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Synthesis, characterization and biological evaluation of some glutathione inducing amino acid conjugates of valproic acid with reduced hepatotoxicity

Saurabh K Sinha, B Praveen, Prabhat K Shrivastava, Sushant K Shrivastava*

Pharmaceutical Chemistry Research Laboratory, Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi, U.P., India-221 005 and Research and Development Centre, UP, India

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ABSTRACT

Objective: To synthesis and evaluate three glutathione inducing amino acid conjugates of valproic acid (VPA) and compare with the same VPA to diminish its hepatotoxicity. **Methods:** Purified synthesised prodrugs were subjected to thin layer chromatography, melting point, solubility studies and characterised by UV, FTIR, 1H, 13C NMR and elemental analysis. The synthesised prodrugs were subjected to *in vitro* hydrolysis in various buffer solution (pH 1.2, 7.4, 9.0) and *in vivo* anticonvulsant, hepatotoxic activity studies. **Result:** Three synthesized conjugates were assumed to be in agreement with the anticipated structures. All the three conjugates were also able to prevent seizures in experimental rats with a comparable activity as the parent drug, VPA. Among the three conjugates, the glycine conjugate showed better anticonvulsant activity compared to glutamic acid and cystine conjugates. **Conclusions:** Significant reduction in hepatotoxicity and comparable anticonvulsant activities were obtained in all synthesised prodrugs as compared to VPA.

1. Introduction

Valproic acid (VPA; 2-propylpentanoic acid or dipropylacetic acid) is used to treat the petit mal and generalized seizures. Its principal mechanism of action is believed to be the inhibition of GABA transaminase and inflate the concentration of GABA in brain[1,2]. However, hepatotoxicity, which can cause liver failure, is the major threat associated with the use of this drug[3,4]. Hepatotoxicity caused by VPA is mainly due to its metabolites 4-ene VPA and (E)-2,4-diene VPA[5]. These metabolites are normally detoxified by glutathione and N-acetylcysteine conjugation reaction but due to more generation of metabolites, the level of glutathione goes abnormally low and hepatotoxicity is then produced[6]. These metabolites accumulate and form covalent binding with liver macromolecules and cause oxidative stress[7,8] with hepatic cell damage[9]. Patients receiving chronic treatment with VPA in the form of single or polytherapy are at high risk of hepatic enzymes elevation[10]. In pervious study we have synthesized dextran and aminoacid conjugate of lamotrigine to minimize its hepatotoxicity[11] and dextran conjugate of VPA for the same purpose[12]. The number of amino acid conjugates were reported for reducing toxicity and improving physicochemical properties[13,14]. Amino acids are normal dietary constituents and non- toxic compared to other carrier. The highly water soluble amino acids have ability to increase the water solubility of low soluble or insoluble drugs, and therefore, enhances the drug bioavailability and improves the pharmacokinetics. The glutathione tripeptide macromolecule is produced by its precursor amino acids glutamic acid, cysteine, and glycine. It exists in its reduced form (GSH), it is enzymatically or non-enzymatically oxidised to a disulfide dimer (GSSG) and used to detoxify hydrogen peroxide and lipid hydroperoxides. Therefore, the elevation of GSSG in tissues has been used as a marker for oxidative stress[15]. This current research work is designed to minimise the hepatotoxicity of VPA to a great extend by its conjugation with glutathione inducing amino acids. The synthesised VPA-amino acid conjugates showed similar activity as their parent drug molecule with significantly

*Corresponding author: Dr. Sushant K. Shrivastava
Tel: 0542-2307049, Fax - 0542-2368428
E-mail address- skshrivastava.phe@itbhu.ac.in

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reduced hepatic cell damage.

2. Materials and methods

VPA (Sodium valproate) standard drug was obtained as a gift sample from Sun Pharma (Baroda, India). Amino acids [glycine (Gy), glutamic acid (Gu), cysteine (Cy)] were purchased from Sigma-Aldrich Chemical Ltd, USA. Methanol, ether, *n*-propanol, methylene chloride, chloroform, acetone, and hexane were purchased from Fisher Scientific, USA and thionyl chloride was purchased from Merck, India. Melting points were determined using a BI 9300 Bumstead/electrothermal Stuart (SMPIO) melting point apparatus. Thin layer chromatography was performed on TLC silica gel 60 F254 aluminium sheets (Merck, India). The MeOH: water (2:8) was used as mobile phase. UV spectra were obtained using a JASCO (Model 7800) UV-VIS spectrophotometer. FTIR spectra were recorded on a Shimadzu FT-IR 8400S spectrophotometer at the scanning range of 400-4000 cm^{-1}. 1H and 13C NMR spectra were recorded on a JEOL AL 300 FT-NMR spectrophotometer in DMSO-d6. Elemental analysis was carried out by EXETER CE-440 elemental analyzer.

2.1. Synthesis of VPA -amino acid ester conjugates

2.1.1. Esterification of amino acids

The methyl ester hydrochloride of glycine, glutamic acid and cysteine were prepared by general method[17]. The resulting solid product obtained by the given method was collected and dried under high vacuum to yield 85%-99% crude methyl ester hydrochloride of amino acids. The crude material was recrystallized from hot methanol by slow addition of ether followed by cooling at 0 ℃. The crystals were collected, washed twice with ether: methanol (5: 1) solution, followed by pure ether and dried under high vacuum. Yields; 75-85%, R_f ; 0.44 - 0.50 in water : *n*-propanol (1:1).

2.1.2. Synthesis of VPA amide conjugates with amino acid methyl ester

VPA (10 mmoL) was dissolved in distilled thionyl chloride solution (40 mmoL) and mixture was refluxed with stirring at 70 ℃ for 2 h. The reaction mixture was cooled and dissolved in 30 mL of dry methylene chloride. This solution was slowly added to amino acid methyl ester (10 mmol) in 5 % $NaHCO_3$ solution. The mixture was stirred at 4 ℃ for 12-16 h. The organic layer was separated and washed three times with distilled water and 10 % aqueous citric acid. The organic layer was dried over $MgSO_4$ and solvent was evaporated under reduced pressure. The obtained product was purified using methylene chloride: acetone (1:1) and recrystallized using chloroform: hexane mixture (1:3)[16].

2.2. In-vitro Hydrolysis

In-vitro hydrolysis study was performed for VPA amino acid ester conjugates in different buffer solution. The 100mg dried samples of VPA amino acid ester conjugates were mixed separately with 5ml of 0.2M hydrochloric acid buffer pH 1.2, 0.2M phosphate buffers solution pH 7.4 and 0.1M borate buffer pH 9.0. These sample solutions were packed in cellophane membrane dialysis bag. The different dialysis bag tagged in to the 50 mL of same buffer solution maintaining the temperature at (37±1.0) ℃ with continuous stirring. The 1 ml sample solutions were withdrawn at predetermined time interval from different buffer solution and replaced with same amount of buffer solution. The concentration of VPA release from conjugates was analyzed by UV spectrophotometer. The rate of hydrolysis and half-life of the prepared conjugate were calculated by the following equation.

Where k is the rate constant, t is the time in h, 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.

2.3. Biological evaluation

Wistar rats of either sex (weighing120-150 g) obtained from Central Animal House, Institute of Medical Sciences, Banaras Hindu University, Varanasi (Registration No. 542/02/ab/CPCSEA). They had *ad-libitum* access to water and semi synthetic balanced diet, with occasional supply of green vegetables (salad leaves). Rats were caged six per Perspex experimental cages at room temperature (22 - 25 ℃). Twelve hours of light and dark cycles was strictly followed in a fully ventilated room. Animals were divided into 5 groups with five animals in each group i.e control-I and experimental II, III, IV and V. The group-I received vehicle (1 % gum acacia) whereas, the group-II received VPA drug suspension (conc.200mg/kg, *p.o.*) and group-III received VGyM conjugate, group-IV received VGuM conjugate and group-V received VCyM conjugate (conc. 200 mg/kg, *p.o.* drug equivalent) for seven days. The food was withdrawn half day before to commencement of the experiment, while water was withdrawn immediately before the experiment.

2.3.1. Anticonvulsant activity

Anticonvulsant activity of VPA amino acid ester conjugates was determined by maximum electroshock seizure (MES) method. 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 and conjugates solution were administered interaperitoneal (*i.p.*) and after 30 minute an electrically induced seizure was applied. The rats were examined 0.5 and 4h 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[17].

2.3.2. Hepatotoxicity

On the seventh day all the rats were sacrificed by cervical dislocation and the blood was collected by cardiac puncture in centrifuge tube. Liver function tests were carried out immediately after the separation of serum using Span diagnostic reagent kits for ALP, ALT, AST, bilirubin, and direct bilirubin estimation[18,19]. Animals were sacrificed and the liver sections were subjected to histopathological observation at the Institute of Medical Sciences, BHU, Varanasi. Coloured microscopical images of the liver sections were taken on a Nikon ECLIPSE 50i optical microscope (Nikon Corporation, Japan) with the resolution of 400X attached to a camera.

3. Results

Prior to the conjugation reaction, the free acid group of amino acids was protected by esterification to give the corresponding amino acid methyl ester. The acyl chlorides of VPA and free amino group of esterified glycine, glutamic acid and cysteine were conjugated and formed VPA amino acid methyl ester conjugates VGyM, VGuM and VCyM respectively (Scheme I) physico-chemical parameters of thease conjugates were shown in Table 1. It was observed in FTIR spectra of amino acid methyl esters that there was a strong C=O stretching at 1740 cm^{-1} and in 13C NMR spectra showed δ 170-172 ppm for CO indicating the formation of ester linkage. The amino acid methyl ester-VPA conjugates spectra showed an amide linkage due to the presence of C=O stretching in the range of 1610-1690 cm^{-1} and C-N stretching in 1000-1125 cm^{-1}. The 1H NMR spectra of amino acid methyl ester-VPA conjugates has shown a signal of amino group (1H, NH) at δ 5.9-6.7 ppm and absence of proton signal of COOH group at δ 10.57-11.41 ppm and 13C NMR spectra showed δ 175-178 ppm for CONH indicating that amino acid methyl esters were engaged as an amide linkage with the VPA. All the above spectral data (Table 2) shows that the synthesized conjugates were assumed to be in agreement with the anticipated structures. The amino acid methyl ester conjugates of VPA were estimated for the percentage of elements like C, H, and N. The obtained and calculated percentage of C, H and N elements in the VGyM, VGuM, and VCyM were found to be (60.40, 56.20, 54.14), (8.45, 7.12, 6.64), (6.05, 4.15, 5.00) and (61.39, 59.80, 55.17), (9.76, 8.97, 8.81), (6.51, 4.65, 5.36) respectively. The obtained and calculated percentage of 'S' element in VCyM was found to be 12.0 and 12.27. The synthesized amino acid conjugates of

Table 1
Physico-chemical parameters of synthesized prodrugs of VPA.

Compound	Molecular formula	Molecular weight	M.p / ℃	Yield %	Color	R_f [a] value	λ_{max}/nm
VGyM	$C_{11}H_{21}NO_3$	215.29	71-73	60	off-white	0.35	215.0
VGuM	$C_{15}H_{27}NO_5$	301.38	65-67	55	light brown	0.42	225.5
VCyM	$C_{12}H_{23}NO_3S$	261.38	76-78	70	yellow	0.48	217.0

a- TLC was developed in the solvent system of MeOH: water (2:8).

Table 2
Characterization of synthesized VPA-amino acid ester and prodrugs of VPA.

Compound	Spectral data
GyM	IR, /cm-1: KBr); 1743.40 (C=O str), 3200. (N-H str), 1071.23 (C-N str).1H NMR (DMSO-d6), δ 2.1(2H,t, NH_2), 3.9 (3H, s, CH_3), 3.1(2H, t, CH_2), 13C NMR (DMSO-d6), δ 170 (CO), 52(OCH_3), 41 (CH_2)
GuM	IR, /cm-1: KBr); 1722.59 (C=O str), 3009.20 (N-H str), 1574.06 (N-H bending), 1134.25 (C-N str).1H NMR (DMSO-d6), δ 2.1(2H,t, NH_2),2.2-2.3(4H,m, CH_2-CH_2),3.5(1H,m,CH), 3.9 (3H, s, CH_3), 3.1(2H, t, CH_2), 11.2 (1H, s, OH), 13C NMR (DMSO-d6), δ 178(COOH),172 (CO), 59(CH), 52(OCH_3),30,31(CH_2)
CyM	IR, /cm-1: KBr); 1733.40 (C=O str), 3150.0 (N-H str), 1505.61 (N-H bending), 1054.53 (C-N str), 2580.00 (S-H str).1H NMR (DMSO-d6), δ 1.7(1H, s, SH), 3.0-3.3 (2H, m, CH_2), 2.1(2H,t, NH_2),3.7 (1H, s, CH), 3.9 (3H, s,CH_3), 13C NMR (DMSO-d6), δ 172 (CO), 57(CH), 52(OCH_3),31(CH_2)
VGyM	IR, /cm-1: KBr); 3307.9 (N-H str); 2935.4, 2930.4 (C-H str); 1770.7 (ester C=O str); 1609.7 (amide C=O str); 1105 (C-N str) 1H NMR (DMSO-d6), δ 0.95 (6H, t, $-CH_3$, J = 7.2 Hz); 1.255-1.589 (8H, m, $-CH_2$, J = 6.9 Hz); 2.189 (1H, m, =CH); 3.05 (1H, s, $-OCH_3$); 4.09 (2H, d, $-CH_2N$, J = 5.1 Hz); 6.097 (1H, t, -NH), 13C NMR (DMSO-d6), δ 175(CONH), 170(COO), 52(OCH_3), 45(=CH) 43($NHCH_2$), 35,21(CH_2-CH2), 15(CH_3)
VGuM	IR, /cm-1: KBr); 3288.93 (N-H str); 2947.5 (C-H str); 1738.02 (ester C=O str); 1641.57 (amide C=O str); 1537.4 (N-H bending); 1209.48 (C-O str); 1124.6 (C-N str), 1H NMR (DMSO-d6), δ 0.892 (6H, t, $-CH_3$); 1.257-1.572 (8H, m, $-CH_2$, J = 3 Hz); 1.98 (2H, m, $-CH_2$); 2.125 (2H, m, $-CH_2CO$); 2.439 (1H, m, =CH); 3.762 (6H, s, $-OCH_3$); 4.146 (1H, m, =CHN, J = 6.9 Hz); 6.241 (1H, s, -NH), 13C NMR (DMSO-d6), δ 178(COOH), 175(CONH), 172(COO), 53(NHCH), 52(OCH_3), 45(=CH) 35,21(CH_2-CH_2), 15(CH_3)
VCyM	IR, /cm-1: KBr); 3300 (N-H str); 2958.6, 2873.6 (C-H str); 2560 (S-H str); 1733.9 (ester C=O str); 1684.3 (amide C=O str); 1541.8 (N-H bending); 1241.6 (C-O str); 1003.5 (C-N str), 1H NMR (DMSO-d6), δ 0.914 (6H, t, -CH3, J = 6.6 Hz); 1.399-1.594 (8H, m, $-CH_2$); 2.162 (1H, br, -SH); 2.372 (1H, m, =CH); 3.204 (2H, d, $-CH_2S$); 3.772 (3H, s, $-OCH_3$); 4.873 (1H, t, =CHN); 6.386 (1H, s, -NH), 13C NMR (DMSO-d6), δ 175(CONH), 172(COO), 56(NHCH), 52(OCH_3), 45(=CH) 35,21(CH_2-CH_2), 29(CH_2SH), 15(CH_3)

Table 3
In vitro hydrolysis study of VPA- amino acid ester conjugates in different buffer solution.

Prodrug code	0.2 M Hydrochloric acid buffer pH 1.2		0.2 M phosphate buffers pH 7.4		0.1 M borate buffer pH 9.0	
	k (h^{-1})	$t_{1/2}$ (h)	k (h^{-1})	$t_{1/2}$ (h)	k (min^{-1})	$t_{1/2}$ (min)
VGyM	-	-	0.1070	6.47	0.01100	63.00
VGuM	-	-	0.0820	8.45	0.00824	84.10
VCyM	-	-	0.0954	7.26	0.00970	71.44

VPA subjected for in-vitro hydrolysis studies in different buffer solutions pH medium (pH 1.2, 7.4, 9.0) at 37 ± 1 ℃. The synthesized conjugates of VPA were showed negligible hydrolysis in acidic medium (pH 1.2) for 6 h. The half-life (t1/2 'h') of the synthesized conjugates VGyM, VGuM and VCyM in the pH 7.4 buffer was found to be 6.47, 8.45 and 7.26 h. The conjugates were showed a slow rate of hydrolysis following the first order kinetics in pH 7.4 buffer solution. The half-life (t1/2 'min') of the synthesized conjugates VGyM, VGuM and VCyM in pH 9.0 buffer was found to be 63.0, 84.1 and 71.44 min respectively. The conjugates were showed a faster rate of hydrolysis following the first order kinetics in pH 9.0. The results of in-vitro hydrolysis studies were shown in Table III. The VPA-amino acid conjugates were analysed for anticonvulsant activity using the electroshock method and they were found to be active as anticonvulsants. VPA-treated rats were protected from tonic seizures (% protection = 100). Among the three conjugates, the glycine conjugate (% protection = 95) showed better anticonvulsant activity compared to glutamic acid (% protection = 90) and cystine conjugates (% protection = 90). Better anticonvulsant profile of the glycine conjugate is due to its antagonistic action of glycine itself on the metabotropic glutamate receptor[23] (Table IV). Hepatotoxicity of VPA and its amino acid conjugates was evaluated by estimating biochemical parameters (ALP, AST, ALT, bilirubin, and direct bilirubin) of rat serum (Table 4).

Table 4
Anticonvulsant activity of VPA and its amino acid ester conjugates in Wistar rats.

Compound[a]	Rats protected/rats tested				Protection/%
	Time/h				
	1	2	3	4	
Control	0/5	0/5	0/5	0/5	0
VPA	5/5	5/5	5/5	5/5	100
VGyM	5/5	5/5	5/5	4/5	95
VGuM	5/5	5/5	4/5	4/5	90
VCyM	5/5	5/5	4/5	4/5	90

a- 200 mg/kg was used.

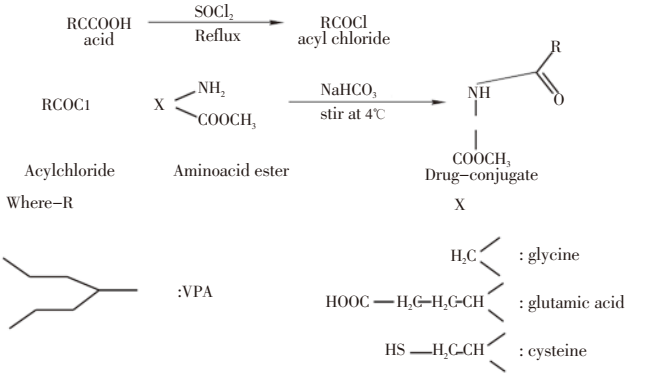

Figure 1. Synthesis of VPA amino acid prodrug.

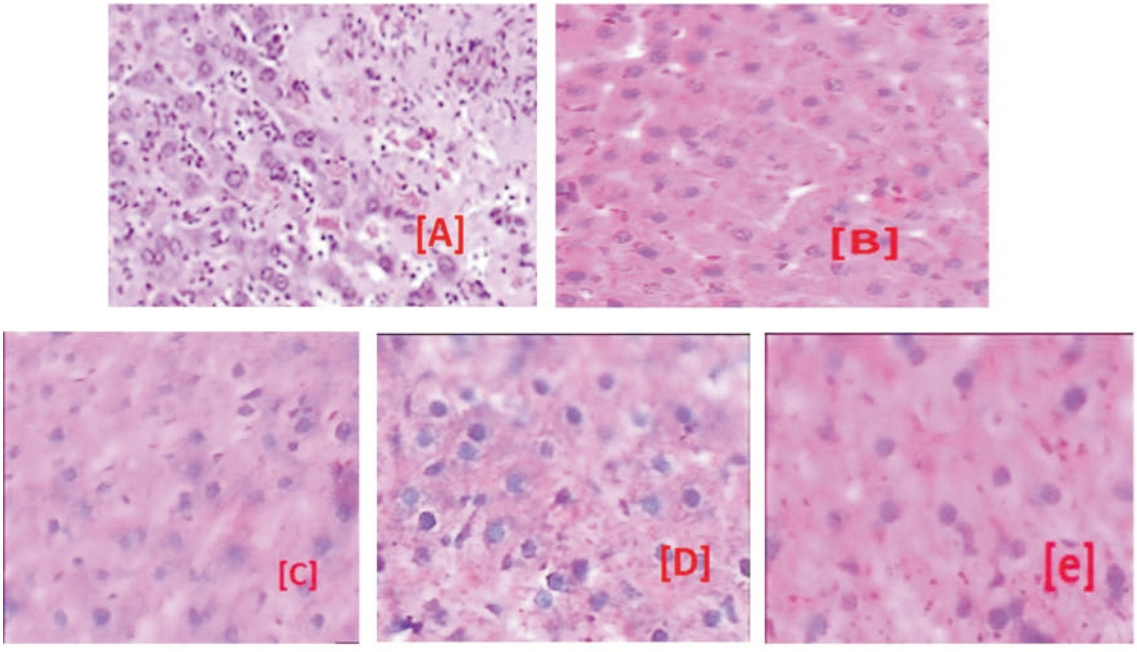

Figure 2. Histopathological examination of liver section in normal and experimental rats at the magnification of 400 ×.
Normal rat liver parenchyma in control group (a); rat liver treated with VPA (b) (arrows show hepatocyte necrosis); rat liver treated with: VGyM conjugate (c), VGuM conjugate (d), and VCyM conjugate (e).

4. Discussion

All the three conjugates were also able to prevent seizures in experimental rats with a comparable activity as the parent drug, VPA. Among the three conjugates, the glycine conjugate showed better anticonvulsant activity compared to glutamic acid and cystine conjugates. Better anticonvulsant profile of the glycine conjugate is due to its antagonistic action of glycine itself on the metabotropic glutamate receptor[20]. The level of ALP, AST, ALT, bilirubin, and direct bilirubin in the VPA-conjugates-treated group did not increase significantly, whereas in the VPA-treated group there was significant increase in the level of the above parameters. On basis of these results it can be concluded that VPA-conjugates show lower hepatotoxicity, and due to

significant increase in the level of ALP, AST, ALT, bilirubin, and direct bilirubin, hepatotoxicity is evident in the VPA-treated group. Hepatotoxicity of VPA is attributed to its 4-ene VPA and (E)-2,4-diene VPA metabolites which are detoxified by glutathione. Higher concentration of these metabolites leads to depletion of glutathione because of this reactive oxygen species increases and damages hepatic cells and causes hepatotoxicity, which is indicated by the elevated level of liver and serum enzymes. Amino acids (glycine, glutamic acid and cysteine,) are the precursors of glutathione and generate more glutathione as required for the detoxification of these metabolites. In the present Hepatotoxic studies of three VPA-amino acid ester conjugates, the cystine conjugate was more effective than the other two amino acid conjugates due to its transformation into N-acetyl cystine. It is a well established fact that N-acetyl cystine prevents liver damage by inhibiting apoptotic pathway and oxidative stress[21]. Histopathological examination of rat liver was also performed in order to study pathological changes in liver tissue. The histopathology report also confirmed that VPA-amino acid ester conjugates have lower hepatotoxicity than the parent drug, VPA. Anticonvulsant activity of the VCyM conjugate comparable to that of VPA and the significantly lower damage to liver, shown by biochemical results, prove this conjugate to be a most promising alternative for the treatment of convulsions at clinical level.

Conflict of interest statement

We declare that we have no conflict of interest.

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