Antinociceptive properties of neurosteroids III: experiments with alphadolone given intravenously, intraperitoneally, and intragastrically

R. Nadeson and C. S. Goodchild*

Department of Anaesthesia, Monash University, Clayton, Victoria, Australia 3168
*Corresponding author

The veterinary neurosteroid anaesthetic Saffan® has the same formulation as Althesin® now withdrawn from human use and is a mixture of two neurosteroids, alphadalone, and alphaxalone. The molecular structures of these two pregnanes and their properties as i.v. anaesthetics were reported to be similar. Preliminary experiments showed that alphadalone caused powerful antinociceptive effects without sedation when given i.p. In this study, alphadalone was given to rats (weight 100–200 g) i.v., i.p., and intragastrically. I.v. injections of alphadalone (25 mg kg⁻¹) caused anaesthesia and sedation, whereas i.p. (0.1–100 mg kg⁻¹) and intragastric administration (750 mg kg⁻¹) produced no such effects. Intragastric alphadalone caused antinociceptive effects assessed with the electrical current threshold test (response 2.2 ± pre-drug control values) without sedation. These effects were reversed at the level of the spinal cord by intrathecally-administered bicuculline (10 pmol). We conclude that a metabolite of alphadalone acetate produced in the liver leads to antinociceptive effects after i.p. and intragastric administration of the parent compound. This antinociception involves spinal cord GABAA receptors, even though the drug was administered via a non-spinal route.

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After a long search for a new safer i.v. anaesthetic agent, Althesin® was introduced into clinical practice in 1970 and proved to be a highly successful i.v. agent with a very wide therapeutic margin.¹ Much research on the compound followed, and it was used widely for anaesthetic practice all over the world. It is interesting to note, in light of the contents of this report, that all active properties of the mixture of the two steroids called Althesin® were attributed to the alphaxalone content.¹ This assumption has been made repeatedly in the literature based on the statement that alphadalone was present in the mixture merely to improve the solubility of alphaxalone in Cremophor.² Thus, no references to the specific physiological properties of alphadalone exist. Althesin® was withdrawn from clinical practice in the late 1980s because of major anaphylactic reactions to Cremophor EL.³ The mixture is still available for veterinary use (Saffan®).

Experiments reported previously, showed that subanaesthetic doses of Saffan® produce powerful antinociception in rats.⁴ This is because of an interaction of one of the components of the mixture with spinal cord GABAA receptors even though the drug was given i.p.⁴ Investigations showed that all the sedative and anaesthetic properties of the mixture are a result of the alphaxalone content and all of the antinociceptive properties are a result of the alphadalone content. It is possible that the difference in the activity profile of alphadalone described in those experiments compared with what was reported in the literature during the development of Althesin® may be because of the route of administration. In the early reports on the development of the i.v. anaesthetic combination of alphaxalone and alphadalone the individual agents were probably tested by i.v. administration. By contrast, the experiments reporting antinociceptive effects with no sedation utilized i.p. administration of alphadalone.⁵ Thus, in the latter experiments the parent drug would have to pass through the liver and be prone to metabolic transformation before gaining access to the general circulation and the rest of the body. The experiments reported here were performed, first of all to ascertain if i.v. alphadalone did cause anaesthesia as no documentation exists for the effects of this drug given alone. The second aim was to produce a dose response curve for the antinociceptive effect of i.p. alphadalone and to determine if much higher doses than
those used previously could cause sedation when given i.p. Finally, intragastric administration was investigated as this also delivers the drug first to the liver prior to the drug gaining access to the rest of the body. These experiments might indicate a potential use of the drug as an orally-administered analgesic. The highest dose of alphadolone that could be administered intragastrically was investigated for sedative and antinociceptive effects.

Materials and methods
This work was carried out with permission of the Monash University Standing Committee on Ethics in Animal Experimentation (SCEAE Project No. 93017).

I.v. administration
Four male Wistar rats (weight 100 g) were given 0.25 ml i.v. injections of alphadolone acetate solution (10 mg ml⁻¹; dissolved in 10% Cremophor EL®), into the lateral tail vein using a Hamilton microsyringe and 27-gauge needle. A stopwatch was used to time the following scoring of sedation every 15 s:
0—no righting reflex;
1—righting reflex present but no spontaneous locomotion;
2—spontaneous locomotion observed but uncoordinated;
3—normal locomotion and exploratory behaviour.
These scores were plotted against the time of the observation for each rat.

I.p. administration

Dose response relationship for antinociception
Thirty male Wistar rats (weight 150–200 g) were given a range of doses of alphadolone (0.06, 0.6, 6.0, and 60 mg per kg⁻¹ i.p.; n=4, 10, 15 and 4 experiments at each dose, respectively). The drug was suspended in saline solution and injected in a volume of 2 ml kg⁻¹. The rats were observed continuously for signs of sedation such as normal startle reactions to tapping on the Plexiglass restrainer in which they were placed and attempts at exploratory behaviour in the confined space. Antinociceptive thresholds were measured in the tail using noxious electrical current (ECT) as described previously.⁵ ECT measurements were made every 5 min until three stable readings were obtained. At that stage either saline or alphadolone 750 mg kg⁻¹ suspended in saline was instilled into the stomach via an oesophageal needle. Five minutes later and every 5 min thereafter, ECT measurements were performed in the skin of the tail and neck. At 60 min, 10 pmol of bicuculline (a GABA₅ receptor antagonist) dissolved in 5 µl 6% dextrose solution was injected intrathecally down a Portex catheter that had been implanted previously in the lumbar subarachnoid space to lie adjacent to the most caudal segments of spinal cord responsible for tail innervation. ECT measurements were continued for tail and neck electrodes every 5 min for a further 30 min. ECT results for tail and neck electrodes for each treatment (vehicle or alphadolone) at each time point were combined as means (SEM) to produce time response curves.

Results

I.v. administration
All five rats lost the righting reflex and were heavily sedated after i.v. alphadolone acetate 25 mg kg⁻¹. All four rats scored 0 immediately after the i.v. alphadolone. Three rats recovered fully within 90 s and one 270 s after the i.v. injection of alphadolone.

I.p. administration
It can be seen from Fig. 1 that there was a dose-related increase in antinociceptive effect with a maximum response of approximately 2.2× control (pre-drug) values. There were no overt signs of sedation even at the highest dose of i.p. alphadolone 60 mg kg⁻¹. Rats exhibited normal startle
reflexes and exploratory behaviour, even after release from the restrainer 30 min after the i.p. injection when the full antinociceptive effects assessed by tail ECT were still present.

**Intragastric administration**

As shown in Fig. 2 the intragastric vehicle produced no antinociceptive effects in either the tail or neck electrodes. By contrast intragastric alphadolone 750 mg kg$^{-1}$ produced a statistically significant increase in the electrical current thresholds in the skin of the tail and the neck ($P<0.01$; $n=5$; Student’s $t$-test). Observation of the animals during this time revealed no differences between the groups with regard to sedation. None of the rats showed overt signs of sedation or a decrease in exploratory behaviour. Intrathecal bicuculline produced no changes in the electrical current threshold in the tail or the neck in those experiments where intragastric vehicle had been administered ($P>0.05$; Student’s $t$-test). However, intrathecal bicuculline injection (10 pmol) onto the caudal segments of spinal cord responsible for tail innervation completely reversed the antinociceptive effects of intragastric alphadolone assessed by ECT measurements in the tail. This change was statistically significant when values of ECT readings were compared before and after bicuculline administration (Student’s paired $t$-test; $P<0.01$). No change occurred in the neck thresholds after intrathecal bicuculline indicating that the actions of this GABA$_A$ antagonist were confined to caudal segments of the spinal cord.

**Discussion**

The present study confirms that alphadolone, given i.v. is an anaesthetic. However, the compound seems to be devoid of anaesthetic and sedative properties when it is given by the i.p. and intragastric routes. It is not uncommon for drugs to have different effects when given by different routes. Before drawing a conclusion about i.p. alphadolone being devoid of sedative properties it is important to determine that enough drug has been given i.p. to achieve an adequate blood level for the effect. The dose of Saffan$^R$ that causes anaesthesia in rats is 2 ml kg$^{-1}$ which translates to alphaxalone 18 mg kg$^{-1}$ and alphadolone 6 mg kg$^{-1}$, a total of 24 mg kg$^{-1}$ neurosteroid. In previous experiments it was shown that alphaxalone 18 mg kg$^{-1}$ given i.p. caused sedation equal to Saffan$^R$ 2 ml kg$^{-1}$ and alphadolone 6 mg kg$^{-1}$ caused no sedation.$^2$ By contrast, alphaxalone given alone caused no antinociceptive effects but alphadolone at 6 mg kg$^{-1}$ produced ECT antinociceptive effects at 2.2× control, a value equal to that caused by Saffan$^R$ 2 ml kg$^{-1}$ and also equal to the maximum value of the dose response curve for i.p. alphadolone reported in this paper. It is reported that alphadolone is one-third as potent an alphaxalone in anaesthetic.$^2$ Thus, from this argument it could be expected a dose of alphadolone three times the total dose alphaxalone that caused anaesthesia (i.e. 3×18=54 mg) would be
required for sedation. Doses of alphadolone up to 100 mg kg\(^{-1}\) i.p. caused no signs of sedation for a total observation time of 40 min.

Previous experiments have also shown that the antinociceptive effects of Saffan\(^6\) which were because of the alphadolone content of the anaesthetic mixture were caused by an interaction of the neurosteroid with spinal cord GABA\(_A\) receptors as intrathecal injection of the GABA\(_A\) receptor antagonist bicuculline reversed the antinociceptive effects.\(^4\) GABA\(_A\) antagonists also reversed the spinally-mediated antinociceptive effects of intrathecal injections of water-soluble aminosteroids.\(^7\) Results presented in this paper also show that injection of bicuculline onto the most caudal segments of the spinal cord totally reversed the antinociceptive effects in the tail, the region of the body innervated by the most caudal segments of the spinal cord. As bicuculline is a GABA\(_A\) antagonist and as the neck thresholds remained unchanged, it may be conclude that non-sedating doses of intragastric alphadolone produced long-lasting antinociceptive effects mediated by spinal cord GABA\(_A\) receptors even though the drug was given intragastrically.

The effect of sex and adrenal steroids on the modulation of neuronal activity, dendritic structure, and behaviour has been well documented.\(^8\) These effects result from the regulation of intracellular receptors which direct changes in protein synthesis.\(^10\) Therefore, the physiological action of these compounds occurs slowly over hours or days.\(^12\) Pioneering work by Seyle 50 yr ago demonstrated that certain pregnanes and androstanes have potent anaesthetic and anticonvulsant effects occurring within a few minutes of administration.\(^14\) Since these early findings, it has become apparent that some steroids can interact directly with a surface membrane receptor–complex to cause a change in central nervous system (CNS) activity.\(^15\) Investigators studying the steroid anaesthetic alphaxalone (3\(\alpha\)-hydroxy-5\(\alpha\)-pregnan-11,20-dione) found that this drug can allosterically positively modulate the \(\gamma\)-aminobutyric acid (GABA) receptor. In subsequent studies alphaxalone and other neuroactive steroids were shown to enhance the inhibitory effect on neuronal excitability of GABA acting at the GABA\(_A\) receptor complex.\(^17\)\(^20\)

It is now well established that GABA generally and receptors of the GABA\(_A\) subclass are involved in spinal cord control of nociception.\(^21\)\(^23\) Several different subtypes of GABA\(_A\) receptor involved with spinal cord antinociceptive systems have been described.\(^9\) Thus, there is the possibility for these GABA\(_A\) receptors to be targeted selectively by analgesic compounds. This effect might be achieved without sedation if they are significantly structurally different from the receptors in the brain that are responsible for the sedative, anxiolytic, anticonvulsant, and anaesthetic effects of a wide variety of compounds.\(^9\)\(^24\)\(^28\) This idea is supported by the observation of antinociception after recovery from the anaesthetic and sedative effects of propofol, an i.v. anaesthetic drug that causes its sedative and anaesthetic effects by positive modulation of GABA\(_A\) receptors.\(^29\) It might, therefore, be expected that two structurally different neurosteroids could interact with different receptors. There is also some suggestion from \textit{in vitro} studies that neurosteroid modulation of native GABA\(_A\) receptors obtained from the brain and spinal cord may be different.\(^30\) However, the molecular structures of alphaxalone and alphadolone are very similar and there are no suggestions in published reports of any differences between these two compounds in activity profiles.

Reports in the literature concerned with the development of Althesin\(^6\) suggest that the alphadolone acetate component of Saffan\(^6\) is present in the mixture merely to improve the solubility of alphaxalone (the active ingredient) in Cremophor EL.\(^2\) It is also reported that alphadolone is an i.v. anaesthetic like alphaxalone but with one-third the potency.\(^1\) The results of this study, in which alphadolone was given i.v. and all four rats were anaesthetized support those statements. This seems to conflict with the results of the other experiments reported in this paper which show that doses of alphadolone up to 100 mg kg\(^{-1}\) given i.p. and 750 mg kg\(^{-1}\) intragastrically caused no sedative effects.

It is clear, however, that the drug is absorbed in rats after i.p. and intragastric administration because antinociceptive effects follow such treatment. One possible explanation is that metabolic transformation of the molecule deactivates anaesthetic properties but preserves or imparts antinociceptive properties. In rat brain membranes a well-defined structure-activity relationship has been shown for pregnanes. Studies have shown that the 3\(\alpha\)-hydroxyl group on the steroid is required for anaesthetic activity whilst a carbonyl group at position C17 confers greater potency.\(^31\)\(^33\) Glucuronide and sulfates have been described to be major metabolites of these neurosteroids.\(^34\) These metabolites may be produced by reaction at the 3\(\alpha\)-hydroxyl group in both alphaxalone and alphadolone, and also at the hydroxyl group in the 21 position on the alphadolone molecule after de-acetylation. A drug injected i.p. or given via the gastrointestinal tract is first presented to the liver and neurosteroids are known to have significant first pass metabolism at the liver. As the only difference in the molecular structure between alphadolone and alphaxalone is the hydroxyl group at the 21 position it is suggested that metabolism at this group in the liver is responsible for the differences observed in their biological activity after i.p. and intragastric administration. This metabolite is analgesic and not anaesthetic, a subject worthy of further investigation.

We conclude that the antinociceptive effect of alphadolone after i.p. and gastrointestinal administration may be because of production of a metabolite of the parent compound. This causes spinally-mediated antinociceptive effects by interaction with spinal cord GABA\(_A\) receptors. As this effect occurs in the absence of sedation, this suggests a possible clinical use of alphadolone or its metabolite in the management of pain syndromes.
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