondensation at lower temperature racemization was incomplete, so the final product PHEA had $[\alpha]_D^{21}$ −4.8° (*c* 0.4, H_2O). Weight-average molecular weight of polymer was determined by applying the Mark-Houwink equation (Antoni et al., 1974) $[\eta] = 2.32 \times 10^{-3}\ M^{0.87} = 30.80$ ml g^{-1} and a value of molecular weight $M = 54\ 850$ was obtained. Intrinsic viscosity was determined by measuring the reduced viscosity at different concentrations, in the range 2–10 mg ml^{-1}, and extrapolating to zero concentration.

1-Benzotriazole carboxylic acid chloride (BtcCl) (2) BtcCl was synthesized from benzotriazole and phosgene (Butula et al., 1977).

N-(1-Benzotriazolylcarbonyl)-L-dopa (3) To a suspension of 7.89 g (0.040 mol) L-dopa in 65 ml dioxane a solution of 3.63 g (0.020 mol) of 1-benzotriazole carboxylic acid chloride (**2**) in 65 ml dioxane was added dropwise. The reaction mixture was stirred at room temperature for 24 h. The solution was separated from sticky L-dopa hydrochloride and evaporated under reduced pressure. The yellow oil obtained was dissolved in ethyl acetate and extracted three times with water. The organic layer was dried and evaporated to give 5.98 g (87%) of product **3**. The product crystallized by adding ether and petroleum ether. m.p. 90–92°C. IR(KBr): ν_{max} 3700–2500, 1730, 1615, 1525 cm^{-1}, $[\alpha]_D^{22} + 4.28°$ (*c* 0.35, dioxane). Anal. Calcd for $C_{16}H_{14}N_4O_5$ (342.31): C, 56.14; H, 4.12; N, 16.37. Found: C, 56.23; H, 4.21; N, 16.30.

N-(1-Benzotriazolylcarbonyl)-α-methyldopa (4) To a suspension of 2.11 g (0.010 mol) α-methyldopa in 16 ml dioxane solution of 0.91 g (0.005 mol) of 1-benzotriazole carboxylic acid chloride (**2**) in 17 ml dioxane was added dropwise. The reaction mixture was stirred at room temperature for 20 h and evaporated under reduced pressure. The oil was dissolved in methylene chloride and extracted three times with water, dried over anhydrous sodium sulfate and evaporated. For analysis, **4** was recrystallized from ether/petroleum ether. Yield: 0.80 g (45%). m.p. 67–70°C. IR(KBr): ν_{max} 3600–2800, 1725, 1600, 1500 cm^{-1}. Anal. Calcd for $C_{17}H_{16}N_4O_5$ (356.35): C, 57.30; H, 4.53; N, 15.72. Found: C, 57.55; H, 4.60; N, 15.67.

L-dopa and α-methyldopa esters 5–10: General procedure To a solution of 0.001 mol of *N*-(1-benzotriazolylcarbonyl)-L-amino acid **3** or **4** in 10 ml absolute alcohol, 0.02 g of sodium dithionite and 0.71 g (0.007 mol) TEA was added. For preparation of benzyl esters **7** and **10** 3 ml of benzyl alcohol, 1 ml TEA and 10 ml dioxane were used. The solution was left at room temperature for 24 h. The reaction was run under a nitrogen atmosphere. The solution was evaporated under reduced pressure. The esters **5–10** were separated from benzotriazole on a silica gel column (chloroform/methanol 9:1). Yield: 80–90%. Esters of L-dopa were transformed to HCl salts.

L-dopa methyl ester hydrochloride (5) m.p. 169–171°C; $[\alpha]_D^{22} + 14.0°$ (*c* 11.0, CH_3OH); [(Bodor et al., 1977) m.p. 170.5–171.5°C; $[\alpha]_D^{22} + 14.7°$ (*c* 12.5, CH_3OH)].

L-dopa ethyl ester hydrochloride (6) m.p. 125–127°C; $[\alpha]_D^{22} - 8.2°$ (*c* 1.0, H_2O); [(Losse et al., 1961) m.p. 126–129°C; $[\alpha]_D^{22} - 8.95°$ (*c* 1.053, H_2O)].

L-dopa benzyl ester hydrochloride (7) m.p. 190–191°C; $[\alpha]_D^{22} - 10.5°$ (*c* 0.3, CH_3OH); [(Bodor et al., 1977) m.p. 190.5–191°C; $[\alpha]_D^{24} - 9.5°$ (*c* 1.4, CH_3OH)].

α-Methyldopa methyl ester (8) m.p. 164–166°C; $[\alpha]_D^{22} - 6.6°$ (*c* 0.5, 1 mol l^{-1} HCl); [(Hajos and Sohar, 1967) m.p. 165–166°C; $[\alpha]_D - 7°$ (*c* 0.5, 1 mol l^{-1} HCl)].

α-Methyldopa ethyl ester (9) m.p. 154–156°C; $[\alpha]_D^{22} - 9.5°$ (*c* 0.4, 1 mol l^{-1} HCl); [(Hajos and Sohar, 1966) m.p. 156–157°C; (Reinhold and Sletzinger, 1968) $[\alpha]_D - 10°$ (*c* 1, 1 mol l^{-1} HCl)].

α-Methyldopa benzyl ester (10) IR: ν_{max} 3680–2200, 1740, 1605 cm^{-1}.

PHE4-L-dopa adduct (11) Through a solution of 0.5 g PHEA in 20 ml DMF nitrogen was bubbled for 20 min. 0.34 g (0.001 mol) Btc-L-dopa

(**3**) was added and after that a solution of 0.71 g (0.007 mol) TEA in 12 ml DMF was added dropwise. The reaction was run at room temperature for 24 h under a nitrogen atmosphere. Solvent was evaporated in vacuo to a small volume and adduct **11** precipitated by adding an acetone/glacial acetic acid mixture. The product was filtered off and washed several times with a small amount of acetone until benzotriazole was completely washed off (TLC control). Yield: 0.55 g (80%). IR(KBr): ν_{max} 3280, 1740, 1650, 1525 cm^{-1}. UV λ_{max}: 280.6 nm ($c = 392$ μg ml^{-1}, Tris-HCl buffer pH 7.4).

PHEA-α-methyldopa adduct (12) An analogous procedure to that for **11** was applied. Yield: 0.41 g (60%). IR(KBr): ν_{max} 3270, 1725, 1650, 1525 cm^{-1}. UV λ_{max}: 279.6 nm ($c = 71.9$ μg ml^{-1}, Tris-HCl buffer pH 7.4).

In vitro experiments

Drug content in PHEA-L-dopa (11) and PHEA-α-methyldopa (12) A solution of 26.10 mg of adduct **11** and 35.20 mg of adduct **12**, respectively, in 10 ml 6 mol l^{-1} HCl was refluxed in a nitrogen stream for 6 h and left for 24 h at room temperature. Each solution was quantitatively transferred to a measuring flask and diluted with water to 100 ml. The concentrations of L-dopa and α-methyldopa in these solutions were determined spectrophotometrically and from the results obtained the load of drug in each adduct was calculated (17.4 and 14.0%, respectively).

Release of drugs from PHEA-L-dopa (11) and PHEA-α-methyldopa (12) A solution of adduct **11** ($c = 366$ μg ml^{-1}) or adduct **12** ($c = 710$ μg ml^{-1}), in Tris-HCl buffer (pH 7.4) in a well stoppered silica cell was thermostated at 37 ± 0.1°C. The drug release from adduct by hydrolysis was measured by UV spectrophotometry (280 nm) at suitable time intervals. Rate constants were computed using a nonlinear least-square fitting program.

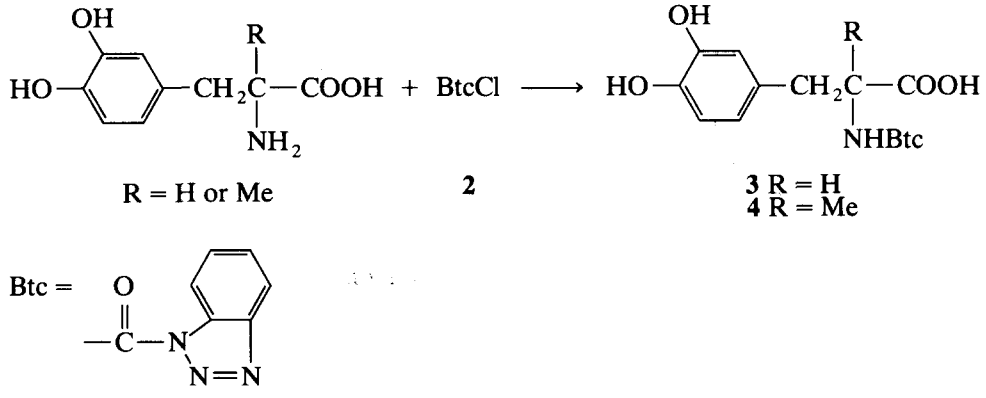
In vivo experiments

Release of L-dopa from PHEA-L-dopa (11) Six male Wistar rats (Charles River, U.S.A.) weighing 250–275 g with free access to food and water ad libitum were used in these experiments. Animals were anesthetized with Equithesin (3 ml/kg) and placed into stereotaxic apparatus (Kopf). A hole was drilled through the skull for the implantation of the cannula guide. PHEA-L-dopa adduct (500 $\mu g/5$ μl) was injected into the corpus striatum (coordinates: AP 1.21, L 2.6 from bregma and V 6.9 from dura). A microdialysis probe (4 mm, CMA 10, Carnegie Medicine AB, Stockholm) was inserted immediately after the administration of PHEA-L-dopa adduct. The probe was secured with the screw and connected with a peristaltic micropump (CMA 100, Carnegie Medicine AB, Stockholm) delivering artificial cerebrospinal fluid at a constant rate (2 μl/min). The fractions were continuously collected every 20 min for 4 h and 20 min, and again after 24 h using the same fraction collecting schedule. The samples were kept at −80°C until analysis by HPLC.

Determination of L-dopa 20-μl aliquots of mycrodialysates were directly injected into a reverse phase ion pair HPLC system with UV absorbance detection preset at 280 nm. L-dopa was well separated from the peaks of endogenous compounds on a 250 × 4.6 mm i.d. column prepacked with Ultrasphere ODS 5 μm (Altex). The mobile phase consisted of 0.15 mol l^{-1} sodium dihydrogen orthophosphate containing 0.1 mmol l^{-1} EDTA, 0.65 mmol l^{-1} octyl sodium sulfate (Kodak) and 10% methanol. The pH was set at 3.8 using concentrated phosphoric acid. The flow rate was 1.0 ml/min. The retention time for L-dopa under these conditions was 3.8 min and the run was completed within 25 min. The amounts of L-dopa in microdialysates were calculated using L-dopa as an external standard by determining the peak area ratios (Data-Jet integrator, Spectra Physics). The limit of determination of L-dopa in microdialysates was 0.2 μmol L^{-1}.

Results and Discussion

A previously developed method for preparation of amino acid esters (Matijević-Sosa et al., 1985) has been applied to L-dopa and α-methyldopa. The method is simple, proceeds under mild conditions, and requires three single steps: (i) preparation of BtcCl; (ii) coupling of BtcCl with amino acids (e.g., L-dopa, α-methyldopa); (iii)

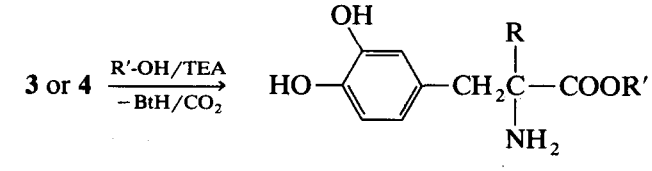


Scheme 1.

reaction of *N*-Btc-amino acids with alcohols. In this method the Btc group serves both as an *N*-protecting and C-activating group.

N-(1-Benzotriazolylcarbonyl)-L-dopa (**3**) and *N*-(1-benzotriazolyl-carbonyl)-α-methyldopa (**4**) were synthesized from the corresponding amino acid and 1-benzotriazole carboxylic acid chloride (**2**). The molar ratio was 2:1, since the second mole of the amino acid was necessary as an acceptor of hydrochloride separated during the reaction (Scheme 1). The compounds **3** and **4** reacted with alcohols (methanol, ethanol, benzyl alcohol) giving the corresponding amino acid esters **5–10** (Scheme 2). The reactions were accelerated with triethylamine (TEA). In order to prevent the oxidation of L-dopa and α-methyldopa derivatives (they are rapidly oxidized in alkaline solutions by atmospheric oxygen and turn dark), a small amount of sodium dithionite was added to the reaction mixtures and the reactions were carried out under a nitrogen atmosphere. Analytical results on the synthesized esters were in good agreement with literature data (see Materials and Methods).

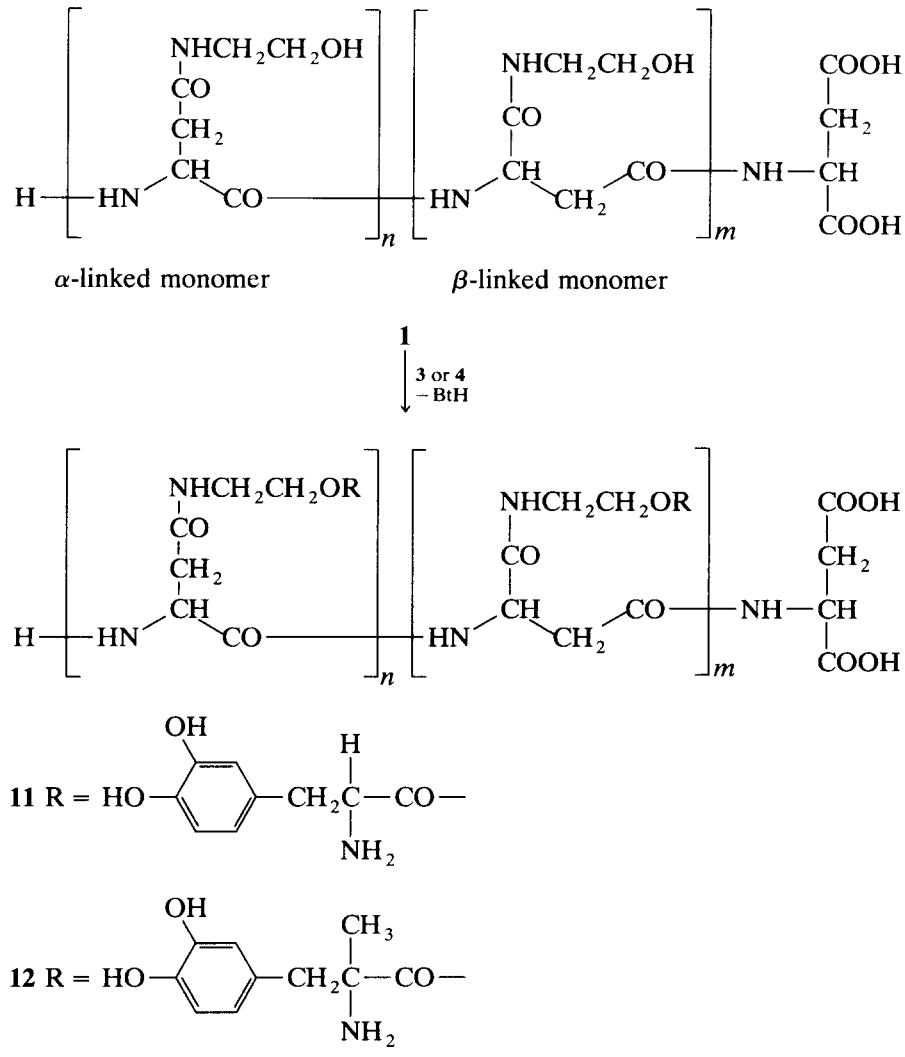
This method of ester bond formation could also be applied to PHEA, a polyhydroxy polymer (Scheme 3). The existence of an ester bond in PHEA-L-dopa (**11**) and PHEA-α-methyldopa (**12**) was confirmed by IR and UV spectroscopy. The IR spectra of adducts **11** and **12**, beside absorption bands characteristic for hydroxyl, amide I and amide II, showed an ester carbonyl bond at 1740 and 1725 cm^{-1}, respectively. UV spectra of L-dopa and α-methyldopa adducts exhibited one maximum at 280.6 and 279.6 nm, respectively (PHEA had no absorption in the range of 220–400 nm; the absence of the free drugs in **11** and **12** was checked by TLC).



BtH = benzotriazole
TEA = triethylamine

5 R = H, R′ = Me
6 R = H, R′ = Et
7 R = H, R′ = Bzl
8 R = Me, R′ = Me
9 R = Me, R′ = Et
10 R = Me, R′ = Bzl

Scheme 2.



Scheme 3.

The content of the active agent in the polymeric prodrugs depends on the applied molar ratio of the reactants **3** or **4** and PHEA. We have chosen the molar ratio allowing a substitution of approx. 33% of the available hydroxyl groups of PHEA. The molar percentage of L-dopa and α-methyldopa in the polymeric adducts was determined by hydrolysis in 6 mol l^{-1} HCl. Under the same reaction conditions free drugs did not undergo any degradation. The load of L-dopa in **11** was 17.4%, the amount of α-methyldopa in **12** being 14%.

Release of drugs from PHEA-L-dopa (**11**) and PHEA-α-methyldopa (**12**) was studied in vitro in order to evaluate the possible time span in which the drugs would be available free of adduct for their originally intended purpose, i.e., pharmacological activity in organism. The results are presented in Figs 1 and 2 showing two features of the drug release, i.e., the percentage of drug incorporated in adduct that is released and the amount (mg) of drug available from 100 mg of adduct. The latter data are suitable to calculate eventual dosage regimens in in vivo experiments.

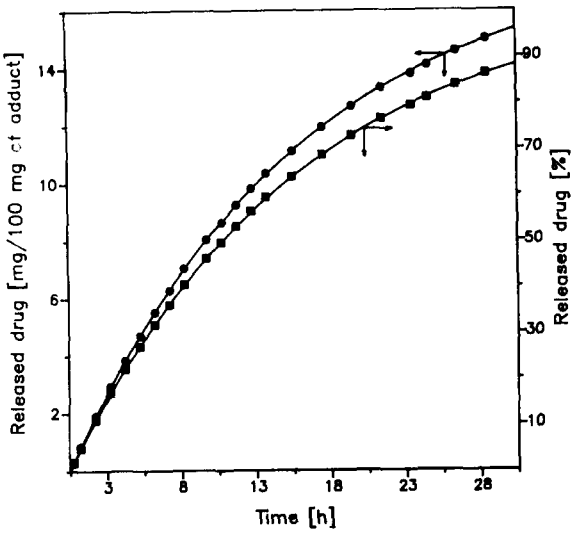


Fig. 1. Release of L-dopa from adduct **11** in Tris-HCl buffer (pH 7.4; 37±0.1°C). Points are experimental (mean values of three measurements), and curves are the best fit for first order kinetics ($r^2 = 0.999$).

The release data conformed to (pseudo) first order kinetics, and the rate constants computed were 1.06×10^{-3} min^{-1} and 6.91×10^{-4} min^{-1} (correlation coefficient not less than 0.999) for

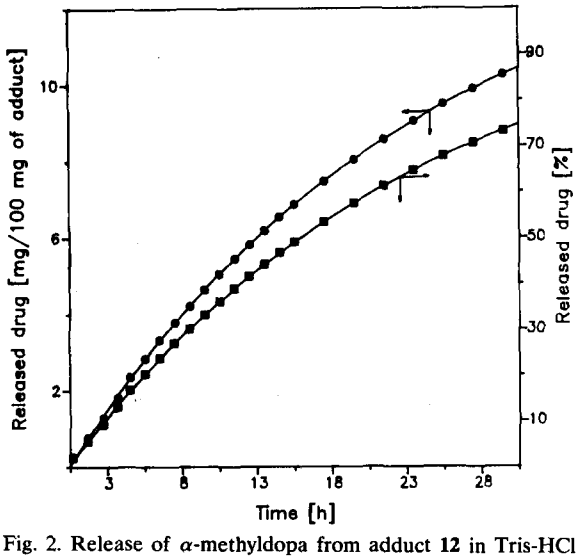


Fig. 2. Release of α-methyldopa from adduct **12** in Tris-HCl buffer (pH 7.4; 37±0.1°C). Points are experimental (mean values of three measurements), and curves are the best fit for first order kinetics ($r^2 = 0.999$).

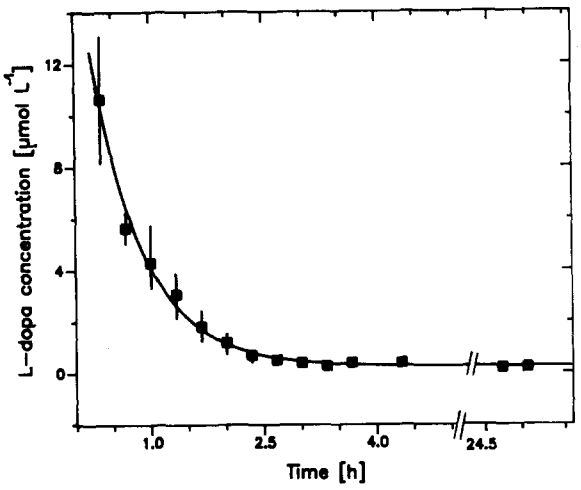


Fig. 3. In vivo concentration of L-dopa released from adduct **11** obtained by brain microdialysis. Bars indicate range of values ($n = 4$).

L-dopa and α-methyldopa, respectively. These values correspond to half-times in vitro of 10.9 and 16.7 h. These results show on the one hand that hydrolysis of drug-PHEA adducts follow the kinetics of simple ester hydrolysis, and, on the other, that the adducts studied can be considered as typical examples of 'drug reservoir' that might sustain drug to be available in the body for longer time periods. It might be speculated that a rather long half-time of 10.9 h for L-dopa to be released from its polymer adduct should increase the chance for less degradation by metabolism and thus improve drug bioavailability. The results of L-dopa release in vitro from its polymer adduct cannot be considered conclusive for the drug behaviour in vivo. Therefore, as a preliminary step in the study of polymer-adduct behaviour in vivo an additional study was performed. Freese et al. (1989) have shown that the microdialysis technique can be used to monitor the in vivo drug release from controlled release formulations. From the results shown in Fig. 3 it is evident that a certain prolongation was achieved although not on a scale comparable with the release in vitro. As was noted previously (Maysinger et al., 1992) the implantation of a microdialysis system in vivo causes destabilization of extracellular fluid around the semipermeable membrane and therefore the results for fraction collected within 60 min follow-

ing implantation are not reproducible. Therefore, the results of control samples after the free L-dopa application were not included in Fig. 3. Also (in two control rats) it was shown that no endogeneous L-dopa was released in the brain region by the process of microelectrode implantation. Bodor et al. (1977) hawe shown better availability of prodrug L-dopa preparations when compared to free or 'enteric' drug formulations. Thus, the described experiments and results in this contribution suggest that the polymer-L-dopa adduct can be interesting in further studies.

Conclusion

Several simple esters of L-dopa and α-methyldopa were prepared by means of the benzotriazole method. The same method of esterification was applied for binding these drugs to α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) as a drug carrier. Release on the basis of hydrolysis of L-dopa and α-methyldopa from thus prepared polymer-drug adducts under conditions similar to physiological (pH 7.4, 37°C) followed (pseudo) first order kinetics (L-dopa: k 1.06×10^{-3} min^{-1}, $t_{1/2} = 10.9$ h; α-methyldopa: $k = 6.91 \times 10^{-4}$ min^{-1}, $t_{1/2} = 16.7$ h). The results obtained confirmed that sustained release of these drugs was achieved in vitro. It can be concluded that PHEA can be considered to be promising as a drug carrier. From preliminary experiments in vivo (brain microdialysis) it can be concluded that further studies with ester type drug polymer adducts appear to hold promise.

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