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In-Vitro release and pharmacological study of synthesized valproic acid-dextran conjugate

B. Praveen, Prabhat Shrivastava, Sushant Kumar Shrivastava*

Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi, U.P., India-221 005

Abstract

The valproic acid – dextran conjugate was synthesized by preparing valproic acid acyl imidazole with N, N carbonyldiimidazole and *in- situ* condensation with dextran of molecular weight 110,000. The degree of substitution was estimated to be 16% and molecular weight was determined by viscometery method. *In-vitro* hydrolysis study of valproic acid dextran conjugate was performed in different buffer solutions (pH 1.2, 7.4 and 9.0). The hydrolysis followed first order kinetics and a faster hydrolysis was observed at pH 9 compared to pH 7.4. The conjugate was evaluated for anticonvulsant, hepatotoxicity and ulcerogenic activities. The results showed a remarkable reduction in hepatotoxicity and ulcerogenicity along with comparable anti-convulsant activity as parent drug valproic acid.

Key words: Macromolecular prodrug, characterization, *in-vitro* hydrolysis, hepatotoxicity, ulcerogenic activities.

Introduction

In macromolecular or polymeric conjugates, the drug is either linked by physical entrapment or by chemical linkage to polymeric carriers. The main advantages of this polymeric drug conjugate include: (1) an increase in water solubility of low soluble or insoluble drugs, and therefore, enhancement of drug bioavailability; (2) an improvement in pharmacokinetics; (3) the ability to provide passive or active targeting of the drug specifically to the site of its action; (4) the possibility to form an advanced complex drug delivery system (Hoste 2004, Khandare and Minko 2006). Dextran was investigated as macromolecular carrier delivering for drugs and proteins and also demonstrated that it is useful to target the therapeutic agents to the liver (Sato 1989).

Valproic acid is one of the major antiepileptic drug having a broad-spectrum activity against different kind of epilepsy (Levy and Shen 1989, Zaccara et al. 1988). Numerous studies reported that valproic acid has shown potent antitumor effects in a variety of *in vitro* and *in vivo* system and emerged as a promising drug for cancer treatment (Blaheta et al. 2002, Duenas-Gonzalez et al. 2008. Despite of broad spectrum antiepileptic activity, its use is restricted because of rare but potentially-life threatening side effects e.g. hepatotoxicity, teratogenisity, gastrointestinal irritation and pancreatitis (Sobol et al. 2004, Grauso-Eby et al. 2003). Acute valproic acid-induced liver toxicity is fatal for the majority of patients. Especially for very young children the risk is high (Bryant and Dreifuss 1996). Valproic acid liver toxicity was also reported while receiving adequate L-carnitine supplementation (Bohan et al. 2001). The

*Corresponding author: sushant_itbhu@rediffmail.com

combination of valproic acid, topiramate and acetaminophen given for the children with Dravet syndrome developed a serious rise of liver toxicity. (Nicolai et al. 2008).

Valproic acid shows low ability to cross the blood brain barrier because it is more efficiently transported from brain to blood. Valproic acid has the shortest half life (10-15 h) among the all existing antiepileptic drugs (Badir et al. 1991). As a result of this short half-life, valproic acid has to be administered several times in a day, which causes gastric irritation. Generally, pharmaceutical and chemical approaches are used to minimize the fluctuation of drug in plasma level. In the pharmaceutical approach, one develops a sustained- release dosage form by which sustained release or prolonged absorption minimize fluctuations of drug in plasma (Bialer et al. 1985). In chemical approach, a prodrug is designed and rate of biotransformation of prodrug to the parent drug is used to obtain sustained level of drug in plasma (Haj-Yehia and Bialer 1990).

In present work, dextran molecular weight (M_W 1, 10,000) is used as promoiety for the preparation of prodrug of valproic acid due to their excellent physicochemical properties and physiological acceptance (Virnic et al. 1975). The number of dextran conjugates was reported for reducing toxicity, improving physicochemical properties and targeting the drug to the specific site (Larsen 1990). We have previously reported that the dextran is a potential macromolecular carrier for the delivery of anti-inflammatory drugs (Shrivastava et al. 2003a, 2003b). It is demonstrated that dextrans may be of great value in targeting therapeutic agents to the liver (Mehvar et al. 1994). The dextran-glutathione conjugate was also reported for treating hepatopathies (Kaneo 1989). It increases the half-life of therapeutic agents in circulation (Tu et al. 2004, Mehvar 2000). The polymeric prodrug of valproic acid is synthesized to improve its physicochemical properties to reduce gastrointestinal irritation and hepatotoxicity.

Materials and Methods

Material and Instrument

Dextran (M_W 110, 000) and N, N′ carbonyl di-imidazole (CDI) were purchased from Fluka, Sigma-Aldrich, USA. Valproic acid (Sodium valproate) was supplied by Sun Pharma (Baroda, India). All other reagents and solvents were of analytical grade from Merck Chemical and Reagents, Mumbai. The methanol (HPLC grade) used in HPLC analysis was obtained from Merck Chemical and Reagents, Mumbai.

Fourier transform IR spectra were recorded on a JASCO FT-IR 5300 spectrophotometer. ^{1}H NMR spectra were recorded on a JEOL AL 300 FT-NMR spectrophotometer in DMSO-d6 using TMS as internal standard. *In vitro* hydrolysis study and degree of substitution of valproic acid –dextran conjugate was studied on water HPLC system (Rexdale, Canada) consisting of model 6000A pump, a 710B WISP auto injector and a 490 multiple- wavelength detector operated at ambient temperature. The column was stainless steel (10 cm X 4.6mm id) octadecyl-bonded silica (5μ particle, ODS-3, Whatman Inc. Clifton, N.J.) along with 5.0 cm guard column of the same material with particle size of 10 μm. The mobile phase consisted of methanol: water: phosphoric acid (50:50:0.5 v/v) and delivered at a flow rate of 1.0 ml/min at λ_{max} 212 nm.

2.2 Synthesis of valproic acid –dextran conjugate

Accurately weighed 2.047 mmol valproic acid (0.33 ml corresponding to 0.3 g) were dissolved in 3.0 ml dry DMSO and 2.047 mmol (0.332 g) of N, N′ carbonyl di-imidazole (CDI) was added slowly in portions. The temperature during reaction was maintained at 10 °C. The reaction mixture was then kept at room temperature for 30 minutes to get valproic acid acyl imidazole (VAI) *in situ.*

Accurately weighed 0.3 g of dextran (M_W 110,000) was dissolved in 5.0 ml dry DMSO added in reaction mixture of VAI (*in situ*) with stirring (Figure 1). The whole reaction mixture was set-aside in a desiccator for 72 h at room temperature with occasional shaking. After 72 h, absolute alcohol (20 ml) was poured in reaction mixture and precipitate was collected. The precipitation step was repeated three times to avoid possibly entrapped free drug in dextran conjugate. The concentrated aqueous solution was applied to gel

filtration column (Sephadex G-50 column, 50×2.5 cm) for the elution with 20 mM sodium phosphate buffer (pH 7.4). The flow rate of elution was 1.0 ml/min and effluent was monitored at 212 nm by JASCO model 7800 UV/Vis spectrophotometer. High molecular weight fraction of valproic acid – dextran conjugate was collected and lyophilized as conjugated product. The purity of conjugates was checked by thin layer chromatography (TLC) using silica gel G as stationary phase and MeOH: $CHCl_3$ as mobile phase. The spots were visualized by spraying 5% H_2SO_4 in ethanol followed by heating at 105 °C for 15 minutes.

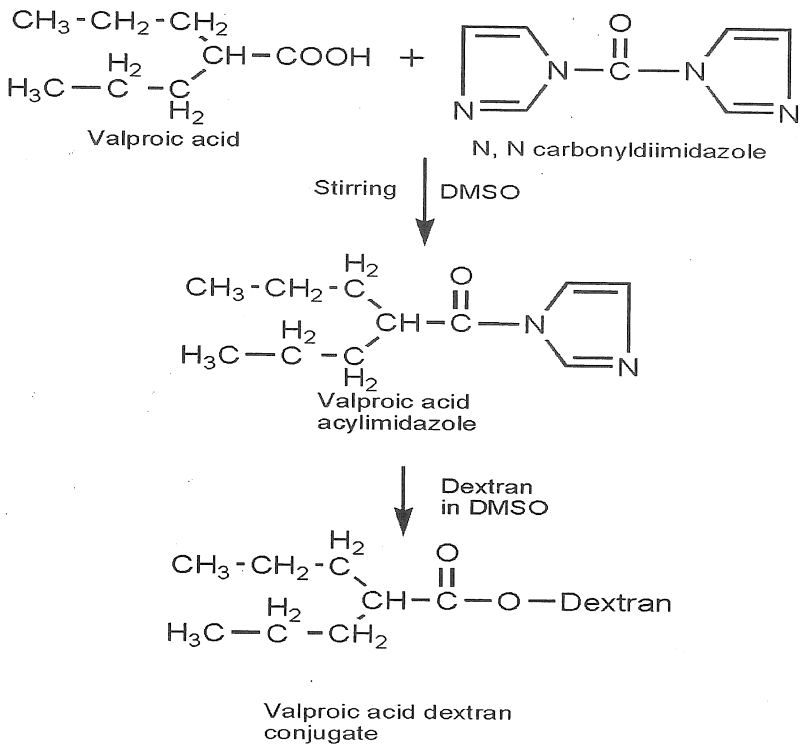

Figure 1. Schematic design for the synthesis of valproic acid- dextran conjugate.

Molecular weight determination

Molecular weight of synthesized dextran conjugate was determined by viscometery method. Ostwald viscometer was used in the determination of viscosity at constant temperature (20°C) and pressure. The change in the viscosity of solution is correlated with molecular weight of polymer as defined by Mark-Houwink Sakurada equation (Misra 1993).

$$[\eta] = kM^{\alpha}$$

Where k and α are constant having values of 7.24×10^{-4} (dl/g) and 0.52 respectively.

η = Intrinsic viscosity, M = Molecular weight (dl)

The values were determined by plotting the graph between η_{sp}/ C and C and molecular weight was determined by using above equation.

Degree of substitution (DS)

The degree of substitution (Bansal et al. 2001, Soane 1992) of valproic acid –dextran conjugate was determined by the above mentioned HPLC method. Accurately weighed dextran conjugate (20.0 mg) was dissolved in conical flask containing 0.1 M borate buffer pH 9.0 (20.0 ml). The solution was stirred at 37± 1.0 °C for 3 h and left aside for 24 h for complete hydrolysis. The hydrolyzed drug was extracted with chloroform and extracted chloroform layer was filter with 0.45 µm membrane filter and injected in HPLC system. The total amount of hydrolyzed drug was estimated through standard calibration curve.

Partition coefficient

The partition coefficient of valproic acid –dextran conjugate was studied by shake- flask method using 1-octanol/ water system under constant temperature 37±1.0 °C. It was determined by the following equation $P = Va/Vo\ [C_1/C_2 - 1]$, where, Va correspond to volume of aqueous phase, Vo to the volume of octanol phase, C_1 to the concentration of drug in aqueous phase before extraction and C_2 to the concentration of drug in aqueous phase after extraction.

In vitro hydrolysis studies

In vitro hydrolysis studies were performed for valproic acid – dextran conjugate. Valproic acid dextran conjugate was dissolved in three different buffer hydrochloric acid buffer (0.2M) pH 1.2, phosphate buffers (0.2M) with pH 7.4 and borate buffer (0.1M) with pH 9.0 and solution was stirred at 37±1.0°C. The samples were withdrawn at predetermined time intervals and concentration of valproic acid and conjugate were analyzed by HPLC. HPLC analysis was performed in the condition mentioned above. The rate of hydrolysis and half-life of the prepared conjugate were calculated by the following equation.

$$k = \frac{2.303}{t} \times \log \frac{a}{a-x} \quad \text{..... (1)}$$

$$t_{1/2} = \frac{0.693}{k} \quad \text{..... ....(2)}$$

Where k is the rate constant (h^{-1}), t is the time in hours, a is the initial concentration of conjugate, x is the amount of the conjugate hydrolyzed into free parent drug, a-x is the amount of parent drug remained in conjugate form and $t_{1/2}$ is the half life of conjugate.

Anticonvulsant activity

Anticonvulsant activity of valproic acid- dextran conjugate was determined by maximum electroshock seizure (MES) method (Krall et al. 1978, David et al. 2004). Wistar rats of either sex weighing about 150±20g were obtained from central animal house, I.M.S., B.H.U., Varanasi, India (Registration No. 542/02/ab/CPCSEA) and they were divided into three groups each group containing four animals. The food was withdrawn half day before the commencement of the experiment, while water was withdrawn immediately before the experiment. The valproic acid (200 mg) was dissolved in physiological saline (20.0 ml) giving valproic acid concentration of 10.0 mg/ml. The 10.0 mg equivalent amount of valproic acid –dextran conjugate was dissolved in physiological saline to give the same concentration of valproic acid. The dose 10.0 mg/kg was administered orally to the animals of first and second group with valproic acid and valproic acid –dextran conjugate respectively, whereas animals of third group (control group) was administered only physiological saline solution. The convulsion was produced by ear clip electrodes using alternating current 150 mA in a pulse of 60 Hz for 0.2 second. The standard drug (valproic acid) solution prepared in physiological saline was administered orally and after 30 minute an electrically induced seizure was applied. The animals were examined 0.5 and 4 h after the injections were made and produced convulsion was observed. The anticonvulsant activity was determined by measuring the change in duration of hind limb tonic extensor spasm.

Hepatotoxic activity

For hepatotoxic activity, Wistar rats were randomly distributed in three experimental groups, each group contains six animals. All the samples solution prepared in 1% gum acacia were administered orally for

seven consecutive days. The animals were fasted for 8 h prior to dosing and 4 h post dosing, on the seventh day all the animals were sacrificed by cervical dislocation and the blood was collected by cardiac puncture in centrifuge tube. The samples were centrifuged and separated serum was analyzed for SGPT (serum glutamate pyruvate transaminase), SGOT (serum glutamate oxaloacetate transaminase) and ALP (alkaline phosphatase) by using the diagnostic reagent kit. For the estimation of SGPT and SGOT level, all the prepared samples developed brown color corresponding to enzyme activity levels and was measured photometrically against distilled water at λmax 505 nm. In case of ALP, the orange red color developed in sample solutions was measured at λmax 510 nm (Reitman and Frankel 1997, Kind and Kings 1954).

Ulcerogenic activity

The ulcerogenic activity, Wistar rats were randomly distributed in three experimental groups, each group contains six animals. The suspension of standard drug (valproic acid) and it dextran conjugates were prepared in 1% gun acacia and administered orally. The animals were fasted for 8 h prior to dosing and 4 h past dosing were sacrificed and the abdomen was opened at the mid line, the stomach and the first 3 cm of duodenum were removed. The stomach was opened along the greater curvature and washed with saline water. The mucus was wiped off and observed for ulcer in the glandular portion of the stomach. The number of ulcer was noted and the severity of ulcer was scored by means of magnifier (10 X lens). Sub-acute gastrointestinal toxicity studies were done by method reported (Khan and Khan 2002).

Results and Discussion

Dextran conjugates can be synthesized either by direct conjugation or by spacer arm technique. Valproic acid contains a free carboxylic group and therefore can be conjugated directly with dextran through esterification. Valproic acid- dextran conjugate was synthesized by first activating the carboxylic group using CDI to obtain valproic acid acylimidazole (VAI), which was then condensed with dextran Mw110,000 *in situ* to get valproic acid –dextran conjugate (Figure 1). The progress of reaction was monitored by TLC, which was performed on silica gel (Merck No.5554). To remove unreacted valproic acid from the conjugate separation was conducted by gel filtration. Valproic acid – dextran conjugate and unconjugated valproic acid can be separated by gel filtration on a Sephadex G-50 column.

Valproic acid-dextran conjugate was identified by comparing UV, IR and NMR spectra of valproic acid, dextran and valproic acid–dextran respectively. No maximum absorption wavelength can be identified for dextran solution within the range between 220 nm to 600 nm. The maximum absorption wavelength (λ max) of valproic acid-dextran conjugate in water was 207.5 nm as compared with 212 nm of valproic acid. The change in maximum absorption wavelength might be due to formation of ester bond, indicating that valproic acid –dextran conjugate was formed. The FT-IR spectrum of valproic acid showed a strong absorption band at 1709 cm^{-1} attributable to the C=O group stretching where as valproic acid-dextran conjugate showed the shift of peak of (C=O str) 1709 cm^{-1} to 1720 cm^{-1} also indicating the formation of ester bond between valproic acid and dextran. Valproic acid –dextran conjugate also showed a large absorption band at 3600 cm^{-1}-3200 cm^{-1} indicating polymeric O-H stretching and formation of ester bond with dextran. ^1H NMR spectra of valproic acid and valproic acid-dextran conjugate were obtained: valproic acid ^1H NMR (300MHz, DMSO-d_6, δ TMS): 2.431 (m, 1H, CH), 1.502 (m, 4H, 2 CH_2), 1.298 (m, 4H, 2 CH_2), 0.883 (t, 6H 2 CH_3) and valproic acid- dextran conjugate ^1H NMR (300 MHz, DMSO d_6, δ TMS): 2.618 (s, 1H, CH), 1.58 (s, 4H, 2 CH_2), 1.249 (s, 4H, 2 CH_2) 0.921-0.928 (s, 6H, 2 CH_3), 3.723 (s, 2H, OCH_2 polymeric). The significant shifts were observed for all the protons of valproic acid dextran conjugate indicating the formation of ester linkage with polymer.

The degree of substitution (amount of valproic acid in 20 mg of dextran conjugate) of valproic acid in dextran conjugate was also determined by complete hydrolysis method and estimated to 16% by HPLC. The average molecular weight was calculated through viscosity method and

was found to be 108001 and 161652 for dextran and valproic acid dextran conjugate respectively.

The *in vitro* hydrolysis studies were performed on Waters HPLC system. The percent release of valproic acid from dextran conjugate on hydrolysis was determined by peak area of drug with the standard calibration graph of valproic acid. Valproic acid - dextran conjugate showed negligible hydrolysis of valproic acid in acidic medium. It showed a slow rate of hydrolysis at pH 7.4 and much faster hydrolysis at pH 9.0. The release data were fitted well with first order kinetics. The rate constant was calculated as 0.0354 h^{-1} at pH 7.4 phosphate buffer and 0.42 h^{-1} at pH 9.0 borate buffer solutions. The results indicated that *in vitro* release of valproic acid was devoid of acid catalysis and stable in acid condition. The half-life of the conjugate was 19.58 and 1.65 h at pH 7.4 and 9.0 respectively.

Anticonvulsant activity was determined by measuring change in duration of hind limb tonic extensor spasm. It was found that valproic acid –dextran conjugate reflected 93.75% protection in animals with the dose equivalent to valproic acid. The results of the experiment were shown in Table 1.

Table 1. Anticonvulsant activity of VPA and VD conjugates on Wistar rats.

Compound	Dose (mg/kg)	MES Screening				% Protection
		Time in hours				
		1h	2h	3h	4h	
		Number of rats protected/ Number of rats tested				
Control (vehicle)	0.5 ml/100g	0/4	0/4	0/4	0/4	0
VPA	10mg	4/4	4/4	4/4	4/4	100
VD	Equivalent to 10mg of VPA	3/4	4/4	4/4	4/4	93.75

*Number of animals in each group = 4

Valproic acid-dextran conjugate was evaluated for hepatotoxic activity by estimating serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP) level using biotechnological kits, manufactured by Span Diagnostic Pvt. Ltd. India. The SGPT, SGOT, ALP of valproic acid and valproic acid- dextran was found to be 98±7.43, 89±6.23, 21.23±3.3, and 72±3.23, 68±5.28, 12.27±2.38 respectively ($p<0.05$) indicating valproic acid –dextran conjugate is less hepatotoxic than parent drug valproic acid (Table 2).

Table 2. SGPT, SGOT and ALP levels in the serum with the treatment of VPA and VD conjugate Wistar rats[a].

Treatment	Dose (mg/kg)	SGPT (Units/Ml)	SGOT (Units/Ml)	ALP (Ka Units)
Control	0.5 ml/100g	35 ± 2.212	39 ± 2.543	8.23 ± 1.510
VPA	10mg	98 ± 7.432**	89 ± 6.230**	21.23 ± 3.305**
VD	Equivalent to 10mg of VPA	72 ± 3.234**	68 ± 5.286**	12.27 ± 2.382 (ns)

Value are expressed as mean± S.D., ** $P < 0.01$, ns $P > 0.05$ by one-way ANOVA followed by Dunnett test, [a]Number of animals in each group

Ulcerogenic activity of valproic acid and valproic acid-dextran conjugate valproic acid – dextran conjugate was determined by given method through counting the ulcer score, indicating

the least ulcerogenic index of valproic acid –dextran 2.96±0.84, whereas higher ulcerogenic index of valproic acid 31.4 ± 1.14 ($p<0.01$) (Table 3).

Table 3. Ulcerogenic index of chronic administration of VPA and VD conjugates on the gastric mucosa of rats[a].

Treatment	Dose (mg/kg)	No. of ulcers	Ulcer score	Percent incidence	Ulcerogenic index
Control	0.5 ml	0	0	0	0
VPA	10mg	7.4 ± 1.140**	4	100.00	31.4
VD	Equivalent to 10mg of VPA	0.8 ± 0.837 (ns)	0	10.81	2.96

Value are expressed as mean± S.D., ** $P < 0.01$, ns $P > 0.05$ by one-way ANOVA followed by Dunnett test, [a]Number of animals in each group = 6

The results proved that conjugation of valproic acid with dextran showed parallel anticonvulsant activity with dramatically reduced hepatotoxicity and ulcerogenicity due to its slow release from conjugate and sustained level of action.

Conclusion

The dextran prodrug approach is still in building phase. The results reflect that dextran may be exploited as potential macromolecule candidate for the delivery of drugs. *In vivo* kinetics studies are also in progress in our laboratory. The results of anticonvulsant activity study suggest that dextran can successfully be employed as promoity/carrier for valproic acid to reduce its hepatic toxicity and ulcerogenicity.

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