Synthesis and *In-vivo* Anti-convulsant Activity of Prodrug of 3-Alkylglutamic Acid in Albino Rats

Kamta P. Namdeo*[1], Sushant K. Shrivastava[2] and Ramesh Chandra[3]

SLT Institute of Pharmaceutical Sciences, G. G. University, Bilaspur (C.G.) [1]

Department of Pharmacetics, I.T., B.H.U. , Varanasi(U.P.) [2]

Dr. B.R.Ambedkar Institute of Biomedical Sciences and Research, Delhi University, Delhi[3]

ABSTRACT

Submitted: 08/09/2009 Revised: 17/08/2011 Accepted: 13/02/2012

3-Alkyl glutamic acid decrease excitation and glutamate in brain. 3-Alkyl glutamic acid analogues displayed glutamic acid decarboxylase activation activity and anticonvulsant activity. We herein are reporting the result of our studies on the synthesis and evaluation of anticonvulsant activity for prodrug of 3-Alkyl Glutamic Acid. Prodrugs of Glutamic Acid were synthesized by taking the Dihydropyridine compound as carrier molecules and were subjected to preliminary screening for anticonvulsant activity in albino rats. Anticonvulsant activity of all the compounds were tested by chemo- convulsion method and all the compounds were found to be active. All the compounds were also studied for oxidation in various biological fluids and were found to be stable. Oxidized form of the conjugate inside the brain act as prodrug, which on hydrolysis yields parent drug in sustained release pattern. The significance of the work is to treat neurological disorder implicated by Glutamate.

Keywords: Prodrug, 3-Alkyl Glutamic acid, Anticonvulsant, Phenytoin sodium, Dihydropyridine.

INTRODUCTION

The epilepsies or convulsions are a group of disorders characterized by chronic, recurrent, paroxymal changes in neurologic function caused by abnormalities in electrical activity of the brain. They are one of the common neurologic disorders estimated to affect 0.5-2% of the population and can occur at any age. The epileptic attack is initiated by an abnormal focus of the electric discharge, originated either in grey matter or other part of the brain.

This disease is very common throughout the world and pose a great problem to modern society due to its crippling effect, resulting at times in complete invalidity. The problem is further heightened due to lack of any specific treatment. In spite of the discovery of anticonvulsant and the emergence of several such newer agents, the search for drugs producing better anticonvulsant effects is continued because these agents have many known side effects as well as majority of these drugs interfere with their own metabolism and of many other drugs emphasizing the need for plasma level monitoring and adjustment of dosage form time to time. Several Indian laboratories and educational institutions are actively engaged in the anticonvulsant drug research and have shown significant results [1].

***Address for Correspondence:**

Kamta P. Namdeo, SLT Institute of Pharmaceutical Sciences, Guru Ghasidas University, Bilaspur (CG)-495009

E-mail: knamdeo@yahoo.com

Glutamate is a major excitatory neurotransmitter in human brain. Glutamic acid decrease excitation as well as glutamate in brain. 3-alkyl glutamic acid analogues displayed glutamic acid decarboxylase activation activity and anticonvulsant activity. The literature survey revealed the anticonvulsant activity of the 3-Alkyl Glutamic Acid analogues. However, no work has so far been reported on the anticonvulsant activity on the prodrug of 3-Alkyl Glutamic Acid in the literature. So it was thought worthwhile to investigate anticonvulsant activity of the prodrug of 3-Alkyl Glutamic Acid analogues [2,3].

The concept of developing methods for site specific delivery of biological active agents is highly desirable to improve efficacy and decrease toxicity. The delivery of drugs to the brain is often seriously limited by transport and metabolism factors and more specifically by the functional barrier of the endothelial brain capillary wall called the blood brain barrier (BBB). It is generally accepted that the ability of the molecule to pass the blood brain barrier (BBB) is a function of its partition coefficient between lipid and water. The approach of derivatizing the compounds and forming a prodrug that exhibits improved physicochemical properties for the transport through the blood brain barrier (BBB) and improve delivery of the drug to the brain. This Prodrugs approach is based on an interconvertible Dihydropyridine · · pyridinium salt carrier, similar to the endogenous NADH · · NAD coenzyme system is known as redox drug delivery system and also a chemical drug delivery system (CDS)[4].

MATERIAL AND METHOD

All the Chemicals were procured from Qualigen Mumbai, and CDH New Delhi. They were of an analytical grade.

Results were subjected to statistical analysis by ANOVA and results were expressed as mean SEM.

The procedure for the synthesis of Nicotinyl- 3-Alkyl Glutamic Acid analogues was followed as per the method described by Bodor et al.[4,5] (1983) and the synthetic procedure involved the five steps as stated below.

Step-I: Esterification of Glutamic Acid with various alcohols

In a small round bottom flask, 1.0M alcohol (EtOH, Isopropanol, BuOH and Benzyl alcohol**)** was taken, $SOCl_2$ (2 ml, 0.01M) was added to the alcohol slowly with cooling and Glutamic acid (1.46 g, 0.01M) was added to this solution. The solution was refluxed for four hours and solvent was evaporated to get the crude ester HCl, which was triturated with ether at 0.0°C until the excess of dimethyl sulphate was removed. The resulting product was collected and dried under high vacuum to yield crude ester HCl. The crude material was recrystallized from 25 ml of hot methanol by slow addition of 100 ml of ether followed by cooling to 0.0°C. The crystals were collected, washed twice with Ether: Methanol solution (5:1), once with ether and dried under high vacuum[6]. The yield of the diethyl, di-isopropyl, dibutyl and dibenzyl esters of glutamic acid were 90%, 80%, 85% and 90% and the melting points were 160°C, 170°C, 160°C and 120°C respectively.

I.R Data- 1625.7 (C=O), 127.86 (C-N), 1311.36 (C-O), 3326.61 (N-H), 1417.8 (C-H);

^{1}HNMR (500MHZ, CDCl3, TMS) 1.30 (m, 3H, C-CH_3), 2.0 (s, 2H, NH_2), 2.47 (s,1H,-C-CH_2), 4.12 (m, 2H, O-CH_2). 4.2 (m, 2H, O-CH_2), 8.04 (m, 1H, CH).

Step-II: Amidation with carrier (Nicotinic acid) or coupling of esterified Glutamic Acid with carrier molecule:

In a small round bottom flask the ester of glutamic acid (0.01M) and nicotinic acid (0.01M) were taken in 20 ml dry pyridine after cooling to 0-5 °C, a solution of 2.06 g dicyclohexylcarboxa-imide (0.01M) was added and mixture was stirred at 20-25 °C for 24 hrs. After filtering off the precipitated dicyclohexyl urea, the solvent was removed by distillation under reduced pressure, the oily residue was mixed with 25 ml methylene chloride to precipitate more dicyclohexyl urea. The resulting residue was crystallized from water at 0°C. The product was isolated by filtration, dried and recrystallized from 2- propanol. The yield of the N-substituted nicotinyl diethyl, di-isopropyl, dibutyl and dibenzyl esters of glutamic acid were 95%, 95%, 76% and 63% and the melting points were 195 °C, 175 °C, 215 °C and 223 °C respectively.

I.R Data- 1625.7 (C=O), 1270.86 (C-N), 1311.36 (C-O), 3326.61 (N-H), 1417.8 (C-H).

^{1}HNMR (500MHZ, $CDCl_3$,TMS) 0.23 (m, 4H, CH_4), 1.30 (m, 3H, C-CH_3), 2.2 (m, 2H,-C-CH_2), 2.82 (m, 2H,-C-CH_2), 4.12 (m, 2H, O-CH_2). 4.2 (m, 2H, O-CH_2), 0.86 (m, 3H, C-CH_3), 5.0 (s, H), 5.7 (s, H).

Step-III : Alkylation of Glutamic Acid at 3-position by alkyl halide (Methyl iodide and Propyl iodide):

Firstly sodium ethoxide was prepared from 0.35 g of clean sodium and 4.40 ml of super dry ethanol in two litre three necked flask. When the sodium ethoxide solution, which is vigorously stirred, was cooled about 50°C, 0.01M esters of N-nicotinyl glutamic acid and alkyl halide were added, reaction occured almost immediately; much more heat was evolved ,cooled the flask directing under stream of cold water over it and then refluxed the reaction mixture on a water bath for two hours[7]. Ethanol was distilled off. The yields of the N- nicotinyl 3-methyl- diethyl, di-isopropyl, dibutyl and dibenzyl esters of glutamic acid were 60%, 60%, 75% and 73%, N- nicotinyl 3-propyl- diethyl, di-isopropyl, dibutyl and dibenzyl esters of glutamic acid were 80%, 60%, 95% and 80%.. The melting points of esters of N-nicotinyl 3-methyl- derivatives were 212 °C, 205 °C, 222 °C and 215 °C, esters of N- nicotinyl 3-propyl- derivatives were 200 °C, 220 °C, 230 °C and 219 °C respectively.

I.R Data-1625.7 (C=O), 1270.86 (C-N), 1311.36 (C-O), 3326.61(N-H), 1417.8 (C-H).

^{1}HNMR (500MHZ, $CDCl_3$, TMS) 0.23 (m, 4H, CH_4), 1.30 (m, 3H, C-CH_3), 2.2 (m, 2H, -C-CH_2), 2.82 (m, 2H, -C-CH_2), 4.12 (m, 2H, O-CH_2). 4.2 (m, 2H, O-CH_2), 0.86 (m, 3H, C-CH_3), 5.0(s, H), 5.7(s, H)

Step-IV: Quaternization of carrier using methyl iodide:

To a solution of 1.26 g of (0.05M) product obtained from step-III in 10 ml of acetone was added 1.41 g (0.01m) methyl iodide and was refluxed for six hours with stirring. The acetone was removed and residue was recrystallized from methanol. The yield of the esters of N- quaternized nicotinyl 3-methyl-glutamic acid were 88%, 50%, 60% and 63%, esters of N- quaternized nicotinyl 3-propyl- glutamic acid were 88%, 50%, 55% and 77%. The melting points of N-quaternized nicotinyl 3-methyl- glutamic acid were 218 °C, 222 °C, 228 °C and 240 °C, & esters of N- quaternized

nicotinyl 3-propyl- glutamic acid were 230°C, 208°C, 235°C and 227°C respectively.

Step-V: Reduction with sodium dithionite to get 1, 4-dihydropyridine derivatives:

To an ice cold solution of 1g (2.5 mmol) of compounds obtained from step IV in a 200 ml of deaerated water and 200 ml ethyl ether was added, 1.26 g (15 mmol) of sodium bicarbonate. Nitrogen was bubbled into the mixture while stirring. Stirring was continued for one hour and mixture was extracted twice with 50 ml of water, dried with anhydrous sodium sulphate and evaporated to dryness. The yeild of the compounds (X21-X28) were 88%, 50%, 93% 81%, 95%, 54% 88% and 77% respectively. The melting points of the synthesized compounds (X21-X28) are presented in table1.

I.R Data-1625.7 (C=O), 1270.86 (C-N), 1311.36 (C-O), 3326.61(N-H), 1435.74 (C-H), 1574 (C=C), 1435 (alkyl), 1448 (Substitution on 3-C), 1536 (Iso group)

^{1}HNMR (500MHZ, $CDCl_3$, TMS) 2.2 (m, 3H, N-CH_3), 1.33 (m, 2H, -C-CH_2), 1.95 (s, H, - CH), 1.9 (m, 2H, -CH_2). 1.06 (m, 3H, -CH_3 on 3-posision), 1.125 (m, 3H, C-CH_3), 3.5 (s, H, 3C), 1.6(m, 21 H), 7.25(s, H, C2), 3.46(m, 2H, C4), 4.05(s, H, C5), 4.08(s, H, C6).

CHEMISTRY:

The synthetic reaction followed the given path

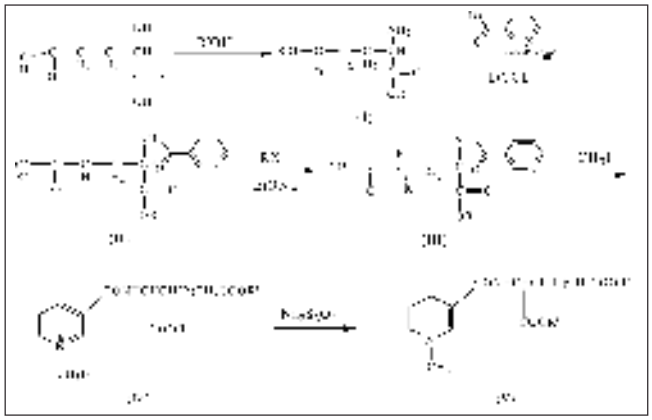
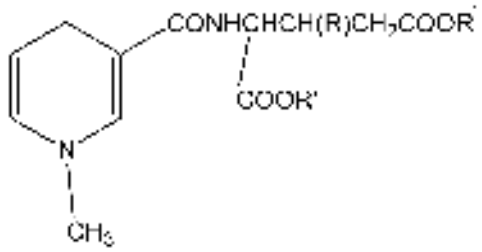


Table1 : List of the Synthesized compounds and Anticonvulsant activity



Cmpd Code	R'	R	MP (°C)	Avg. wt. of animal "g"	Dose (i.p.) mg/kg	%Inhibition of Convulsion	Lipophilicity Rm (Rf)
X21	CH_3CH_2	-CH_3	205	200 ± 10	25	85**	2.6(2.5)
X22	$(CH_3)_2CH$		212	175 ± 10	25	55*	1.06(0.014)
X23	$CH_3CH_2CH_2CH_2$		235	190 ± 10	25	80**	2.55(2.8)
X24	$C_6H_5CH_2$		270	190 ± 10	25	80**	2.0(0.017)
X25	CH_3CH_2	-C_3H_7	212	160 ± 10	25	75*	1.99(0.017)
X26	$(CH_3)_2CH$		228	220 ± 10	25	70*	1.95(0.01)
X27	$CH_3CH_2CH_2CH_2$		225	200 ± 10	25	65*	1.98(0.01)
X28	$C_6H_5CH_2$		232	200 ± 10	25	60*	1.75(0.017)
DPH Na+				190 ± 10	25	80	

** Highly significant at P< 0.001, values are expressed as ± SEM, * Significant at P< 0.01

PHYSICO-CHEMICAL STUDIES

The synthesized N-Nicotinyl- 3-Alkyl Glutamic acid derivatives were evaluated chemically by performing the qualitative chemical tests and thin layer chromatography. They gave positive qualitative tests for Ester and Amide functional groups with Nitrogen as element. The melting points were determined in open capillary tubes in liquid paraffin and were uncorrected, presented in table-1.

All the structure of synthesized compounds were confirmed by UV, IR and NMR Spectroscopy and on the basis of C, H and N analysis, presented in table-2.

EXPERIMENTAL ANIMALS

Healthy albino rats of either sex (Wistar strain) weighing 100-160g were used in present study. The animals had free access to food and water and were maintained under controlled temperature (27±2^{0}C) and 12 h: 12 h light and dark cycle. Initial body weight of each animal was recorded.

ACUTE TOXICITY STUDIES

The synthesized Nicotinyl- 3-Alkyl Glutamic acid derivatives at different doses (50-2000 mg/kg) were administered orally to normal rats. During the first four hours after the drug administration, the animals were observed for gross behavioral changes if any for 7 days. The parameters such as hyperactivity, grooming, convulsions, sedation, hypothermia, mortality was observed. No mortality observed with oral administration of all the extracts even at the highest dose (2000 mg/kg). Institutional Animal Ethics Committee (IAEC) had approved the experimental protocol and care of animals was taken as per the guidelines of CPCSEA, Department of animal welfare, Government of India.

TEST FOR ANTICONVULSANT ACTIVITY

The synthesized Nicotinyl- 3-Alkyl Glutamic acid derivatives were tested for anti-convulsant activity by following the chemo- convulsion method using Strychnine HCl to produce convulsion in albino rats. Healthy albino rats of either sex, weighing 100-160 g were selected and provided standard rat feed and water *ad libitum*. Before the experiment, food was withdrawn overnight but adequate water was given to the rats. The animals were divided into ten groups of 6 animals each . One group was injected with only Strychnine HCl (Control) and the other group received Strychnine HCl but after 4 hrs of injection of the synthesized compounds (or phenytoin sodium). Noted the severity of convulsions in these groups [8,9] and compared the activity of the synthesized compounds to the standard drug and percent reduction in convulsion was calculated, which were represented in table1.

TEST FOR LIPOPHILICITY

Lipophilicity is a essential feature for a chemical delivery system having brain delivery properties. Lipophilicity of the synthesized compounds was determined using the formula $R_m = \log (1/ R_f -1)$.The chromatographic R_m value was correlated with penetrating substance in biological cells and calculated R_m value of these synthesized derivatives were compared to that of respective parent drugs [10,11].

TEST FOR OXIDATION OF SYNTHESIZED COMPOUNDS

The synthesized derivatives were studied for the rate of oxidation in various biological fluids (like whole human blood, plasma liver, and brain tissue homogenates).The methanolic solution of these derivatives were prepared and added to the media. The mixture was kept at 37^{o}C and scanned using UV spectrophotometer from 400-250 nm for every.

10 min till 2 hrs [12]. Then the percentage of the dihydropyridine derivatives and the quaternary compounds were determined in these biological media and was calculated the half lives of these dihydropyridine derivatives (table-3).

Table 2: Carbon, Hydrogen & Nitrogen Analysis report of the Synthesized compounds

Cmpd code	R'	R	Mol. weight	C% Found (cal.)	H% Found (cal.)	N% Found (cal.)
X21	CH_3CH_2	$-CH_3$	$C_{17}H_{26}O_4N$(308)	65.1 (66.23)	8.43 (8.44)	4.5 (4.54)
X22	$(CH_3)_2CH$		$C_{19}H_{30}O_4N$ (336)	67.6 (67.85)	8.83 (8.92)	3.92 (4.16)
X23	$CH_3CH_2CH_2CH_2$		$C_{21}H_{34}O_4N$ (364)	69.0 (69.23)	9.25 (9.34)	3.75 (3.84)
X24	$C_6H_5CH_2$		$C_{27}H_{30}O_4N$ (432)	74.89 (75)	6.89 (6.94)	2.94 (3.24)
X25	CH_3CH_2	$-C_3H_7$	$C_{19}H_{30}O_4N$ (336)	67.7 (67.85)	8.82 (8.92)	3.85 (4.16)
X26	$(CH_3)_2CH$		$C_{21}H_{34}O_4N$ (364)	68.9 (69.23)	8.92 (9.34)	2.96 (3.84)
X27	$CH_3CH_2CH_2CH_2$		$C_{23}H_{38}O_4N$ (392)	69.8 (70.4)	8.62 (9.69)	3.45 (3.57)
X28	$C_6H_5CH_2$		$C_{29}H_{34}O_4N$ (460)	75.6 (75.65)	7.28 (7.39)	2.97 (3.04)

Table 3: The half-live (t½) of the derivatives in various biological fluid

Code	BLOOD		PLASMA		LIVER		BRAIN	
	K "min-1"	t½ "min-1"	K "min-1"	t½ "min-1"	K "min-1"	t½ "min-1"	K "min-1"	t½ "min-1"
X21	0.0495	14	0.0247	28	0.124	5.6	0.0447	15.5
X22	0.161	4.3	0.0256	27	0.2038	3.4	0.1174	5.9
X23	0.2235	3.1	0.030	23	0.288	2.4	0.133	5.2
X24	0.144	4.8	0.0266	26	0.169	4.1	0.157	4.4
X25	0.1136	6.1	0.033	21	0.169	4.1	0.128	5.4
X26	0.0778	8.9	0.0247	28	0.301	2.3	0.0602	11.5
X27	135	5.1	0.033	21	0.277	2.5	0.115	6
X28	0.150	4.6	0.0256	27	0.433	1.6	0.1414	4.9

RESULTS AND DISCUSSION

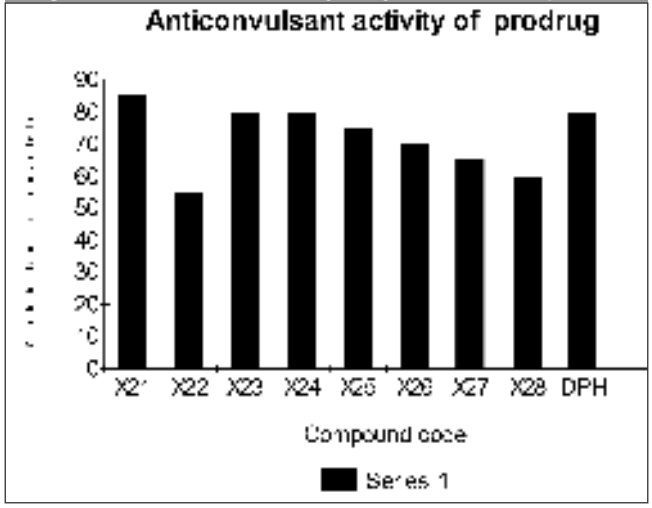
All the synthesized compounds have shown a comparable anticonvulsant activity with that of phenytoin sodium (25 mg/kg) and showed a significant (p0.01) inhibition of convulsion induced by Strychnine HCl (2 mg/kg) in albino rats. The results are presented in Table1 and Figure1.

In the synthesized compounds X21, X23 and X24 were found to exhibit high and equal activity with standard followed by X25, X26, X27, X28 and X22 respectively.

Maximum activity was found in the synthesized compound code X21, even more than standard in the same dose. Anticonvulsant activity of X23 and X24 was comparable to that of Phenytoin sodium and duration of action was found to be almost same as that of standard drug.

The lipophilicity of synthesized compounds were determined using R_m value and it appears that all of them will be able to penetrate the blood brain barrier, the order (most lipophilic → least lipophilic) being X21>X23>X24> X25>X26>X27>X28> X22 . The lipophilicity of synthesized compounds was found to be superior to the parent drug.

Fig.1: Anticonvulsant activity of synthesized compounds



The oxidation of synthesized compounds was examined in various tissue homogenates as well as human blood. All the compounds were found quite reactive in these biological media among which X21 was stable. However, X21 ($t_{1/2}$ =15.5) was shown to get oxidized to its pyridinium derivatives in brain homogenate. The other dihydropyridine in tissues other than brain, did not show any accumulation of its corresponding pyridinium compounds during the incubation period, so no peripheral toxicity could be seen.

The Redox Delivery Prodrug Approach (RDPA) has been applied to several potential 3-alkyl glutamic acid analogues for solving the site and organ delivery problem. The same approach has been used in this work and prepared the Nicotinyl derivatives, that were screened for their anticonvulsant activity and brain specific release.

All the compounds prepared and tested were shown a comparable anticonvulsant activity to that of phenytoin sodium but the compound X21 has higher activity and more lipophilicity.

In oxidation studies all the compounds have shown stability in various biological media. The *in vivo* study reveals that there is in an appearance and disappearance of quaternary compound in blood and brain after the administration of dihydropyridine derivative in a sustained manner. The synthesized compounds effectively antagonised the convulsion induced by Strychnine HCl. These synthesized compounds might be showing its anticonvulsant actions through glycine agonism[13]. These studies showed that this type of Chemical delivery System of Drug (3-alkyl glutamic acid analogues) producing significant and sustained brain specific glutamic acid decarboxylase activation activity by decreasing glutamate and excitation.

However more elaborate work is required to establish the efficacy of the synthesized nicotinyl- 3-alkyl glutamic acid derivatives as potent anticonvulsant drugs.

CONCLUSION

These compounds were synthesized with the objectives of developing better anticonvulsant molecules like Valproic acid with optimal anticonvulsant activity.

From the screening result, it can be concluded that all the synthesized compounds were effective against convulsion induced by Strychnine hydrochloride. The presence of carrier increases the lipophilicity of drug and helps to cross the BBB wich produce brain specific effect in minimum dose. It can also be concluded that compound with methyl substitution were more active than compounds with propyl substitution. Compound X21 was most effective than compound X24 because it is methyl substituted, on the other hand compound X24 is phenyl substituted. Normal alkyl substituted carbon atom exhibited considerable anticonvulsant activity then branched carbon.

We provided a convenient synthetic method for synthesis a new titled compound and results of anticonvulsant screening are encouraging. Further investigations with appropriate structural modification of title compounds may result in therapeutically useful products.

ACKNOWLEDGEMENT

Authors are deeply grateful to the Institute of Pharmacy, Bundelkhand University, Jhansi (U.P.) and Department of Pharmaceutics, Institute of Technology, B.H.U., Varanasi (U.P.) for research facilities.

REFERENCES

1. Sharma A.K, Khosla R., Mehta V. L., Kela A. K., Antiepileptic agents: Newer generation. Indian J. Pharmacol. 1996; 28: 1-10.
2. Cho S.W., Cho E.H., Cho S.Y., Activation of two types of brain glutamate dehydrogenase isoproteins by Gabapentin. F.E.B.S. Letters. 1998; 426: 196-200.
3. Scott K.R., Adesioye S.B., Edafiogho I.O., John D., Kodwin P., Irving T.M., Moore J.A., Nicholson J.M., Synthesis and evaluation of amino analogues of Valproic acid. Pharmaceutical Research. 1994; 11(4): 571-574.
4. Bodor N., Farag H.H., Improved delivery through biological membranes.Brain specific delivery of Dopamine with a Dihydropyridine pyridinium salt type Redox delivery system., J.Med.Chem. 1983; 26(4): 528-34.
5. Pop E., Shek. E, Murakami T., Bodor N., Improved anticonvulsant activity of Phenytoin by redox brain delivery system. J.Pharm.Sci. 1989; 78(8): 609-16.
6. Ronald GW. Malcolm WH. Charle AS., A nuclear magnetic resonance method for distinguishing.alpha- aminoacid from Beta. And gama. Isomers, J. Org. Chem. 1969; 34(3): 576-80.
7. Furniss BS,Hannaford AJ,Smith PWG and Tatchell AR,Vogel's Text Book of Practical Organic Chemistry,5th Edition, An imprint edision Wesley Longman,Inc., 1998, 683.
8. Kulkarni SK. , "Handbook of Experimental Pharmacology", 3rd edn., Vallabh prakashan, New Delhi. 2004, 131- 4.
9. Savatore G. P., Jose H.W., Ewart A.S., Effect of stimulus intensity on the profile of anticonvulsant activity of Phenytoin,ethosuximide and valproate, *J. Pharm. Exp. Therap*. 1985; 232(3): 741- 5.
10. Hansch C., Fujita T. , --. Analysis: A method for the correlation of biological activity and chemical structure, *J. Am. Chem. Soc*., 1964; 86(8): 1616-26.
11. Fujita T., Iwara J., Hansch C., A new sbstituent constant, , derived from partition coefficient, *J. Am.Chem. Soc*,1964; 86(23): 5175-80.
12. Bodor N., Farag H.H., Improved delivery through biological membranes: A redox chemical drug delivery system and its use for brain specific delivery of Phenylethylamine,*J. Med. Chem*., 1983; 26(4): 313-8.
13. Kadam S.S., Mahadik K.R., Bothara K.G., Principle of medicinal chemistry. 10th edn., Nirali prakashan, Pune. 2002, 2: 224-5

****