DETERMINATION OF PROTECTIVE INDICES OF PHENYTOIN AND SODIUM VALPROATE USING MOUSE BEAM WALKING ASSAY FOR MOTOR COORDINATION DEFICIT AS A MEASURE OF TOXICITY

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Abstract

Protective Index is a comparism of the amount of a therapeutic agent that causes the therapeutic effect, to the amount that causes toxicity. Thus, it is the ratio between the Median Toxic Dose (TD50) to that of Median Effective Dose (ED50). It is therefore an important parameter used to establish balanced safety – efficacy profile. The aim of this study was to determine the Median Toxic Dose (TD50), Median Effective Dose (ED50) and Protective Index (PI) of two anticonvulsant agents – phenytoin and sodium valproate, so as to assess their safety margins when motor coordination deficit was evaluated on Mouse Beam-walk model. The efficacy of phenytoin and sodium valproate was demonstrated using maximal electroshock (MES)- and pentylenetetrazole (PTZ)-induced seizure models in mice respectively. Whereas, the toxicity of both drugs was evaluated using beam walking apparatus for motor coordination deficit in mice. The protective index value for each was determined using Log-Probit Analysis according to Miller and Tainter. The ED50 values for phenytoin and sodium valproate were 12.6 ± 2.83 and 154.88 ± 48.53 mg/kg, respectively, while their TD50 values were 151.36 ± 46.38 and 319.98 ± 25.63 mg/kg, respectively. Whereas, the corresponding PI values for phenytoin and sodium valproate were 12.6 and 2.07, respectively. Therefore, it could be said that phenytoin did not induce significant impairment in motor coordination, hence, possess wide margin of safety. However, sodium valproate induced motor coordination deficit; characteristic of its sedative effect, thus, it is said to have narrow margin of safety.

Keywords: Protective Index (PI), Median Effective Dose (ED50), Median Toxic Dose (TD50), Phenytoin, Sodium valproate.

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INTRODUCTION

Protective index (PI) is considered to be an index representing the margin of safety and tolerability between median effective dose (ED\(_{50}\)) and median toxic dose (TD\(_{50}\)) values (Sergio et al., 2013). It is an important parameter in efforts to achieve an appropriate balanced safety-efficacy profile (Patrick et al., 2012). In anticonvulsant studies, value of PI has to be determined to know if the anticonvulsant activity of a certain compound is selective (Raza et al., 2008). Phenytoin is an anticonvulsant drug used primarily in the management of complex partial seizure and generalized tonic-clonic seizure; it acts by causing voltage-dependent blockade of voltage-gated sodium channels (Rogawski and Losher, 2004). Sodium valproate is a widely used antiepileptic drug (AED) with broad spectrum of activity (Raza et al., 2008); it is known to enhance GABA transmission and also blocks T-type calcium ion channels in addition to sodium ion channels blockade (Rang et al., 2006). Several studies have been conducted to determine the protective indices of phenytoin and sodium valproate, and the values obtained seemed to be dependent on the model used for assessing motor coordination function. For instance, according to works of Borowick et al. (2004); Raza et al., (2008); Luszczki et al., (2009), the neurological deficit tests conducted on sodium valproate was using Chimney Test for motor performance. On the contrary, the toxicity assessment of phenytoin; phenytoin and sodium valproate, were done by Shindikar et al., (2006); Begum et al., (2015), respectively, using Rotarod Assay for motor function. Therefore, the aim of this study was to determine and compare the PI values of phenytoin and sodium valproate using Beam-walk Assay for the motor function test in albino mice, which to the best of our knowledge was not previously reported.

MATERIALS AND METHODS

Equipment

Electroconvulsive machine (Ugo Basile, model no. 7801), Beam walking apparatus- Flat ruler (80 × 3 cm) and Wooden beam rod (8 mm × 60 cm), Plastic animal cages were used. Drugs include Phenytoin, Sodium valproate and Pentylentetrazole (Sigma Chemical Company, Louis Mo, USA).

Animals

Swiss albino male mice of both sexes weighing 18-22 g each were the experimental animals used in this study. They were obtained from Animal House, Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria.
animals were housed in groups, kept at room temperature and allowed access to food (a standard Grower Mesh) and water _ad libitum_. The animals were used in compliance with the National Institute of Health Guide for the Care and use of Laboratory Animals (Publication nos. 85-23, revised 1985). The institutional approval number for the protocol was given as DAC/IW-OT/003-10.

**METHODOLOGY**

**Determination of Median Effective Dose (ED\textsubscript{50}) of Phenytoin in Maximal Electroshock-induced Seizure Test**

This was determined using maximal electroshock-induced seizures, according to Swinyard and Kupferberg (1985); Swinyard _et al._, (1989). Phenytoin at doses 20, 15, 10 and 5 mg/kg was administered to four groups of six mice each via intraperitoneal route (_i.p._); such that two points were established between the limits of 100% and 0% protection. Thirty minutes post treatment seizures were electrically induced to each mouse using an Ugobasile electro-convulsive machine (Model No. 7801) connected with corneal electrodes. The shock parameters were 50 (mA), 50 (Hz), 0.3 (s) and 0.4 (ms); episode of tonic extension of the hind limb was regarded as full convulsion while lack of tonic extension of the hind limbs was considered as protection. The percentage protections obtained from each group were converted to probit values while the doses were converted to log dose values.

**Determination of Median Effective Dose (ED\textsubscript{50}) of Sodium Valproate in Pentylenetetrazole-induced Seizure Test**

The method of Swinyard (1969) was adopted. Thirty adult albino mice were divided into five groups of six mice. Each group (Group I-V) received 250, 200, 150, 100 and 50 mg/kg (_i.p._) of sodium valproate, such that two points were established between the limits of 100% and 0% protection. Thirty minutes later 90 mg/kg (_s.c._) of freshly prepared solution of pentylenetetrazole was administered to each mouse. The mice were observed for presence or absence of clonic seizures characterized by loss of righting reflex for at least 5 seconds. The percentage protections obtained from each group were converted to probit values while the doses were converted to log dose values. The median effective dose (ED\textsubscript{50}) was determined as described for phenytoin.

**Determination of Median Toxic Dose (TD\textsubscript{50}) of Phenytoin and Sodium Valproate**

The study was conducted according to method described by Stanley _et al._, (2005). The mice were trained to travel from a start platform along a ruler (80 cm long, 3 cm wide) elevated 30 cm above
the bench by metal supports to a metal box. Trials were performed for each mouse, and were designed such that the mice to be tested would be aware that there was a goal box that could be reached. Five groups of six mice each received phenytoin (250, 200, 150, 100 and 50 mg/kg) while another four groups of six mice each were administered sodium valproate (400, 350, 300 and 250 mg/kg). The drug administration was via i.p and in such that two points were established between limits of 100% toxicity and 0% toxicity. Thirty minutes post treatment, each mouse was placed at one end of wooden beam (8 mm in diameter, 60 cm long and elevated 30 cm above the bench by metal supports), and was allowed to walk to the goal box. The number of falls as an indicator of neurotoxicity for each group was counted and recorded. The percentage falls was found with its corresponding dose and converted into probit and log dose respectively. ED$_{50}$ and TD$_{50}$ values with their corresponding error of mean (SEM) as well as corrected percentages for the two limits of protection and toxicity (0% and 100%), according to Miller and Tainter (1944), were determined as explicitly described by Randhawa (2009). Corrected % Formula for 0 and 100%, both protection and toxicity, is 100(0.25/n) and 100(n-0.25/n), respectively; where n is the number of animals. The probit values are plotted against log-doses and then the dose corresponding to probit 5, i.e., 50%, was calculated.

Calculation of SEM is given by the formula below:

SEM of ED$_{50}$ = (Log ED$_{50}$ 84 – Log ED$_{50}$ 16) / $\sqrt{2N}$; where N is number of animals in each group.

This was similarly applied for SEM of TD$_{50}$.

**Analysis of Data**

ED$_{50}$ value obtained from MEST and PTZ as well as TD$_{50}$ values from Beam-walk assay were estimated by using probit analysis and transformed in standard error of mean (SEM) at 95% confidence limits, as described by Miller and Tainter (1944). The percentage protection against the tonic hind limb extension of MES-induced seizures and clonic phase of PTZ-induced seizures; per dose of phenytoin and sodium valproate, respectively, were fitted by using log-probit linear graphs.

**RESULTS AND DISCUSSION**

Phenytoin (5, 10, 15 and 20 mg/kg) and sodium valproate (50, 100, 150, 200 and 250 mg/kg) were evaluated for anticonvulsant activity using MES- (Table 1) and PTZ- (Table 2) induced seizures respectively. Two points were established between 0% and 100% protections in each case. Respective doses and percentage protections were converted to Log doses and Probits.
respectively. ED$_{50}$ values (probits 5) of phenytoin and sodium valproate were estimated from the linear graphs of Probit versus Log dose as Log 1.08 (12.07 mg/kg) and Log 2.19 (154.88 mg/kg), respectively (Figure 1 and 2). Similarly the two drugs were assayed for motor function using Beam Walking test and their respective TD$_{50}$ values were estimated as above using percentage animal fall as a measure for toxicity (Table 3 and 4). The values were Log 2.18 (151.36 mg/kg) and 2.505 (319.89 mg/kg) as in Figure (3 and 4). Whereas their protective index values (TD$_{50}$/ED$_{50}$) were found as 12.6 and 2.07, each, respectively (Table 5).

Table 1: Log-dose and Probit Values of Phenytoin for the Determination of ED50 in Maximal Electroshock-induced Seizure Test in Mice

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Log Dose</th>
<th>Protection against Seizures (%)</th>
<th>Percentage Corrected (%)</th>
<th>Probit Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHT (20)</td>
<td>1.3</td>
<td>100</td>
<td>95.8</td>
<td>6.70</td>
</tr>
<tr>
<td>PHT (15)</td>
<td>1.18</td>
<td>83.5</td>
<td>83.5</td>
<td>5.97</td>
</tr>
<tr>
<td>PHT (10)</td>
<td>1.0</td>
<td>16.7</td>
<td>16.7</td>
<td>4.03</td>
</tr>
<tr>
<td>PHT (5)</td>
<td>0.7</td>
<td>0</td>
<td>4.2</td>
<td>3.31</td>
</tr>
</tbody>
</table>

PHT = Phenytoin, ED$_{50}$ = Median Effective Dose, n = 6
Log 1.08 = ED$_{50}$ 12.02 mg/kg

Fig. 1: Graph of Log Dose-Curve in the Determination of ED50 Value of Phenytoin

Table 2: Log-dose and Probit Values of Sodium Valproate for the Determination of ED50 in Pentylenetetrazole-induced Seizure Test in Mice

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Log Dose</th>
<th>Protection against Seizures (%)</th>
<th>Percentage Corrected (%)</th>
<th>Probit Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA (250)</td>
<td>2.4</td>
<td>100</td>
<td>95.8</td>
<td>6.69</td>
</tr>
<tr>
<td>VA (200)</td>
<td>2.3</td>
<td>50.0</td>
<td>50.0</td>
<td>5.00</td>
</tr>
<tr>
<td>VA (150)</td>
<td>2.18</td>
<td>33.3</td>
<td>33.3</td>
<td>4.58</td>
</tr>
<tr>
<td>VA (100)</td>
<td>2.0</td>
<td>16.7</td>
<td>16.7</td>
<td>4.03</td>
</tr>
<tr>
<td>VA (50)</td>
<td>1.7</td>
<td>0</td>
<td>4.2</td>
<td>3.31</td>
</tr>
</tbody>
</table>

VA = Sodium Valproate, ED$_{50}$ = Median Effective Dose, n = 6
Log 2.2 = ED$_{50}$ 154.88 mg/kg

Fig. 2: Graph of Log Dose-Curve in the Determination of ED$_{50}$ Value of Sodium Vaproate

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Table 3: Log-dose and Probit Values Phenytoin for the Determination of TD50 in Beam-Walking Test in Mice

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Log Dose</th>
<th>Protection against Seizures (%)</th>
<th>Percentage Corrected (%)</th>
<th>Probit Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHT (250)</td>
<td>2.4</td>
<td>100.0</td>
<td>95.8</td>
<td>6.69</td>
</tr>
<tr>
<td>PHT (200)</td>
<td>2.3</td>
<td>66.7</td>
<td>66.7</td>
<td>5.43</td>
</tr>
<tr>
<td>PHT (150)</td>
<td>2.18</td>
<td>50.0</td>
<td>50.0</td>
<td>5.00</td>
</tr>
<tr>
<td>PHT (100)</td>
<td>2.0</td>
<td>16.7</td>
<td>16.7</td>
<td>4.03</td>
</tr>
<tr>
<td>PHT (50)</td>
<td>1.7</td>
<td>0</td>
<td>4.2</td>
<td>3.31</td>
</tr>
</tbody>
</table>

VA = Sodium Valproate, TD\(_{50}\) = Median Toxic Dose, n = 6

Log 2.18 = TD\(_{50}\) 151.36 mg/kg

![Fig. 3: Graph of Log Dose-Curve in the Determination of TD\(_{50}\) Value of Phenytoin](image)

Table 4: Log-dose and Probit Values of Sodium Valproate for the Determination of TD50 in Beam-Walking Test in Mice

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Log Dose</th>
<th>Protection against Seizures (%)</th>
<th>Percentage Corrected (%)</th>
<th>Probit Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA (400)</td>
<td>2.60</td>
<td>100.0</td>
<td>95.8</td>
<td>6.69</td>
</tr>
<tr>
<td>VA (350)</td>
<td>2.54</td>
<td>66.7</td>
<td>66.7</td>
<td>5.43</td>
</tr>
<tr>
<td>VA (300)</td>
<td>2.48</td>
<td>33.3</td>
<td>33.3</td>
<td>4.58</td>
</tr>
<tr>
<td>VA (250)</td>
<td>2.40</td>
<td>0</td>
<td>4.2</td>
<td>3.31</td>
</tr>
</tbody>
</table>

VA = Sodium valproate, TD\(_{50}\) = Median Toxic Dose, n = 6

Log 2.51 = TD\(_{50}\) 319.89 mg/kg

![Fig. 4: Graph of Log Dose-Curve in the Determination of TD\(_{50}\) Value of Sodium Valproate](image)

Table 5: Median Effective Dose (ED50), Median Toxic Dose (TD50) and Protective Index (PI) Values of Phenytoin and Sodium Valproate

<table>
<thead>
<tr>
<th>Drug</th>
<th>ED50 ± SEM</th>
<th>TD50 ± SEM</th>
<th>PI Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHENYTOIN</td>
<td>12.02 ± 2.83</td>
<td>151.36 ± 46.38</td>
<td>12.60</td>
</tr>
<tr>
<td>ODUIUM VA</td>
<td>154.88 ± 48.53</td>
<td>319.89 ± 25.68</td>
<td>2.07</td>
</tr>
</tbody>
</table>
SodiumVA = Sodium valproate, ED$_{50}$ = Median Effective Dose, TD$_{50}$ = Median Toxic Dose, PI = Protective Index, SEM= Standard Error of Mean

**DISCUSSION**

Protective index is an index of margin of safety and tolerability between anticonvulsant doses and doses of the compounds exerting acute adverse effects e.g., sedation, motor coordination impairment, ataxia or other neurotoxic manifestations (Loscher and Nolting, 1991). MES test identifies those compounds which prevent the spread of seizures; protection against hind limb tonic extension (HLTE) in the model predicts anticonvulsant activity of antiepileptic drugs (e.g. phenytoin, carbamazepine, oxcarbazepine, and lamotrigine) that prevent the spread of seizure discharge from an epileptic focus during seizure activity (Browning, 1992). Thus, phenytoin protected the animals against THLE, an index for its anticonvulsant activity. Pentylenetetrazole (PTZ) seizure threshold test is a well-known chemical test used to evaluate anticonvulsant activity (White et al., 1995). Studies have shown that PTZ induces seizures by blocking the major inhibitory pathways mediated by the predominant inhibitory neurotransmitter GABA, at all levels of the CNS (DeSarro et al., 1999). Sodium valproate, ethosuximide and benzodiazepines exhibit dose-dependent suppression of various seizure patterns induced by PTZ (Raza et al., 2001). This explains why sodium valproate offered protection in this test model. Similarly, the two models were conducted to determine the ED$_{50}$ values of phenytoin and sodium valproate based on their anticonvulsant outcome. The assay for motor function was done using Beam-walk test; this can detect motor deficits due to age, central nervous system lesions, and genetic and pharmacological manipulations in young and older rodents (Luong et al., 2011). This model is more sensitive in the sense that, a significant deficit can be observed at about 25-30% GABA$_A$ receptor occupancy where as in rotarod assay about 70% receptor occupancy is required to provide a significant impairment on the rotarod (Stanley et al., 2005). Thus, median toxic dose (TD$_{50}$) of phenytoin and sodium valproate was determined using this test to measure extent of motor deficit, as an index of neurotoxicity. The values of TD$_{50}$ and ED$_{50}$ were used to determine protective index (PI), and it is an index for safety margin (Brummelen, 2001). The safety margin for the two drugs as determined from the ratio between TD$_{50}$ and ED$_{50}$ was more to phenytoin (PI: 12.6) and narrow for sodium valproate (PI: 2.07), using Beam-walk for motor function test. According to the work of Shindikar et al., (2006), the PI of phenytoin in MEST was found to be approximately 7 while that of sodium valproate as found by Raza et al., 2008 was 1.13, using
rotarod and chimney for motor function tests respectively. Other works conducted by Borowick et al., 2004 and Luszczki et al., 2009, found the PI of sodium valproate as 2.28 and 1.72 using Chimney test, respectively. The PI value of sodium valproate obtained in this work was comparable to those found using Chimney test while that of phenytoin is said to be uncomparable to that found in Rotarod test. The fact that Beam-walk test is more sensitive than the rotarod test (Stanley et al., 2005); means that, motor impairment as a measure of neurotoxicity would manifest at lower doses in beam-walk test as against the rotarod. Also, method of Litchfield and Wilcoxon (1949) was used to determine the PI values in the previous works stated; on the contrary, method of Miller and Tainter (1944) was adopted in the present work. The former method has some limitations making impossible the calculation of probits for 0 and 100% effect and therefore, all experimentally determined effects must be higher and lower than 0 and 100%, respectively (Luszczki et al., 2009). The results of this study showed that phenytoin was less neurotoxic than sodium valproate, thus, lesser tendency to cause damage to the motor cortex.

CONCLUSION

The PI values of phenytoin and sodium valproate could be comparable to those previously determined by other researchers, using Chimney test model; but uncomparable to that found using rotarod test. Therefore, the differences in the methods for the neurotoxicity test as well as the graphical determination of the PI, account for the dissimilarity in the index values of the drugs.

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REFERENCES


