ANTICONVULSIVE PROPERTIES OF PREGNANOLONE EMULSION COMPARED WITH ALTHESIN AND THIOPENTONE IN MICE

S. HØGSKILDE, J. WAGNER, P. CARL, N. ANKER, H. R. ANGELO AND M. BREDGAARD SØRENSEN

Naturally occurring fluctuations in the concentration of progesterone in the brain may decrease the frequency of seizures in patients with epilepsy [1]. Moreover, several of the metabolites of progesterone (reduced in the 5 position on the steroid molecule) are considerably more potent than progesterone in their CNS depressant action [2–6], and it has been suggested that the effect of progesterone on brain excitability is mediated subsequent to a decrease in metabolism [2, 5, 7–9]. Thus it has been proposed that some of these progesterone metabolites could be used in the management of epilepsy [1]. Indeed, the administration of a mixture of two 5-α reduced derivatives of progesterone, alphaxalone and alphadolone (Althesin), has been shown to be effective in the treatment of otherwise drug resistant status epilepticus [10–12].

Pregnanolone, a naturally-occurring 5-β reduced metabolite of progesterone [13], has now been prepared in a stable emulsion and may prove suitable for clinical use. The anaesthetic properties of this preparation have been found to be similar to those of Althesin [14, 15].

The anticonvulsive profile of pregnanolone emulsion has been compared with the profiles of Althesin and thiopentone, using four standard experimental anticonvulsive test procedures.

SUMMARY
The anticonvulsive properties of pregnanolone (as an emulsion) were evaluated in mice and compared with similar properties of Althesin and thiopentone. Pregnanolone emulsion was found to antagonize convulsions induced by the GABA antagonists pentetrazole, bicuculline and picrotoxin and by the specific glycine receptor antagonist, strychnine. The drug was effective in all four convulsive tests at subanaesthetic doses with maximal activity appearing within less than 1 min. The anticonvulsive therapeutic indices of pregnanolone emulsion were superior when compared with the therapeutic indices of Althesin and thiopentone in all four tests. Pregnanolone emulsion might be a useful alternative drug in the management of convulsive states resistant to conventional therapy.

MATERIALS AND METHODS

Animals
Male NMRI mice (weights 26–36 g, ages 8–12 weeks) were used for the investigation. All injections were given over 15 s via a tail vein. The mice were observed for 24 h, after which they were sacrificed.

Drugs
Pregnanolone emulsion. Pregnanolone (5-β-pregnan-3α-ol-20-one) was dissolved in soya bean oil and emulsified (KabiVitrum, Sweden). The pregnanolone emulsion consisted of pregnanolone 4 mg, soya bean oil 200 mg, acetyl triglycerides 70 mg, egg yolk phosphatides 18 mg, glycerol 17 mg, and distilled water to 1 ml. The emulsion is isotonic, stable and has a pH of about 7.5.

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mean particle size is in the region of 0.2-0.5 μm with less than 3% being larger than 1.0 μm.

Alphaxalone/alphadolone. Alphaxalone 9 mg and alphadolone 3 mg per ml were dissolved in saline by the addition of the solubilizing agent Cremophor EL (polyoxyethylated castor oil) 200 mg ml⁻¹ (Althesin, Saffan, Glaxo, England).

Thiopentone. Thiopentone sodium was administered in an isotonic 2.5% aqueous solution (Leo, Denmark).

All drugs were diluted with physiological saline when low doses were tested, to allow an injection volume of 10 ml kg⁻¹ (approximately 0.3 ml/mouse), in order to minimize the possible error of injection of inaccurate volumes.

Testing procedures

Time of peak drug effect (TPE). The determinations were based on signs of neurotoxic activity following the i.v. administration of pregnanolone emulsion 1.2 mg kg⁻¹, Althesin 0.6 mg kg⁻¹ or thiopentone 7 mg kg⁻¹ to groups of eight mice, one group for each drug. Neurotoxic activity was tested by the rotating rod test [16]: the three groups of mice were trained to maintain their equilibrium for long periods of time on a rod 3 cm in diameter which rotated with a speed of 6 rev min⁻¹. After injection of the drugs, the groups were again placed on the rod and continuously tested. The time from drug administration until the mice were unable to stay on the rod was noted. The time when the greatest number of animals in a group exhibited signs of neurotoxicity was determined as the time of peak drug effect.

Anticonvulsive activity (ED). The ED was established against convulsions induced by four chemical convulsants given i.v.: pentetrazole, bicuculline, picrotoxin and strychnine. The details of the anticonvulsant tests, their mechanism of action and their value in predicting the antiepileptic activity of new drugs against various types of epilepsy in man, have been published previously [16-18]. Strychnine is a highly selective competitive antagonist of glycine-induced postsynaptic inhibition. The three other convulsants all interfere with GABA-mediated inhibition: bicuculline blocks GABA receptors, whereas pentetrazole and picrotoxin block GABA-mediated increases in chloride conductance; Pentetrazole, and to a lesser extent picrotoxin, have in addition effects directly on membrane properties related to excitability and stability—effects probably mediated by increases in sodium permeability [17].

All convulsants, diluted to an injection volume of 10 ml kg⁻¹, were administered i.v. over a period of 5 s. The following doses were used: pentetrazole 66 mg kg⁻¹, bicuculline 1.26 mg kg⁻¹, picrotoxin 4.25 mg kg⁻¹ and strychnine 0.65 mg kg⁻¹; in pilot experiments these doses had been shown to induce tonic extension of the hindlegs in more than 97% of the mice. Convulsions appeared within 5–15 s after the administration of pentetrazole, bicuculline and strychnine, and with picrotoxin with a variable delay of several minutes to 30 min. Mice treated with pregnanolone emulsion, Althesin or thiopentone 45 s before injection of one of the convulsants, and which did not show the tonic extensor component, were considered protected [16]. The mice given either pentetrazole, bicuculline or strychnine were observed for at least 30 min for the presence or absence of seizure, while those given picrotoxin were observed for 60 min. Groups of eight mice were tested at different doses of pregnanolone emulsion, Althesin or thiopentone. The dose limits at which all or none of the mice in a batch were protected, plus at least three points between these limits, were established.

Minimal neurotoxic activity (TD). The TD was established by the rotarod procedure. Inability of a mouse to maintain equilibrium more than once during the time period from 30 to 150 s after injection of the drug was used as indication of TD [16]. Dose limits at which all or none of the mice in a batch showed signs of TD, plus at least three points between these limits, were established in groups of eight mice.

Acute toxicity (LD). LD was defined as lethality within 24 h following injection of pregnanolone emulsion, Althesin or thiopentone. LD of thiopentone was determined in groups of eight mice. Dose limits at which all or none of the mice in a batch died, and at least three points between these limits, were established. LD of pregnanolone emulsion and Althesin have been determined in an earlier study [14].

The mean anticonvulsive dose (ED₅₀) against each of the four convulsant tests, the mean lethal dose (LD₅₀) and the mean neurotoxic doses (TD₅₀) of pregnanolone emulsion, Althesin and thiopentone were calculated by the method of Litchfield and Wilcoxon [19], and the protective index (PI) = TD₅₀/ED₅₀ and the therapeutic
TABLE I. Mean anticonvulsant doses (ED_{50}), 95% confidence limits (CL) and slope of regression line (Slope) of i.v. administered pregnanolone emulsion, Althesin and thiopentone against convulsions induced by pentetrazole, bicuculline, picrotoxin and strychnine i.v. in mice. Significant differences (P < 0.05): * compared with pregnanolone emulsion and Althesin; † compared with pregnanolone emulsion.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Pentetrazole</th>
<th>Bicuculline</th>
<th>Picrotoxin</th>
<th>Strychnine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnanolone emulsion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED_{50} (mg kg^{-1})</td>
<td>0.13</td>
<td>0.46</td>
<td>1.15</td>
<td>4.20</td>
</tr>
<tr>
<td>95% CL</td>
<td>0.05-0.35</td>
<td>0.24-0.87</td>
<td>0.52-2.56</td>
<td>2.60-6.77</td>
</tr>
<tr>
<td>Slope</td>
<td>0.18</td>
<td>0.40</td>
<td>0.20</td>
<td>0.42</td>
</tr>
<tr>
<td>Althesin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED_{50} (mg kg^{-1})</td>
<td>0.24</td>
<td>0.64</td>
<td>—</td>
<td>16.20</td>
</tr>
<tr>
<td>95% CL</td>
<td>0.13-0.44</td>
<td>0.33-1.23</td>
<td>ED_{50} &gt; LD_{50}</td>
<td>11.8-22.2</td>
</tr>
<tr>
<td>Slope</td>
<td>0.28</td>
<td>0.39</td>
<td>0.53</td>
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<tr>
<td>Thiopentone</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>ED_{50} (mg kg^{-1})</td>
<td>2.80*</td>
<td>9.50*</td>
<td>13.30†</td>
<td>23.00†</td>
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<tr>
<td>95% CL</td>
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<td>5.70-15.8</td>
<td>7.02-24.1</td>
<td>18.2-29.1</td>
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<tr>
<td>Slope</td>
<td>0.56</td>
<td>0.48</td>
<td>0.28</td>
<td>0.65</td>
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</table>

TABLE II. Mean minimal neurotoxic dose (TD_{50}) and anticonvulsant protective indices (PI) of i.v. administered pregnanolone emulsion, Althesin and thiopentone against convulsions induced by pentetrazole, bicuculline, picrotoxin and strychnine i.v. in mice. * Significant difference compared with pregnanolone emulsion and Althesin (P < 0.05).

<table>
<thead>
<tr>
<th>Substance</th>
<th>TD_{50} (mg kg^{-1})</th>
<th>PI (TD_{50}/ED_{50})</th>
<th>Pente-</th>
<th>Bicuculline</th>
<th>Picro-</th>
<th>Strychnine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnanolone emulsion</td>
<td>1.15</td>
<td>8.8</td>
<td>2.5</td>
<td>1.0</td>
<td>0.27</td>
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<tr>
<td>95% CL</td>
<td>0.81-1.63</td>
<td>Slope</td>
<td>1.86</td>
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<td>Althesin</td>
<td>0.76</td>
<td>3.2</td>
<td>1.2</td>
<td>—</td>
<td>0.05</td>
<td></td>
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<tr>
<td>95% CL</td>
<td>0.58-1.00</td>
<td>Slope</td>
<td>1.62</td>
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<tr>
<td>Thiopentone</td>
<td>8.80*</td>
<td>3.1</td>
<td>0.9</td>
<td>0.7</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>95% CL</td>
<td>6.30-12.3</td>
<td>Slope</td>
<td>1.80</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Peak drug effect (TPE) followed within 15–60 s of the injection of pregnanolone emulsion, Althesin or thiopentone, and for all three drugs the activity decreased rapidly; no activity was apparent by our testing procedure of TPE 3 min after the administration of Althesin or thiopentone, and 4 min after the administration of pregnanolone emulsion.

The results were analysed statistically using Kruskal–Wallis one-way analysis of variance by ranks for group comparisons and Dunn's multiple comparison procedure in case with significance using the Kruskal–Wallis test; Mann–Whitney unpaired rank sum test was used to analyse the results obtained with picrotoxin test. Significance was assigned at P < 0.05.

RESULTS

Peak drug effect (TPE) followed within 15–60 s of the injection of pregnanolone emulsion, Althesin or thiopentone, and for all three drugs the activity decreased rapidly; no activity was apparent by our testing procedure of TPE 3 min after the administration of Althesin or thiopentone, and 4 min after the administration of pregnanolone emulsion.

The mean minimal neurotoxic doses (TD_{50}) and the protective indices (PI) of the drugs are given in Table II. Pregnanolone emulsion had the highest PI, except by the strychnine test, where
ANTICONVULSIVE PROPERTIES OF PREGNANOLONE

PI of thiopentone was higher. None of the drugs was effective by this test in non-toxic doses (PI > 1).

The mean lethal doses (LD₅₀) (including the earlier published LD₅₀ of pregnanolone emulsion and Althesin [14]) and the anticonvulsive therapeutic indices (TI) of pregnanolone emulsion, Althesin and thiopentone are shown in table III. The TI of pregnanolone emulsion were superior to that of Althesin and thiopentone by all four tests.

The differences between ED₅₀, TD₅₀ and LD₅₀ values of pregnanolone emulsion and Althesin were not statistically significant. The ED₅₀, TD₅₀ and LD₅₀ of thiopentone were, however, significantly greater than the values obtained for pregnanolone emulsion and Althesin (P < 0.05), with the exception of the ED₅₀ in the strychnine test, for which the difference between thiopentone and Althesin proved not to be significant.

No anticonvulsant activity could be demonstrated after injection of 0.9% sodium chloride, which was tolerated in large amounts as LD₅₀ was greater than 150 ml kg⁻¹.

DISCUSSION

Status epilepticus is a medical emergency with a high mortality [20], therefore, despite the usefulness of the conventional antiepileptic drugs, adjuncts are needed in its management. From the results presented, pregnanolone emulsion appears promising in this respect.

Pregnanolone emulsion was found to antagonize convulsions induced by the GABA antagonists pentetrazole, bicuculline and picrotoxin [17], and by the specific glycine receptor antagonist, strychnine [17, 21]. Maximal activity of the steroid preparation appeared, after i.v. injection, within less than 1 min and the drug abolished convulsions induced by all four chemical convulsants in subanaesthetic doses (mean anaesthetic dose AD₅₀ = 5.25 mg kg⁻¹ [14]). The anticonvulsive therapeutic indices of pregnanolone emulsion were, on all four convulsive tests, superior to the therapeutic indices of Althesin and thiopentone.

The mechanism of the anaesthetic and anti-convulsive action of steroids is unknown. Structure–activity relationships and the short latency of effect suggest, however, that specific receptor sites, located on the cell membrane, are involved [2–6, 9, 22, 23].

Steroid receptor sites may be associated with the GABA receptor–chloride channel complex and it is possible that steroids enhance the inhibitory action of GABA by binding at a site close to or identical with the binding site of barbiturates [9, 24]. To our knowledge, however, steroids have not been reported to interact with the glycine receptor. On the contrary, it has been shown that anaesthetic steroids are unable to alter glycine-activated chloride conductance in spinal neurones [24].

If CNS depressant steroids interact with the GABA receptor complex and not with the glycine receptor complex, the following questions arise. First, why was pregnanolone emulsion able to antagonize strychnine-induced convulsions, and
second, why was Althesin unable to antagonize picrotoxin-induced convulsions?

GABA and glycine receptors are spatially distinct from one another, but both GABA and glycine activate chloride channels with several conductance states and two of these states may be identical [25]. The receptor channels have been shown to be distributed in a mosaic-like fashion over the membrane surface of spinal neurones [25] and it has been suggested that they interact with each other in some mechanistic manner [21], in which case antagonism of strychnine-induced convulsions by pregnanolone emulsion may be explained by enhancement of glycine-mediated inhibition, secondary to a potentiation of GABA synaptic function by the steroid.

Another possibility could be that the CNS depressant action of steroids is mediated by other receptor systems, eventually, in addition to modulation of the GABA receptor complex. Interaction with the tentative neurotransmitter adenosine is speculative, but it is interesting that adenosine has been shown to possess anaesthetic and anti-convulsive properties and that these properties are thought to be mediated by inhibition of the influx of calcium into synaptosomes or regulation of cyclic AMP, or both [26]. Binding of steroid hormones at the membrane level, resulting in a change of membrane-bound adenylate cyclase activity has been reported [27] and it has also been reported that anaesthetic steroids inhibit calcium conductance in uterine smooth muscle [28].

The lack of effect of Althesin in preventing picrotoxin-induced convulsions was surprising, for at least two reasons. First, the specific binding of the radioactive $^{35}$S-labelled convulsant t-butylbicyclophosphothionate (TBPS), a competitive inhibitor of picrotoxin binding that labels at a site close to or on the GABA-operated chloride channel [29], has been shown to be inhibited competitively by anaesthetic steroids [9]. Second, chloride conductance may be stimulated by a direct action of the steroid itself [25]. The deficiency of Althesin in comparison with pregnanolone emulsion in antagonizing the convulsive action of picrotoxin is, however, most probably explained by the fact that neither of the steroids was tested at peak drug effect because of the slow and variable onset of action of picrotoxin, and by a longer lasting CNS depressant effect of pregnanolone emulsion compared with the effect of Althesin.

Even though the therapeutic index of pregnanolone emulsion against strychnine-induced convulsions was less than the indices against the GABA antagonists, it was still 3.5 times higher than the therapeutic index of diazepam, the drug of choice in the treatment of convulsions resulting from strychnine poisoning [30] and in status epilepticus [20]. A therapeutic index of 3.0 against strychnine-induced convulsions has been found for diazepam (Diazemuls), using the same mice model (unpublished observations).

The present study indicates that pregnanolone in an emulsion form might be a useful new alternative in the management of convulsive states resistant to conventional therapy.

REFERENCES


