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Development and implementation of *in vivo* models for evaluation of drugs with potential analgesic activity

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To Bruno.

"Knowing is not enough, we must apply.
Willing is not enough, we must do."

J. W. v. Goethe

ABSTRACT

A large number of behavioural methods have been developed to evaluate the analgesic effect of drugs in animals, but many authors have been neglecting the importance of the assessment of drug induced neurotoxicity or drug induced motor impairment, sedation or stimulation, prior to the nociception tests. In the present work, two *in vivo* models for evaluation of drugs with potential analgesic activity were developed and fully implemented, using NMRi mice: one model was constituted by the formalin paw test associated with the rotarod test and the open-field test (FRO) and the other model was constituted by the writhing test, also associated with the rotarod test and the open-field test (WRO).

The results obtained in the present study demonstrate that eslicarbazepine acetate (1 - 300 mg/kg) orally administered to NMRi mice 60 minutes before the tests produced dose-dependent antinociceptive activity in the two models tested (FRO and WRO). This effect was significant against chemically (formalin and acetic acid) induced nociception. The doses of the eslicarbazepine acetate, carbamazepine and oxcarbazepine that produced analgesia in the formalin paw and writhing tests were smaller in magnitude than those required to produce effects in both the rotarod and open-field tests. The ED_{50} value obtained for eslicarbazepine acetate in the formalin paw test was 41.1 (38.9 - 44.0) mg/kg and in the writhing test was 108.1 (97.4 - 118.9) mg/kg. Moreover the results obtained for protective index (PI) of eslicarbazepine acetate, when compared with the protective indices for carbamazepine and oxcarbazepine show that eslicarbazepine acetate is just about 3-fold more protective against formalin induced-pain than carbamazepine and oxcarbazepine. In the writhing test, eslicarbazepine acetate has shown a PI between carbamazepine and oxcarbazepine.

In conclusion, one can state that both models FRO and WRO are sensitive tools of great importance for the evaluation of the analgesic activity of both old and new drugs and the rotarod and the open-field tests together play an important role in the developed models.

RESUMO

Um grande número de métodos comportamentais para avaliação do efeito analgésico de fármacos em animais têm vindo a ser desenvolvidos, mas muitos autores têm vindo a negligenciar a importância da avaliação da neurotoxicidade induzida por fármacos ou da deficiência motora, sedação ou estimulação induzidas por fármacos, antes dos testes de nocicepção. No presente trabalho foram desenvolvidos e totalmente implementados dois modelos *in vivo* para avaliação de fármacos com potencial actividade analgésica, usando ratinhos NMRi: um modelo foi constituído pelo teste da formalina na pata, associado ao teste do "rotarod" e ao teste de campo aberto (FRO) e outro modelo foi constituído pelo teste das contorções abdominais, também associado ao teste do "rotarod" e ao teste de campo aberto (WRO).

Os resultados obtidos no presente estudo demonstram que o acetato de eslicarbazepina (1 - 300 mg/kg) administrada oralmente a ratinhos NMRi 60 minutos antes dos testes produziu actividade antinociceptiva dependente da dose nos dois modelos testados (FRO e WRO). Este efeito foi significativo após nocicepção induzida por agentes químicos (formalina e ácido acético). As doses de acetato de eslicarbazepina, carbamazepina e oxcarbazepina que produziram analgesia nos testes da formalina na pata e das contorções abdominais foram mais reduzidas do que as necessárias para produzir efeito em ambos os testes de "rotarod" e de campo aberto. O valor de ED_{50} obtido para o acetato de eslicarbazepina no teste de formalina na pata foi 41,1 (38,9 - 44,0) mg/kg e no teste das contorções abdominais foi 108,1 (97,4 - 118,9) mg/kg. Os valores obtidos para o índice de protecção (PI) do acetato de eslicarbazepina, quando comparados com os índices de protecção para a carbamazepina e oxcarbazepina, mostram que o acetato de eslicarbazepina é cerca de 3 vezes mais protector contra a dor induzida pela injecção de formalina do que a carbamazepina ou a oxcarbazepina. No teste das contorções abdominais, o acetato de eslicarbazepina apresentou um PI entre o da carbamazepina e o da oxcarbazepina.

Em conclusão, pode constatar-se que ambos os modelos FRO e WRO são ferramentas sensíveis de grande importância para a avaliação da actividade analgésica de fármacos clássicos e contemporâneos e que os testes de "rotarod" e de campo aberto em conjunto, desempenham um papel importante nos modelos desenvolvidos.

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LIST OF ABBREVIATIONS

ANOVA - Analysis of variance

CBZ – Carbamazepine

CL – Confidence interval

cm - Centimetre

CMC – Carboxymethyl cellulose

CYP – Cytochrome

EC – European Commission

ED_{50} - The dose that was antinociceptive in 50 % of animals tested

ESL - Eslicarbazepine Acetate

FELASA - Federation of European Laboratory Animal Science Associations

FRO - The formalin paw test associated with the rotarod test and the open-field test

kg – Kilogram

M – Morphine

mg - Milligram

min - Minute

ml – Milliliter

mm – Millimeter

N - Sample size

NMDA - *N*-methyl-D-aspartate

NSAID - Nonsteroidal anti-inflammatory drug

OXC – Oxcarbazepine

PI – Protective Index

p.o. - Oral administration

R^2 – Coefficient of determination

sec - Second

S.E.M. - Standard error of the mean

t_{max} - The time that it takes to reach the maximum concentration of drug

TD_{50} - The dose that induces motor impairment in 50% of animals tested

V - Vehicle

v/v - Volume in volume

w/v - Weight in volume

WRO - The writhing test associated with the rotarod test and the open-field test

µl - Microliter

CHAPTER I

INTRODUCTION AND OBJECTIVE

1 Pain

Pain has been part of the human experience for more than 50,000 years. Pain has been a very real and immediate concern in all ages, but the attitudes and responses of people to pain have been shaped by magical, theological, demonological, philosophical, and practical influences in varying degrees with shifting emphasis (Kucharski and Todd, 2008).

Pain is the most frequent reason patients seek medical advice. Pain is the body's mechanism of self-preservation. There is no consensual definition for pain, but it was described by the International Association for the Study of Pain, in November 2007, as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Pain tells you when your finger is touching a hot surface or when a fall has resulted in an injury that requires your attention. In this way, pain acts as a warning sign to alert you when damage to your body is occurring or may occur. Also, pain promotes the healing process as we take great care to protect an injured body part from further damage to minimize the experience of more pain. In fact, the inability to experience pain is a dangerous condition, because injury can occur and go unnoticed. Those individuals who are born with conditions in which they lack nociceptors (receptors for noxious *stimuli*), tend to live short lives due to their inability to recognize the pain *stimuli* as a signal for potential or ongoing tissue damage.

Pain can be classified in different ways. From the point of view of its aetiology, pain can be nociceptive induced or it can be neuropathic. Nociceptive pain refers to pain arising from direct exogenous (mechanical, chemical, thermical, and electrical) or endogenous (inflammation, and tissue ischemia) stimulation of nociceptors, such as occurs with trauma or inflammation. Neuropathic pain refers to pain arising from damage to the nervous system. Pain can also be spontaneous, associated with psychological factors and so called psychogenic (Dubner and Hargreaves, 1989). Some types of mental or emotional problems can cause, increase, or prolong pain.

From the point of view of its duration, pain can be classified in acute pain and chronic pain. Acute pain occurs for brief periods of time and it is always an alarm signal that something may be wrong. Chronic pain (also referred to as "persistent pain") is continuous and recurrent and is one of the symptoms of chronic diseases. The boundaries between acute and chronic pain are

not defined. Acute pain can range from momentary pain (may last a few seconds or as long as the *stimulus* is applied) to several hours or days after an injury (also referred to as "clinical pain") while chronic pain is defined as pain that lasts more than three months, whether it is due to a continuous inflammatory process or a neuropathy (a disease or abnormality of the nervous system) (Purves et al., 2008) .

Scientific evidence gathered over the years made it clear that many different mechanisms modulate the transmission of nociceptive information at every level of the nervous system. The first evidence of the existence of a pain modulation system was the observation that electrical stimulation of discrete areas of the brain inhibited responses to painful *stimuli* (Fields and Adams, 1974). At about the same time, the discovery of opiate receptors and of endorphins provided insight into the manner in which narcotic analgesics relieve pain. It is now generally accepted that pain modulation is achieved through a series of complex interactions between inputs from the periphery, interneurons in the spinal cord and descending control systems from the brain (Fields, 1984).

In the last twenty years, extraordinary progress has been made on understanding the physiology of pain (Purves et al., 2008). The discovery of a complex nervous system network for the modulation of pain, and the isolation and description of endorphins had a great contribute to our understanding of pain transmission (Steeds, 2009). Nevertheless there are still many areas of the physiology of pain that remain unknown. The physiology of pain involves nociceptors, combined with an intricate system of afferent and efferent neuronal connections. Nociceptors are relatively unspecialized nerve cell endings that initiate the sensation of pain (*noci-* is derived from the Latin for "hurt") (Purves et al., 2008). All nociceptors are free nerve endings that are sensitive to painful mechanical *stimuli*, extreme heat or cold, and chemical *stimuli*. Most pain originates when nociceptors are stimulated and nerve impulses are transmitted to the brain through the pain pathways. It is important to note that almost all body tissues are equipped with nociceptors, an important fact considering pain has primary warning functions. Nociceptors can be classified in two types: unimodal receptors and polymodal receptors (Nathan, 1976; Bonica, 1990). Unimodal receptors, also known as mecanothermal nociceptors, are mainly present in the skin and respond to strong pressure applied to the skin and strong *stimuli* as pinprick or sudden heat (greater than 45°C). This type of receptor is mainly associated with small myelinated primary afferent neurons designated Aδ type that transmits impulses rapidly. Stimulation of this

type of receptor results in pain that occurs early after injury and is usually sharp, well-localized, and pricking (Nathan, 1976; Bonica, 1990). Polymodal receptors are the free nerve endings of unmyelinated primary afferent neurons of the C type which are widely distributed throughout most tissues and respond to tissue damage (Nathan, 1976; Bonica, 1990). The best known theory describing how painful *stimuli* may be altered at the spinal level is the Gate Control Theory (Melzack and Wall, 1965). The Gate Control Theory is illustrated in Figure 1. This theory postulates that painful *stimuli* have to pass through a gate in order to be communicated to the central nervous system, lacking one-to-one correspondence between the severity of the *stimulus* and the severity on pain experience. According to this theory, the peripheral nervous system communicates with the central nervous system through a complex interplay: upon injury, pain messages originated in the nociceptors associated with the damaged tissue flow along the peripheral nerves to the spinal cord where they encounter "nerve gates" that open or close depending upon a number of factors, after which the modulated pain messages reach the brain. So, the modulation of the pain messages occurs at the spinal cord level. When the gates are open, pain messages reach the brain more or less easily and pain can be intense. When the gates are closed, pain messages are prevented from reaching the brain without even being experienced. The spinal cord receives inputs from 2 types of nerve fibers: Aδ fibers and the C-fibers (Steeds, 2009). A typical example of the Gate Control Theory is that after bumping one's knee, rubbing the area seems to provide some relief because this action activates other sensory nerve fibers that are even "faster" than Aδ fibers which send information about pressure and touch that reach the spinal cord and brain, overwriting some of the pain messages carried by the Aδ fibers and the C-fibers (Steeds, 2009).

One of the most critical aspects in pain research is its assessment; pain is a highly complex phenomenon that by its nature presents assessment difficulties. Pain is a subjective experience which is difficult to express and quantify, and is not directly measurable. Further difficulties occur since individual patients react to similar painful *stimuli* in many different ways. In addition many factors are known to influence pain response. They include ethnicity, sex, age, culture, personality, the type, duration and intensity of pain, and other psychological variables such as fear, anxiety and stress. However, the measurement of pain is essential for the study of its mechanisms and for the evaluation of pain control methods.

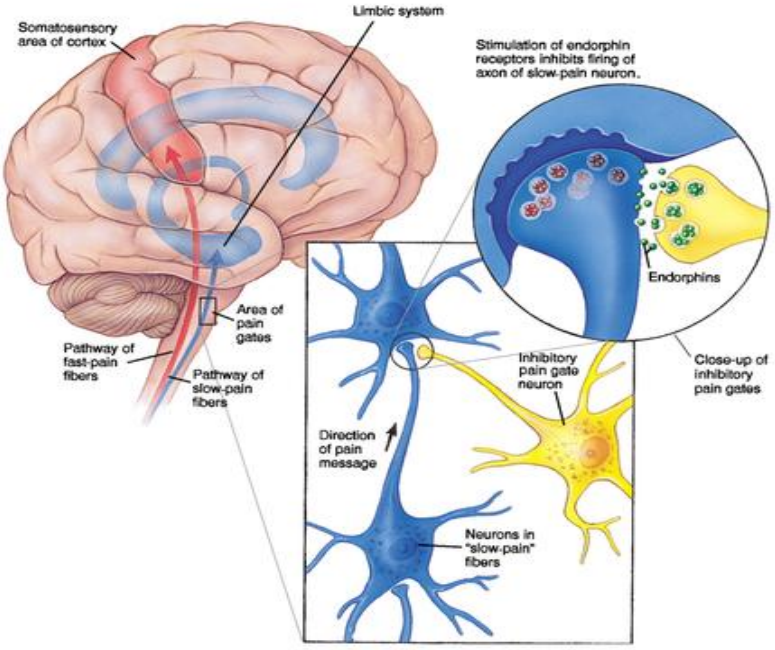

Figure 1: Illustration of the Gate Control Theory proposed Melzack and Wall in the 1960's. Available from http://www.garysturt.free-online.co.uk/pain.htm

2 Animal Models of Pain

Throughout history, scientists have performed experiments on animals to understand animal and human biological structure and function. Practical, economical and scientific reasons make preliminary studies in animals the best solution for studies of a human biological phenomenon. A laboratory animal model describes a biological observable fact that the species has in common with the target species, being "analogy" the key word for understanding the concept of animal models (Rose and Woodbury, 2001). What is generally understood by the term animal model is modelling humans. The focus of research is not the used animal but the analogy of physiological behaviour of this animal to our own species. The practice of studying biological phenomena and diseases in laboratory animal models is well established in biomedical sciences (Rose and Woodbury, 2001). The valid "extrapolatability" of results generated in an animal model depends on the selection of a suitable animal model. Induced animal models involve healthy animals in which the condition to be investigated is experimentally induced.

Animal models of pain have been invaluable tools for understanding pain mechanisms. To study pain transmission, identify new pain targets and characterize the potential analgesic profile of novel compounds, an array of experimental animal pain models has been developed (mainly in rodents) attempting to replicate the many human pain conditions, including inflammatory, neuropathic, visceral and cancer pain states (Walker et al., 1999; Joshi and Honore, 2006). The experience of pain is necessary for survival, so it is reasonable to assume that the mechanisms of pain perception are highly conserved across species (Liebeskind, 1991). This assumption has led to a vast array of important findings concerning pain via experiments conducted in rodents (Liebeskind, 1991). Animal models of pain are mostly models of nociceptive pain (pain arising from an identifiable lesion causing tissue damage, accompanied by stimulation of nociceptors in somatic or visceral structures) (Steeds, 2009). In animals, as well as in humans, in order to study pain of any kind, the appropriate *stimuli* must be applied in order to provoke the desired pain sensation. Different nociceptive pain tests use different *stimuli* with different characteristics: (i) *stimulus* may be thermal, mechanical or chemical; (ii) may or may not produce tissue damage; (iii) and may produce a short or a long lasting pain response (Franklin and Abbott, 1989).

Animal models of nociceptive pain have been developed as assays of pain behaviour and fall mainly into two categories: (i) those that employ an acute nociceptive *stimulus* and that are

concerned with short-term nociceptive responses; (ii) and those that are concerned with the long-term responses (Walker et al., 1999; Rose and Woodbury, 2001).

There are two main types of methods for assessing pain, based on the duration of the applied *stimulus*: (i) the response to the stimulation is fixed and stimulation intensity increases until a defined standard response occurs; (ii) the *stimulus* is standardized, and the strength and duration of response is measured (e.g. the formalin paw test and the writhing test) (Meyer and Svendsen, 2003). The formalin paw test and the writhing test constitute methods for the measurement of pain by behavioural responses to stimulation of nociceptors by injection of irritant substances and are amongst the most frequently used contemporary models of pain (Rose and Woodbury, 2001).

2.1 Formalin Paw Test

The formalin paw test represents a model of moderate continuous pain, suitable for the evaluation of mild analgesics (Hunskaar et al., 1985). The chemical stimulation involving the administration of formalin is a progressive stimulation, with a duration in the order of minutes and with an inescapable character once it has been applied (Le Bars et al., 2001). In 1939, Lewis and Kellgren injected small volumes of hypertonic saline to produce experimental pain in humans (Tjolsen et al., 1992). This kind of work preceded the use of formalin as a noxious agent for animals (Tjolsen et al., 1992). Dubuisson and Dennis (1977) introduced the formalin test, administering 50 µl of 5 % formalin under the dorsal surface of a forepaw of rats and cats.

Following subcutaneous injection of formalin into the hindpaw of an animal, it displays spontaneous pain behaviour that is increased hindpaw licking/biting. The formalin paw induced-pain response is difficult to assess in the forepaws of rodents, because normal grooming behaviour is typically based on animals licking the forepaws. The response to licking the hindpaws is assumed to be nociceptive (Tjolsen et al., 1992). The formalin-induced pain has been reported to be quantified in many different ways. Dubuisson and Dennis originally created behavioural categories that were used to assess formalin-induced pain by the weighted score method of behavioural rating (Coderre et al., 1993). Others used cumulative score methods to

assess pain-evoked responses, using a single parameter, such as scoring the total amount of time spent licking, biting or shaking the injected paw (Hunskaar and Hole, 1987).

Characteristically the formalin paw test produces a biphasic response, with the two phases being distinguished pharmacologically. An early acute phase (first phase) occurring immediately after the injection of the diluted formalin solution lasts for about 3 - 5 minutes, which is thought to be caused by direct chemical stimulation of nociceptors (Abbott et al., 1995). A 10 - 15 minutes gap between the two phases occurs until the second phase begins (Tjolsen et al., 1992). The first phase is considered by some authors to be the early acute phase together with the gap between the two phases lasting for about 15 minutes (Abbott et al., 1995). A later tonic phase (second phase) starting 15 minutes after the formalin injection and lasting for no more than 40 minutes, is believed to occur due to a peripheral inflammatory process mediated by prostaglandins (Tjolsen et al., 1992).

Formalin is the most common substance used as noxious *stimuli* for intradermal injections in several animal species. (Wheeler-Aceto and Cowan, 1991). Formalin is an aqueous solution of 37 % (w/v) formaldehyde. A range of 0.02 to 5 % formalin solution concentrations have been reported in mice, with the most often injected volumes ranging from 20 to 25 µl (Tjolsen et al., 1992). It has been demonstrated that the subcutaneous injection of different concentrations of formalin may induce the first phase or both phases; formalin concentrations of 0.05 to 0.2 % induce high licking/biting activity only in the first phase and formalin concentrations of 1 % or higher induce high licking/biting activity on both the first and the second phases (Rosland et al., 1990).

It has been reported that there are sex differences in the pain–related responses. Female mice show lower pain–related responses compared to males in the formalin paw test (Capone and Aloisi, 2004). Experimental results suggest that estrogen reduces the efficacy of endogenous pain modulation mechanisms, triggering an increase in spinal nociceptive neuronal activity (Spooner et al., 2007).

Many publications refer to the mouse paw formalin test being done using mice habituated and not habituated to the testing environment and handling (Capone and Aloisi, 2004). Exposure to handling-induced stress or to a new environment strongly influences the formalin-induced

behaviour in mice (Tjolsen et al., 1992). Animals should be well adapted to the test observation chamber because the occurrence of the typical exploratory activity of animals in a new environment could distract them from the formalin-induced pain response. They should also be well habituated to handling and restraining because any abnormal discomfort may provoke stress in the animals which can mask the formalin-induced pain results.

2.2 Writhing Test

The writhing test consists of intraperitoneal injection of agents that irritate serous membranes, inducing a very typical behaviour in the mouse and rat which is characterized by abdominal contractions, movements of the hindpaws and twisting of dorsoabdominal muscles (Hendershot and Forsaith, 1959). Some authors consider these behaviours as resulting from visceral pain and others reject this hypothesis and consider the pain as arising from peritoneal pain (Le Bars et al., 2001). Several compounds are used, the most common being the acetic acid. Several modifications to this method have been made over the years, mainly concerning the chemical agent which causes the *stimulus*, its concentration and the volume of the injected solution.

Acetic acid is an algogenic substance that directly activates peripheral nociceptors on the sensory nerve fibers by inducing capillary permeability and releasing endogenous substances that excite pain nerve endings (pro-inflammatory substances) producing inflammation of visceral (subdiaphragmatic) and subcutaneous (muscle wall) tissues (Cervero, 1995). This produces the characteristic ‘writhing response’: lengthwise stretches of the torso with a concomitant concave arching of the back and extensions of the hind limbs (Collier et al., 1968; Bentley et al., 1981). Mice and rats present a very stereotyped behaviour when administered intraperitoneally with agents that irritate the serous membranes (Rose and Woodbury, 2001).

The acetic acid-induced writhing is a pain model widely used for detecting peripheral analgesia (Du et al., 2007). The original test was described in 1957 by Siegmund et al. and used phenylbenzoquinone as irritating agent but since then many modifications have been made to the method mainly concerning the chemical agent that, in turn, determines the duration of the effect:

acetylcholine, dilute hydrochloric acid, bradykinin, adrenaline, potassium chloride, tryptamine, ocytocin and acetic acid have all been used (Le Bars et al., 2001). Modifications to the concentration, temperature, and volume of the injected solution, the experimental conditions, and ways of monitoring behavioural changes have also been made in order to simplify the test and increase its sensitivity (Le Bars et al., 2001). This method has the advantage of allowing evidence to be obtained for effects produced by weak analgesics (Le Bars et al., 2001). Because all analgesics inhibit abdominal cramps, this method is useful for sorting through molecules whose pharmacodynamic properties are unknown (Le Bars et al., 2001).

3 Other Behavioural Models

3.1 Rotarod Test

The loss of motor coordination is one of the most promptly observed effects of drug intoxication. The rotarod test is widely used in biomedical research as one of the most commonly used tests of motor incoordination in rodents. Dunham and Miya (1957) were the first to describe a fixed speed rotating rod, called by these authors "rolling roller apparatus" for detecting neurological deficits in rats and mice. It is frequently used in early stages of drug development to screen-out drugs that might later cause impairment in human motor behaviour. Ataxia (loss of motor coordination) is a frequent endpoint for studies of drug intoxication. One of the most commonly used behavioural models of ataxia is the rotarod test (Rustay et al., 2003). The rotarod test assesses the effect of drugs on an animal's balance and coordination or fatigue resistance on mice and rats, by measuring the amount of time that an animal is able to remain on a longitudinally rotating rod. Animals have to keep their balance on a rotating textured rod. When an animal drops onto the individual sensing platforms below, test results are recorded. Performance is measured by the time that an animal stays on the rod as a function of drum speed.

Many different rotarod systems are available from a number of companies, but almost all apparatus will automatically record the time at which the animal falls, and allow several animals to be tested at the same time (Carter et al., 2001). Many significant modifications have been made to the basic design of the first described rotarod test, like accelerating the rod continuously until the mouse or rat can no longer balance and fall, changing rod diameters from 3 - 8 cm, using fixed-speed rates of 3 - 31 rpm and acceleration rates of 3.5 - 60 rpm/min (Rustay et al., 2003). There are two basic versions of the rotarod test that are widely used: the fixed-speed and the accelerating-speed rotarod (Rustay et al., 2003; Monville et al., 2006). There is still little information on their equivalence or the relative power, reliability and sensitivity of the two protocols. The fixed-speed rotarod system allows changing parameters as rod diameter, maximum time limit for performance and rate of rotation, and requires extensive training of the animals. The accelerating-speed rotarod system eliminated the existence of a maximal time limit for performance, providing a model that starts at a slow speed allowing all animals to stay on and

accelerating at a constant rate until it becomes difficult enough so that all animals would eventually fall off (Rustay et al., 2003). This model allows for the measurement of drug-induced increased in performance, which is difficult to observe using a low rotation rate in the fixed-speed model.

Some strains of rodents are not suitable for usage in the rotarod test due to their propensity to jump from the rod instead of running on top. Also some strains have a propensity to hold onto the rod and instead of actively completing the task, they passively rotate around. Rustay et al. (2003) engineered a way to circumvent this confounding factor by using a rod of 6.3 cm of diameter, which was large enough to prevent most mice from being able to hold to the rod, avoiding passive rotation. These authors also recommend that, independently of the usage of a fixed-speed or accelerating-speed rotarod system, mice should be trained to a stable level of performance before drug administration so that changes seen after drug administration are the result of drug action and not due to effects on learning on the apparatus (Rustay et al., 2003).

3.2 Open-field Test

Open-field test is a common measure of general locomotion activity and exploratory behaviour in rodents (Walsh and Cummins, 1976). It was first developed by Hall in 1934 (Choleris et al., 2001) and it is commonly used to assess the sedative, toxic, or stimulant effects of compounds (Gould et al., 2009). The open-field test was defined by Choleris et al. (2001) as "an enclosed open area where an animal is placed and some form of behaviour, usually activity, is measured". The open-field test was widely used in psychology and it has gradually spreaded out to neurosciences and psychopharmacology (Tvrdeic and Kocevski, 2008). The original studies were in rats, but this test has also been extensively used in mice (Gould et al., 2009).

This test allows the measurement of both qualitative and quantitative activity. But, although sometimes the open-field test is considered a "standard test", many factors can vary among studies like size of the open field arena, shape, level of illumination, single exposure versus repeated exposure to the open-field arena, duration of testing, time of day of testing, food and water deprivation, sex of the animals, predator odour in the arena (Choleris et al., 2001). Most of the automated open-field test systems allow the measurement of the locomotor activity

by determining the amount of distance travelled as well as the various horizontal, vertical and stereotyped behaviours like time spent along the walls compared to time spent in the centre, distance moved over different time periods, and rearing. Typically, in psychopharmacological studies, animals are naive to the open-field arena and the effects of the pharmacological agents on the duration and frequency of some behaviour like locomotion and rearing are assessed (Choleris et al., 2001).

4 Antiepileptics as Adjuvant Analgesics

Pain can be treated in several different ways. Analgesics are drugs that relieve pain without blocking the conduction of nerve impulses or markedly altering the function of the sensory apparatus (Álamo and López-Muñoz, 2007). Analgesics are taken both to control acute and chronic pain. Analgesics are classified is three categories: (i) opioid analgesics, also called narcotics; (ii) non-opioid analgesics, also called non steroidal anti-inflammatory drugs – NSAIDs; and (iii) adjuvant analgesics, which are drugs with a primary indication other than pain but that have analgesic properties (Lussier et al., 2004; Becker and Phero, 2005).

Throughout the clinical usage of antiepileptic drugs, it has been observed that these drugs can be used in many diseases other than epilepsy. Antiepileptic drugs are already used in the treatment of bipolar disorder (Dilsaver et al., 1996), impulse control disorder (Berlin, 2008), eating disorders (Kaplan et al., 1983) and specially to manage pain (McQuay et al., 1995). Antiepileptic drugs have been used in the management of pain since the 1960s (McQuay et al., 1995). The presence of common clinical and physiopathological characteristics between epilepsy and several pain types made possible the usage of different antiepileptic drugs as they would appear in the market (Álamo and López-Muñoz, 2007). In 1912, phenobarbital, the first antiepileptic drug was marketed, but the appearance of more antiepileptic drugs has occurred at a very slow step (Álamo and López-Muñoz, 2007). One group of antiepileptic drugs containing carbamazepine and that is called nowadays classic antiepileptic drugs, was commercialized just before 1970 (Álamo and López-Muñoz, 2007). In 1990 more antiepileptics such as oxcarbazepine were marketed, being more effective, presenting less adverse effects and showing better pharmacokinetic and pharmacodynamic characteristics than the previously marketed antiepileptic drugs (Álamo and López-Muñoz, 2007). In 2009, about twenty years later, eslicarbazepine acetate was developed as a new antiepileptic drug which is structurally related to carbamazepine and oxcarbazepine, but with improved tolerability profiles and efficacy (Dulsat et al., 2009).

4.1 Carbamazepine

Carbamazepine (Figure 2) was first introduced in 1963 in Switzerland and in the United

Kingdom for clinical treatment of epileptic seizures and has become the most frequently prescribed first-line drug for the treatment of partial and generalized tonic-clonic epileptic seizures all over the world (Araujo et al., 2004).

Although it is very effective in the majority of cases, soon it started showing a number of unwanted side effects. In clinical trials, about 10 to 25% of patients administered with carbamazepine showed adverse effects, with a higher incidence in the elderly (Sillanpaa et al., 2009). Carbamazepine active epoxide metabolite is the responsible for a number of adverse effects associated with carbamazepine therapy (Grant and Faulds, 1992). The main metabolic pathway is epoxidation which is catalysed primarily by the cytochrome P450 and results in the formation of a pharmacologically active carbamazepine epoxide metabolite, contributing to both therapeutic and adverse effects (Sillanpaa et al., 2009). Carbamazepine clinical application is complicated by the potent induction of hepatic oxidative metabolism, the potential for adverse reactions and its slow and variable absorption (Hainzl et al., 2002).

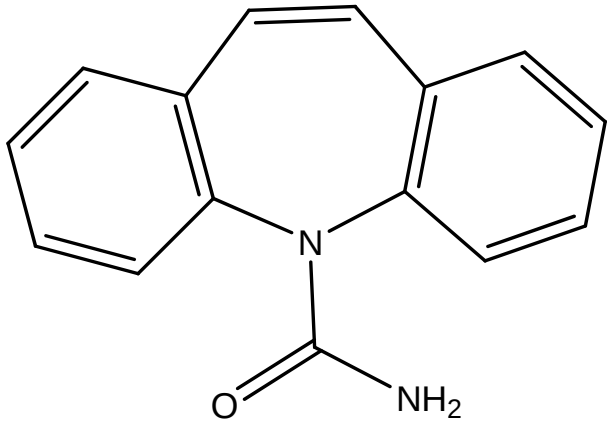

Figure 2: Chemical structure of carbamazepine.

Carbamazepine has been used for the treatment of neuropathic pain in humans. Recently, several studies have reported that carbamazepine has antinociceptive activity, for it has shown to be effective in different animal models of acute pain induced by inflammatory, thermal and mechanical nociceptive *stimuli* (Aoki et al., 2006).

4.2 Oxcarbazepine

Oxcarbazepine (Figure 3) was introduced commercially in Denmark in 1990, has been available throughout the European Union since 1999 and was launched in 2000 in the USA for the management of seizures (Faught and Limidi, 2009). Oxcarbazepine is an antiepileptic drug derived from carbamazepine.

Oxcarbazepine is the 10 - keto analogue of carbamazepine with a differing metabolic profile that manages to circumvent the carbamazepine adverse effects, retaining similar therapeutic utility (Grant and Faulds, 1992). After administration, oxcarbazepine is rapidly reduced to an active metabolite that is present in human plasma at much higher concentrations than the parent drug and that is the principal active agent (Faught and Limidi, 2009). Oxcarbazepine and its metabolite are neutral lipophylic agents that might be expected to easily pass through the blood-brain barrier and are widely distributed in the body without any significant affinity to a particular site (Álamo and López-Muñoz, 2007). The elimination of the double bond between C-10 and C-11 prevents oxidative attack by an epoxidase, which is what occurs with carbamazepine. It has a better side-effect profile and many fewer metabolic drug-drug interactions than carbamazepine, because it is metabolized by a non cytochrome P450 pathway (Grant and Faulds, 1992).

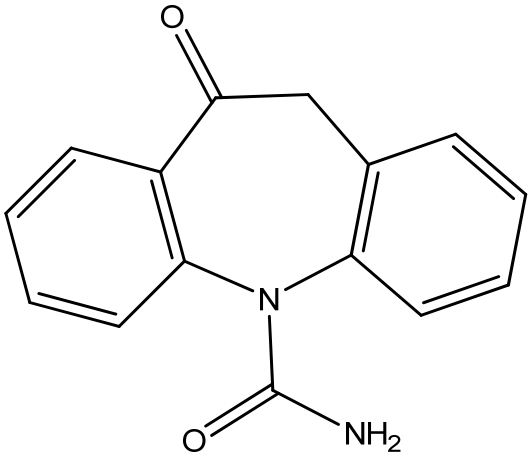

Figure 3: Chemical structure of oxcarbazepine.

Oxcarbazepine exerted analgesic effects in animal models of neuropathic, inflammatory, somatic, and visceral pain (Tomic et al., 2010). The mechanisms of analgesia of oxcarbazepine are not completely understood but it was demonstrated that it suppresses peripheral sensory nerve firing, probably by blockade of sodium currents, and also affects some receptors involved in pain modulation to produce antinociception (Tomic et al., 2010).

4.3 Eslicarbazepine Acetate

Eslicarbazepine acetate (Figure 4) is a drug clinically developed for the treatment of epilepsy, with EMEA recently granting marketing authorization to BIAL (Dulsat et al., 2009). It is currently under clinical development for the treatment of neuropathic pain (Almeida et al., 2008; Almeida et al., 2009).

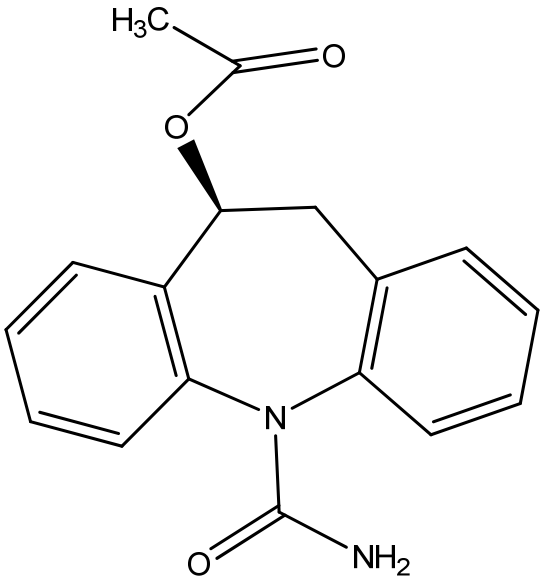

Figure 4: Chemical structure of eslicarbazepine acetate.

Eslicarbazepine acetate is a novel voltage-gated sodium channel blocker, designed to share with carbamazepine and oxcarbazepine the dibenzazepine nucleus bearing the 5-carboxamide substitute, but it is structurally different at the 10,11-position (Benes et al., 1999; Hainzl et al., 2001; Almeida and Soares-da-Silva, 2007). This molecular variation results in differences in metabolism which, among other things, prevents the formation of toxic epoxide metabolites (Almeida and Soares-da-Silva, 2003). Eslicarbazepine acetate mainly undergoes metabolic hydrolysis followed by glucoronidation with minimal CYP-mediated metabolism (Almeida et al., 2009). Oral administration of eslicarbazepine acetate to humans results in very low plasma concentrations of parent compound which is extensively biotransformed to S-licarbazepine by first-pass hydrolytic metabolism, being clearly the major circulating drug (Almeida et al., 2008).

There have been no concerning findings for human use of eslicarbazepine acetate, based on conventional preclinical studies of safety, pharmacology, toxicology, genotoxicity, reprotoxicity and carcinogenity, which makes believe that its use will undoubtedly increase in the future (Almeida et al., 2009).

5 Objectives

5.1 General Objective

Development and implementation of animal models for evaluation of drugs with potential analgesic activity, in NMRi mice.

5.2 Specific Objectives

- Development and implementation of the formalin paw test in mice;
- Development and implementation of the writhing test in mice;
- Development and implementation of the rotarod test in mice;
- Development and implementation of the open-field test in mice;
- Examination of the dose-dependent effect of eslicarbazepine acetate on antinociception using the formalin paw and the writhing tests, on motor function using the rotarod test and on spontaneous exploratory behaviour and general activity using the open-field test;
- Determination of the protective index of eslicarbazepine acetate.

CHAPTER II

MATERIAL AND METHODS

1 Reagents

All reagents and solutions used in all experimental procedures were of at least standard laboratory grade. The water used was obtained from an ultra-pure water producing system.

The reagents used and their respective suppliers are presented next:

- Acetic Acid (Sigma-Aldrich®);
- Carbamazepine (Sigma-Aldrich®);
- Carboxymethyl cellulose (Sigma-Aldrich®);
- Eslicarbazepine Acetate (BIAL-Portela & C^{a}, S.A.);
- Ethanol (Sigma-Aldrich®);
- Formalin solution 10 % (Sigma-Aldrich®);
- Morphine (Sigma-Aldrich®);
- Oxcarbazepine (ChemPacific);
- Sodium Chloride (Sigma-Aldrich®).

2 Drugs, Solutions and Treatment Groups

Administered vehicle was carboxymethyl cellulose (CMC) 0.5 % in ultra-pure water.

The test drug eslicarbazepine acetate, the comparison drugs carbamazepine and oxcarbazepine and the reference drug morphine were dispersed in CMC 0.5 %.

Formalin (5 %) was obtained by dilution of the formalin solution 10 % in sodium chloride 0.9 % in ultra-pure water.

Acetic acid (0.8 %) was obtained by dilution of acetic acid reagent in sodium chloride 0.9 % in ultra-pure water.

Animals administered with the vehicle substance were considered to be the control group. Animals administered with the different concentrations of the test drug eslicarbazepine acetate (1 - 450 mg/kg), the comparison drugs carbamazepine (100 and 300 mg/kg) and oxcarbazepine (100 and 300 mg/kg), and the reference drug morphine (64 mg/kg) were considered to be the treated groups.

3 Laboratory Equipment

The laboratory equipment used is presented next:

- 26 gauge needle attached to a micro syringe by PE10 tubing;
- 29 gauge needle attached to a micro syringe by PE10 tubing;
- Automatic Pipettes Gilson PIPETMAN®;
- Balance Kern® ABJ;
- Balance Mettler Toledo® XP26;
- Chronometer;
- Magnetic Stirrer IKA®;
- Manual counter;
- MilliQ water system (Millipore ®);
- Mirror;
- Plexiglass observation chambers (20 x 26 x 26 cm);
- Potentiometer WTW® pH 3000 with coupled electrode SenTix® 21;
- Rotarod apparatus with a rotating cylinder with a 6.3 cm diameter rod (Ugo Basile Biological Research Apparatus);
- San Diego Instruments Photobeam Activity System – Open Field composed by:
    - Clear acrylic enclosure (40 cm wide x 40 cm deep x 38 cm high);
    - Photobeam mounding frame (with 16 photobeams in each direction, spaced by 2.5 cm);
    - Rear frame;
    - Control unit;
- Ultrasonic bath;
- Vortex mixer SA8 Stuart®.

4 Softwares

The softwares used for data collection and data analysis are the ones described next:

- GraphPad Prism® 5.02 (GraphPad Software, Inc.);
- Microsoft® Office Excel 2007;
- Photobeam Activity System Control software for Windows 2000/XP (version 2.0).

5 Animals

5.1 Species Used

Male NMRi mice, 30 - 40 g body weight range were used. They were supplied by Harlan Interfauna Ibérica, S. L., Spain.

5.2 Animal Housing

Animals were delivered to the laboratory (Laboratory of Pharmacological Research of the Department of Research and Development at BIAL - Portela & Cª S. A.) 2 weeks before the experiment, during which time they were acclimatized to the laboratory conditions. They were housed in groups of 5 in macrolon cages (25 x 19 x 13 cm) on wood litter (Litalabo - SPPS, 95100 Argenteuil, France) with free access to food (food certified by Harlan Teklab Global Diet®) and water until the day before being tested. The animal house was maintained under artificial lighting (12 hours) between 8 am and 8 pm, at controlled ambient temperature of 22 ± 2 ºC, and relative humidity of 60 ± 10 %.

5.3 Ethical Aspects

The EC directive states that an animal should not be kept alive after an experiment if pain is experienced. At the end of the experiment, animals were sacrificed by the mechanical-physical method of dislocation of the cervical vertebrae, by stretching the animal and rotating the neck, away from the presence of other animals, which constitutes a painless method (Hellebrekers et al., 2001). These procedures were executed in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA), which is in accordance with the European Directive no. 86/609 and with the Portuguese legislation (Decreto-Lei 129/92, Portarias 1005/92 and 1131/97). The number of animals used was the minimum possible in compliance with current regulations and scientific integrity.

5.4 Justification for the Choice of Species, Route of Administration and Dose Levels

The choice of the species for a chosen animal model depends on the homology and evolutionary similarity between morphological structures and physiological processes between animals and humans (Beynen and Hau, 2001). Although mice and rats have many concurrent biological characteristics, they do not necessarily serve equally well as models of human malady. Mouse was chosen as animal model because of economical advantages and also because mice have shown the most similar metabolism to humans after administration of eslicarbazepine acetate (Alves et al., 2008).

The route of administration of compounds and vehicle were chosen based on the intent of oral administration of the compounds to humans. Mice animals were deprived of food for 18 hours prior to the experiment to avoid any possible food-drug interaction.

The study included several doses for the assessment of pain. The chosen doses were based on the envisage determination of the effective doses for analgesic effect on the developed models.

To avoid any possible effect of the circadian rhythm in the animal models of pain developed during this experimental work, half of the tested animal for each group were tested in the morning and half in the afternoon.

6 Rotarod Test

The rotarod test allows the assessment of motor coordination and balance in rodents. Using rotating rods allows one to evaluate the duration that mice and rats can maintain balance at a defined rotating speed. Dunham and Miya (1957) described the first rotating rod for detecting neurological deficit in rats and mice.

6.1 Habituation to Test Environment

The newly acquired mice were housed for one week prior to being brought to the testing room for habituation. Adaptation was carried out in the testing room by transferring the animals, in their home cages, from the housing room to the testing room 2 hours before the training trials or before the test (Carter et al., 2001). For habituation to the rotating rod (Figure 5), two days and on the day before the experiment, each mouse was placed individually on the rotating rod and allowed to habituate to the forced motor activity for up to 60 seconds. Upon fall, each animal was returned to its home cage and allowed an inter-trial interval of 5 minutes. Mice were trained until they were able to remain on the rod for 2 consecutive trials (Carter et al., 2001). On the day of the experiment all animals were tested for their ability to stay on the rod for at least 60 seconds, to obtain the pre-drug time spent on the rod. Habituation to restraining and handling was performed between 8.00 am and 5.00 pm on the day before the rotarod test.

6.2 Experimental Procedure

The rotarod apparatus had a 6.3 cm diameter rod, suitably machined to provide grip and a fall high of 17 cm (Rustay et al., 2003). In this apparatus, a motor sets the rotor in motion via the gear belt at a selected speed. When the mouse falls off its cylinder section, the plate below trips and the corresponding counter disconnects, thereby recording the animal's endurance time in seconds. The machine was set at a revolving consistent speed of 15 rpm; always before training the animals or testing them, the rotarod apparatus was manually calibrated so that it was revolving at a consistent speed of 15 rpm (Stepanovic-Petrovic et al., 2008).

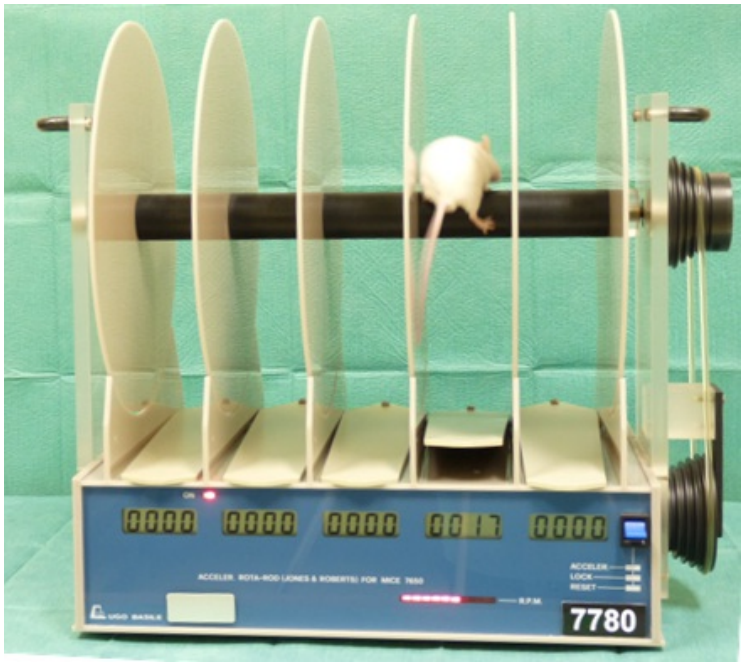

Figure 5: Rotarod apparatus for testing motor coordination.

During the training sessions and at the end of testing each animal, the apparatus was thoroughly cleaned with 70 % (v/v) ethanol. The reason for this last described procedure is that animals defecate and urinate while on the rod and urine and faeces on the rod can impair the performance of the animals.

After adaptation of the animals to the testing room, the animals were separated in groups and each group was constituted by 10 animals under study, being each animal tested only once. Mice were placed on the rod while it was moving. The vehicle, test, comparison or reference drugs were administered orally 59 minutes before the test. All groups were administered by oral gavage at dosing volume of 8 ml/kg of body weight. Motor impairment was assessed for 60 seconds.

The time that the animal remained walking on the rod was scored in seconds, with the task ending when the animal fell off the rotarod or completed the 60 seconds trial. Many authors are concerned about how to score passive rotation when the animal grips on to the rotarod. That problem was circumvented here by using a 6.3 cm diameter rod, as proposed by Rustay et al. (2003). This author observed that using rods with 6.3 cm diameter or greater, the number of animals gripping on to the rotarod was neglectable.

6.3 Data Presentation

The results are expressed as mean ± S.E.M. (standard error of the mean) of the time spent on the rotarod in seconds. MS Excel was used to calculate mean and S.E.M..

The TD_{50} (the dose that induces motor impairment in 50 % of animals tested) was calculated from a corresponding quantal dose–response curve, with 95 % confidence limits. The Boltzmann sigmoidal equation was used to derive the TD_{50} value. GraphPad Prism was used to calculate the TD_{50} value.

7 Open-Field Test

The open-field test was introduced in the 1930s by Calvin Hall (Leroy et al., 2009). The open-field test is a common measure of spontaneous exploratory behaviour and general activity in rodents that is often used to assess the sedative, toxic and stimulant effects of drugs. Recent open-field test systems allow registering general motor activity, locomotion, rearing and the speed of locomotion.

7.1 Habituation to Test Environment

The newly acquired mice were housed for one week prior to being brought to the testing room for habituation. Adaptation was carried out in the testing room by transferring the animals, in their home cages, from the housing room to the testing room 2 hours before the test and by transferring the animals from the rotarod to the open-field arena. Habituation to handling was performed between 8.00 am and 5.00 pm. The open-field test system is shown in Figure 6.

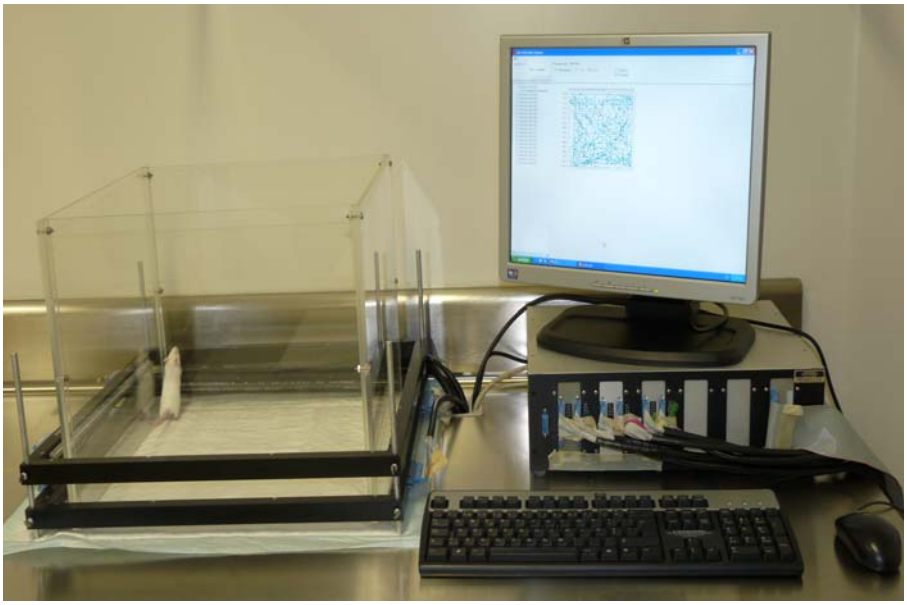

Figure 6: Photobeam Activity System – Open Field.

7.2 Experimental Procedure

The open-field arena consisted of an empty and transparent square arena, surrounded by walls that prevent the animal from escaping. A setup frame was mounted to fit a clear acrylic enclosure (40 cm wide x 40 cm deep x 38 cm high). The frame contains 2 rows of photobeams (16 photobeams mounted in each direction), spaced by 2.5 cm. Above the photo beam frame a rear frame was mounted to allow quantification of the number of rears that the animal performed. The photobeam frame was placed 30 mm above the top of the bench and the rear frame placed 80 mm above the top of the bench. Interruptions of the photobeams were collected by a computer by means of Photobeam Activity System Control software. Two parameters were evaluated: ambulation and rearing. Ambulation allows the assessment of normal motor activity and rearing allows the assessment of exploratory behaviour. The software identifies these two parameters as:

- Ambulation - all interruption of the photobeams in the lower rows;
- Rearing - interruption of the photobeams in the upper rows (rear frame).

After adaptation of the animals to the testing room, the animals were separated in groups of 10 mice with each animal being tested only once. The vehicle, test, comparison or reference drugs, were administered orally 60 minutes before the test by oral gavage at dosing volume of 8 ml/kg of body weight. The same grouped mice tested for the rotarod test were tested in the open-field test. So, immediately after the rotarod test, mice were placed in the centre of the arena and allowed to explore for 15 minutes.

7.3 Data Presentation

The results are expressed as mean ± S.E.M. of the ambulation frequency and of the rearing frequency. Frequencies were defined as the number of occurrences per 15 minutes test time. MS Excel was used to calculate mean and S.E.M..

8 Formalin Paw Test

The formalin paw test was used to assess the antinociceptive effect of the administered drugs. The test described here is a modification of the one proposed by Hunskaar et al. (1985).

8.1 Habituation to Test Environment

Newly acquired mice were housed for one week prior to being brought to the testing room for habituation. Adaptation was carried out in the testing room on the day before the experiment by placing the animals singly in the observation chambers for 2 hours and on the day of the experiment by transferring the animals, in their home cages, from the housing room to the testing room 2 hours before the formalin paw test. The mouse observation chambers were clear plexiglass chambers with a mirror placed behind the chamber wall to allow an unobstructed view of the tested paw. Habituation to restraining and handling was performed between 8.00 am and 5.00 pm on the day before the formalin paw test. The observation chamber is shown in Figure 7, as well as a counter and a chronometer.

Figure 7: Observation chamber, chronometer and counter.

8.2 Experimental Procedure

After adaptation of the animals to the testing room, testing chamber, handling and restraining, using a 26 gauge needle attached to a micro syringe by PE10 tubing, 25 µl of the 5 % formalin was injected subcutaneously into the left dorsal part of the hindpaw of the mouse (Figure 8). Each animal was manually gently restrained in such a way that allowed the referred injection to occur. After the injection of the nociceptive *stimulus*, the animals were allowed to move freely during the observation period.

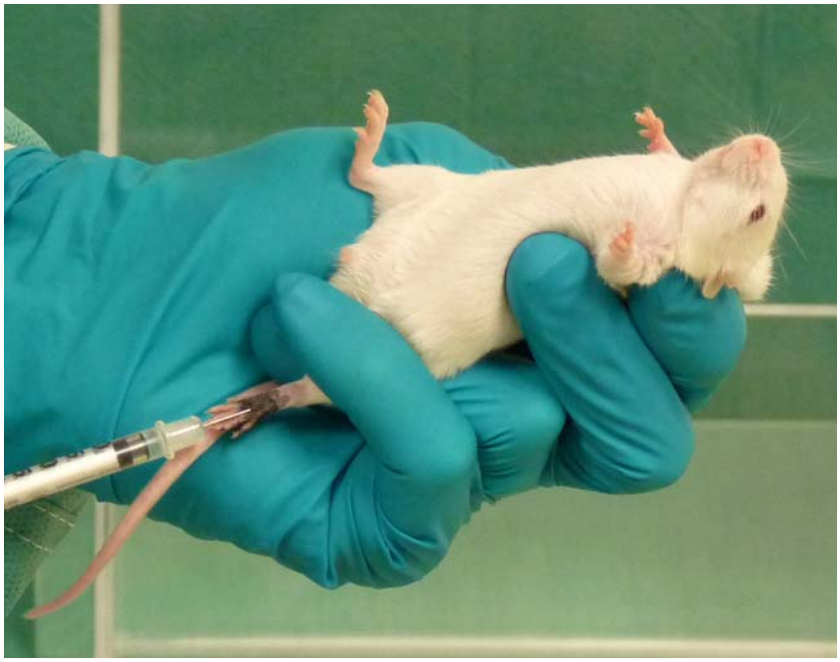

Figure 8: Subcutaneous injection of formalin solution in the dorsal part of the left hindpaw of the mouse.

The vehicle, test, comparison or reference drugs, were administered orally 60 minutes before the test (i.e. 45 minutes before formalin) by oral gavage at a dosing volume of 8 ml/kg of body weight. Each group was composed of 10 animals under study. Each animal was tested only once and the tested paw was marked with a colour marker prior to the formalin injection, to allow

a clear observation of the formalin pain-evoked behaviour.

The mice were observed from 15 to 30 minutes following formalin injection. During the first 15 minutes post formalin injection, pain behaviour reflects the early phase of the formalin paw test and readings were neglected. The late phase of the formalin paw test is observed via pain behaviour in the 15 to 30 minutes time period following formalin injection and the pain-evoked behaviour was scored for this phase. The amount of time that each animal spent licking or biting the injected paw (pain-evoked behaviour) was timed with a chronometer. No food or water was available during the test. Figure 9 shows a mouse executing the typical pain-evoked behaviour caused by subcutaneous injection of formalin in the dorsal part of the left hindpaw.

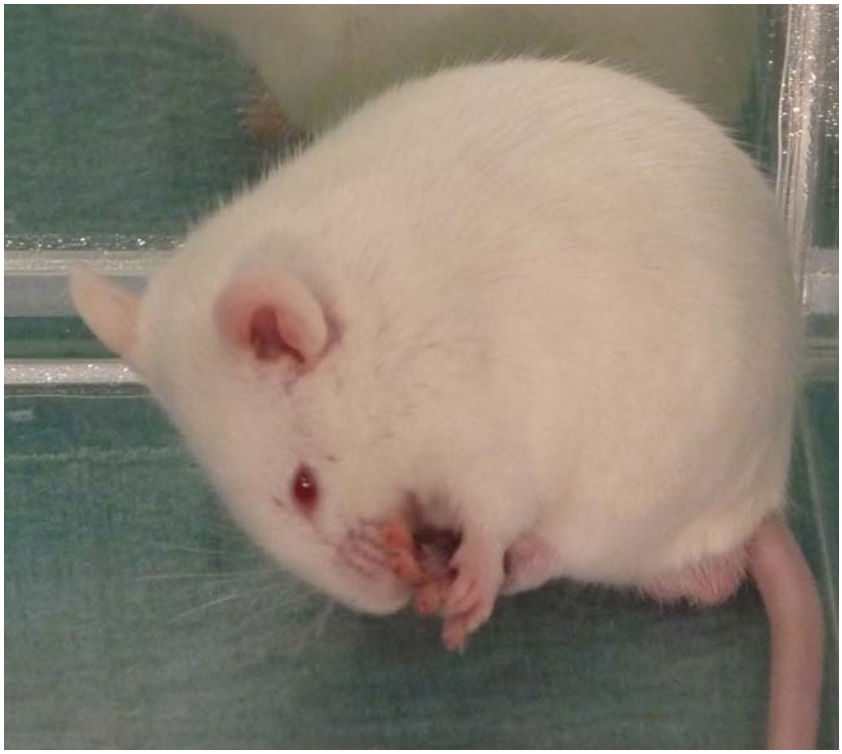

Figure 9: Mouse licking formalin injected paw after subcutaneous injection of formalin solution in the dorsal part of the left hindpaw.

8.3 Data Presentation

The results are expressed as mean ± S.E.M. of the amount of time that each animal spent licking or biting the injected paw after subcutaneous injection of formalin into the left dorsal part of the hindpaw of the mouse or as % protection conferred by orally administered drugs versus the vehicle administered group. The % protection was calculated as follows:

$$\% \text{ Protection} = \frac{\text{FCmean - FTmean}}{\text{FCmean}} \times 100 \quad (1)$$

"FCmean" represents the mean result for the amount of time that the control group mice spent licking/biting the injected paw following formalin injection.

"FTmean" represents the mean result for the amount of time that the treated group mice spent licking/biting the injected paw following formalin injection.

MS Excel was used to calculate mean, S.E.M. and % protection values.

The values of ED_{50} (the dose that was antinociceptive in 50 % of animals tested) were estimated from the corresponding log dose-response curve, with 95 % confidence limits. GraphPad Prism was used to calculate the ED_{50} values.

9 Writhing Test

The antinociceptive effect of the administered drugs was assessed using the acetic acid-induced writhing test. The test described here is a modification of the one proposed by Collier et al. (1968).

9.1 Habituation to Test Environment

As for the formalin test, newly acquired mice were housed for one week prior to being brought to the testing room for habituation. Adaptation was carried out in the testing room on the day before the experiment by placing the animals singly in the observation chambers (Figure 7) for 2 hours and on the day of the experiment by transferring the animals, in their home cages, from the housing room to the testing room 2 hours before the writhing test. A mirror was placed behind the observation chamber wall to allow an unobstructed view of the acetic acid pain-evoked behaviour revealed by the animal. Habituation to restraining and handling was performed between 8.00 am and 5.00 pm on the day before the writhing test.

9.2 Experimental Procedure

After adaptation of the animals to the testing room, testing chamber, handling and restraining, using a 29 gauge needle attached to a micro syringe by PE10 tubing, mice were injected intraperitoneally with a 0.8 % (v/v) acetic acid solution (10 ml/kg of body weight) as an irritant *stimulus* and placed in the observation chamber for observation. Each animal was manually gently restrained in such a way that allowed the referred injection to occur. After the injection of the nociceptive *stimulus*, the animals were allowed to move freely during the observation period. Figure 10 shows the intraperitoneal injection of acetic acid solution to the mouse.

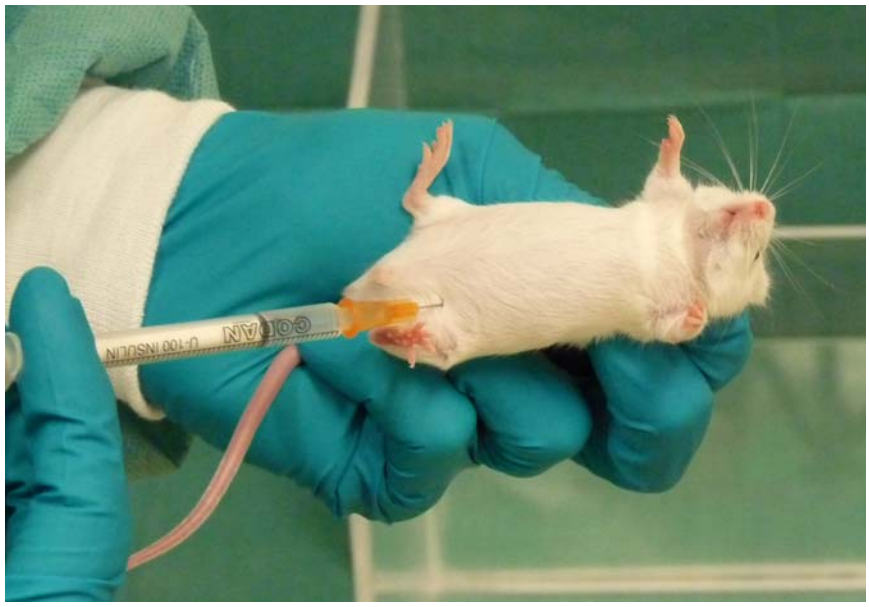

Figure 10: Intraperitoneal injection of acetic acid solution.

For the assessment of antinociception using the acetic acid-induced writhing test, the animals were separated in groups of 10 animals and each animal was tested only once. The vehicle, test, comparison or reference drugs were administered orally 60 minutes before the test and their ability to reduce the acetic acid pain-evoked behaviour was determined. All groups were administered by oral gavage at dosing volume of 8 ml/kg of body weight.

After the intraperitoneal injection of the acetic acid solution, the acetic acid pain-evoked behaviour was assessed. Five minutes post acetic acid injection, the number of accumulated writhes was recorded for 10 minutes, using a manual counter. A writhe consists of the contraction of the abdominal muscles accompanied by stretching of the hind limbs (Collier et al., 1968; Bentley et al., 1981). Figure 11 shows a characteristic writhe of a mouse injected intraperitoneally with acetic acid.

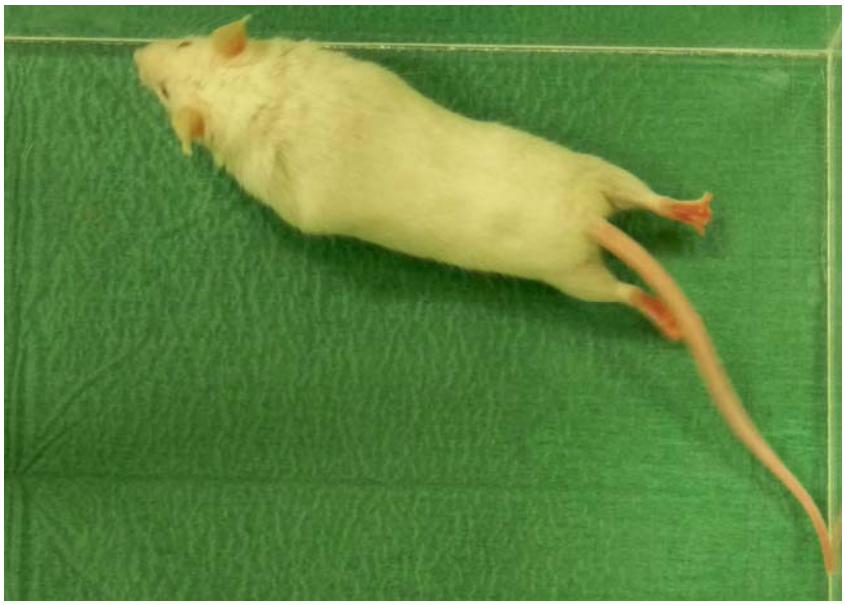

Figure 11: Mouse performing a writhe in the writhing test.

9.3 Data Presentation

The results are expressed as mean ± S.E.M. of the number of writhes induced by the intraperitoneal injection of acetic acid or as % protection conferred by orally administered drugs versus the vehicle administered group. The % protection was calculated as follows:

$$\% \text{ Protection} = \frac{\text{WCmean - WTmean}}{\text{WCmean}} \times 100 \quad (2)$$

“WCmean” represents the mean result for the number of writhes performed by the control group mice following acetic acid injection.

“WTmean” represents the mean result for the number of writhes performed by the treated group mice following acetic acid injection.

MS Excel was used to calculate mean, S.E.M. and % protection values.

The value of ED_{50} was estimated from the corresponding log dose-response curve, with 95% confidence limits. GraphPad Prism was used to calculate the ED_{50} value.

10 Statistical Analysis

Statistical analysis was done using software GraphPad Prism.

Statistical analysis of the data was evaluated by means of one-way ANOVA, applied to pairs of data for unpaired samples. For comparisons made between the treated and control groups, one-way ANOVA was followed by Dunnett's test. For comparisons made between all pairs of data, the post hoc Bonferroni test was applied.

All differences between pairs of data were considered significant when the null hypothesis could be rejected at a risk α of less than 0.05 ($p < 0.05$).

CHAPTER III

RESULTS

1 Rotarod Test and Open-Field Test

1.1 The Effect of Eslicarbazepine Acetate, Carbamazepine and Oxcarbazepine in the Rotarod Test in Mice

Mice were examined for motor incoordination in the rotarod test. All mice were trained to a stable level of performance before drug administration so that changes seen after drug administration are the result of drug action and not due to effects on learning on the apparatus.

In the rotarod test in mice, eslicarbazepine acetate (1 - 450 mg/kg) did not cause a reduction of the time spent on the rotarod, compared with the control group, except at the highest administered dose that caused a significant reduction. Carbamazepine (100 and 300 mg/kg) and oxcarbazepine (100 and 300 mg/kg) produced significant toxic effects on motor performance in mice compared with the control group but only at the highest tested dose (300 mg/kg). The effect on motor performance of mice orally administered with eslicarbazepine acetate (1 - 450 mg/kg.), carbamazepine (100 and 300 mg/kg.) and oxcarbazepine (100 and 300 mg/kg) are presented in Figure 12.

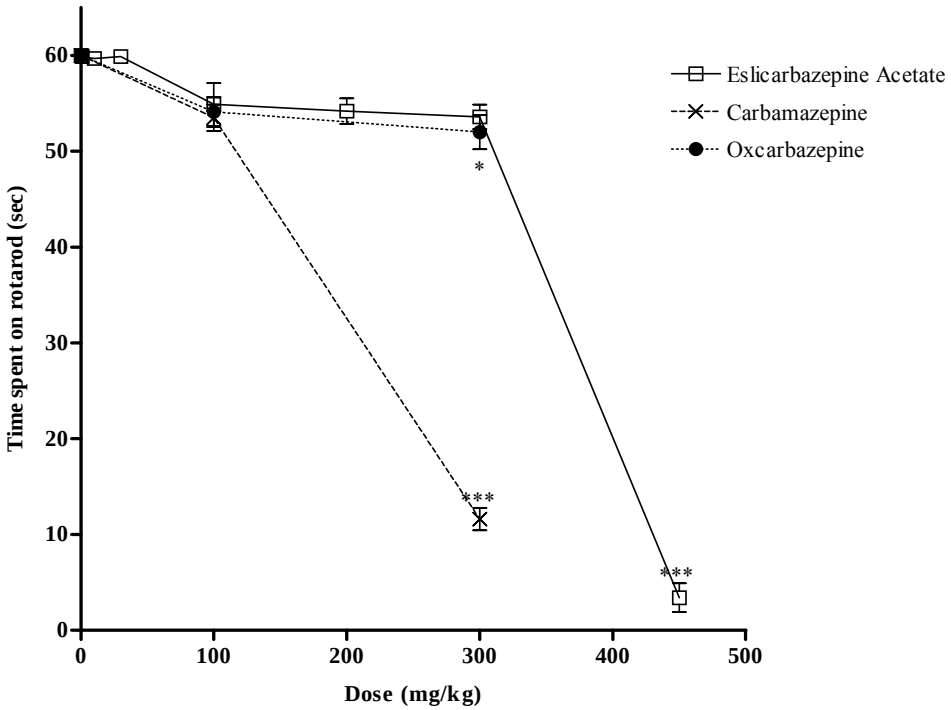

Figure 12: Effect of eslicarbazepine acetate, carbamazepine and oxcarbazepine orally administered doses on motor performance of mice expressed as time spent on rotarod (seconds). Each point represents the mean ± S.E.M. of time spent on the rotarod obtained for 10 animals. Significant differences between vehicle and all doses administered for each treated group are indicated by * ($p < 0.05$) and *** ($p < 0.001$) (Dunnett's test subsequent to one-way ANOVA).

The median toxicity dose (TD_{50}) value for eslicarbazepine acetate-induced motor impairment is shown in Figure 13. The TD_{50} in mice for eslicarbazepine acetate was 342.5 mg/kg, a value that is superior to that of carbamazepine (91.0 mg/kg) and of oxcarbazepine (239.4 mg/kg), accordingly with previously published data (Stepanovic-Petrovic et al., 2008). These values indicate that the motor incoordination caused by eslicarbazepine acetate is much lower than that caused by carbamazepine or oxcarbazepine.

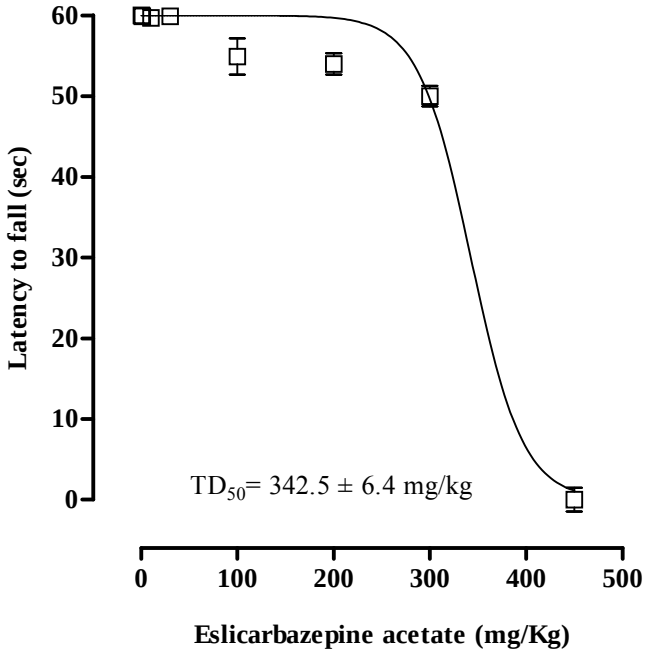

Figure 13: The median toxicity dose (TD_{50}) value for eslicarbazepine acetate-induced motor impairment in mice. Data was fitted using nonlinear regression with goodness of fit (R^2) of 0.980. The Boltzmann sigmoidal equation was used to derive the TD_{50} value of 342.5 ± 6.4 mg/kg (TD_{50} value ± standard error) with 95% confidence intervals of 329.8 to 355.3. The number of mice for each dose group was ten.

1.2 The Effect of Eslicarbazepine Acetate, Carbamazepine and Oxcarbazepine in the Open-Field Test in Mice

In order to assess sedative, stimulant or general behaviour impairment effects on drug treated mice, animals were tested in an open-field arena, immediately after the rotarod test. The behavioural parameters - ambulation and rearing - were observed for 15 min. Ambulation and rearing reflect mostly exploratory activities. Typical behaviour in mice is high ambulation and high rearing when exposed to the open-field.

Exposure to all dose levels of eslicarbazepine acetate, shown by the number of ambulation and number of rearing in the open-field arena during the observed period, caused no significant behavioural effects in mice except for the highest tested dose 450 mg/kg. The ambulation and rearing frequencies (number of occurrences per 15 minutes) for eslicarbazepine acetate in the open-field test are shown in Figures 14 and 15, respectively.

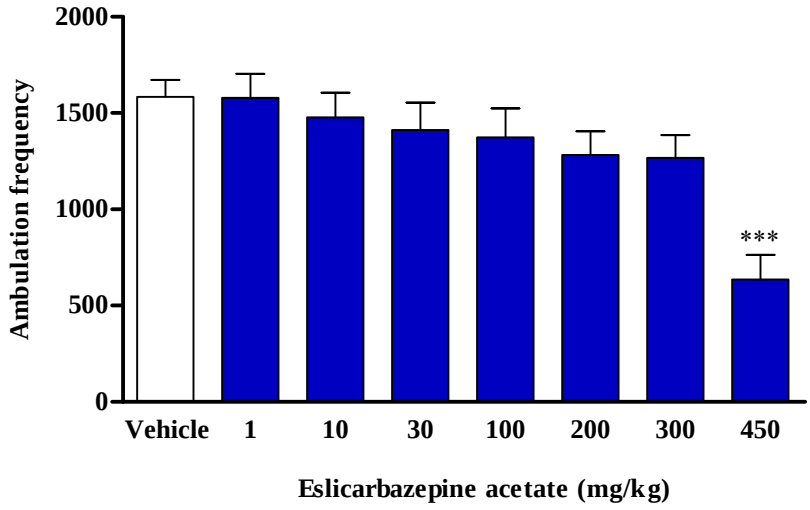

Figure 14: The ambulation frequency in the open-field test after oral administration of eslicarbazepine acetate (1 – 450 mg/kg). Each bar represents the mean ± S.E.M. of number of ambulations during 15 minutes observation time obtained for 10 animals. Significant differences between vehicle and all administered doses are indicated by *** ($p < 0.001$) (Dunnett's test subsequent to one-way ANOVA).

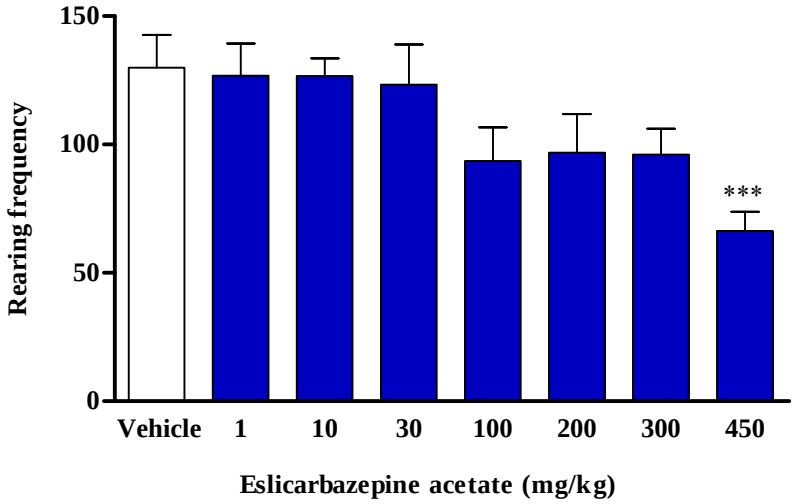

Figure 15: The rearing frequency in the open-field test after oral administration of eslicarbazepine acetate (1 – 450 mg/kg). Each bar represents the mean ± S.E.M. of number of rearing during 15 minutes observation time obtained for 10 animals. Significant differences between vehicle and all administered doses are indicated by *** ($p < 0.001$) (Dunnett's test subsequent to one-way ANOVA).

As for eslicarbazepine acetate, the intact nature of motor functions and exploratory behaviour was also observed for the lower tested dose of carbamazepine (100 mg/kg) but on the other hand this was not observed for the highest tested dose of carbamazepine (300 mg/kg). Also there were no significant differences in any of the parameters evaluated in animals 60 minutes after treatment with oxcarbazepine 100 and 300 mg/kg. Results for ambulation and rearing frequencies for carbamazepine and oxcarbazepine tested doses are presented in Figure 16.

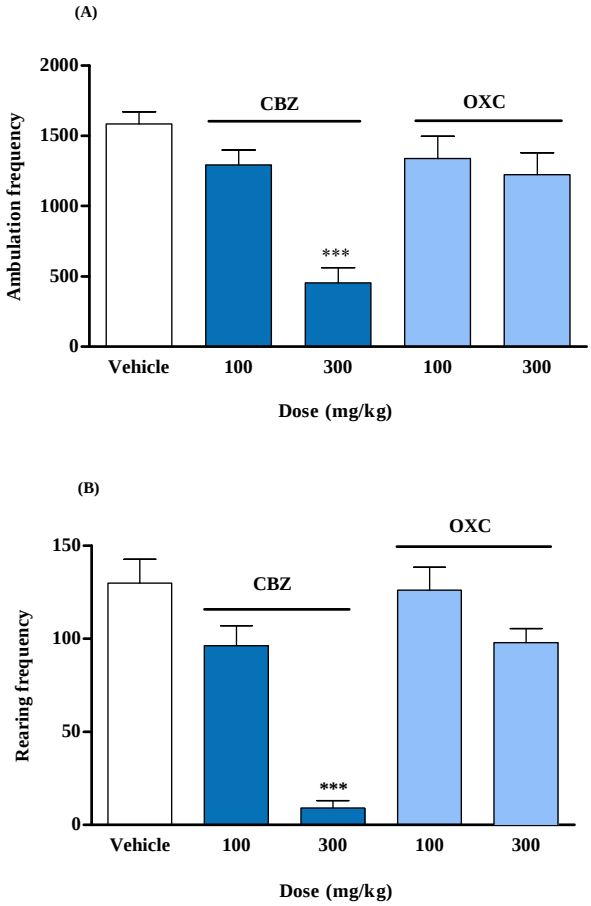

Figure 16: The open-field test for carbamazepine (100 and 300 mg/kg) and oxcarbazepine (100 and 300 mg/kg) orally administered. (A) Number of ambulations during 15 minutes observation time. (B) Number of rearing during 15 minutes observation time. Each bar represents the mean ± S.E.M. for 10 mice. Significant differences between vehicle and drug administered groups are indicated by *** ($p < 0.01$) (Dunnett's test subsequent to one-way ANOVA).

Orally administered eslicarbazepine acetate did not alter the motor behaviour in the open-field and rotarod tests at the doses of 1 mg/kg to 300 mg/kg neither did carbamazepine 100 mg/kg or oxcarbazepine 100 mg/kg.

2 Formalin Paw Test

The formalin paw test is a classical model of nociception in mice. Two phases of behaviour follow injection of formalin into the hindpaw. The spontaneous pain behaviour in mice consists of intense licking or biting of the injected paw. In this study only the late phase of the formalin test was used to assess the analgesic activity of eslicarbazepine acetate, because it is described as being related to a peripheral inflammatory pain, while the early phase is due to a direct effect on nociceptors (Collier et al., 1968; Hunskaar and Hole, 1987). Pain behaviour was scored as the amount of time spent licking or biting the injected paw, in seconds.

2.1 The Effect of Eslicarbazepine Acetate, Carbamazepine and Oxcarbazepine in the Formalin Paw Test in Mice

The formalin paw test developed in this experimental work was validated using morphine orally administered at a high dose (64 mg/kg) to obtain a maximal effective response (Grognet et al., 1982). The anticonvulsants carbamazepine and oxcarbazepine were included as comparison substances to the effect of eslicarbazepine acetate in the late phase of formalin paw test in mouse (Shannon et al., 2005). The late phase was evaluated between 15 and 30 minutes after injection of formalin.

Morphine completely abolished the formalin-induced licking or biting ($p < 0.001$). Both carbamazepine and oxcarbazepine significantly decreased licking or biting the injected paw, conferring protection of 93.3 % and 84.5 % respectively, compared with control vehicle. As morphine, eslicarbazepine acetate (300 mg/kg) conferred a 100 % protection against pain, as compared with control vehicle. Individual values (mean ± S.E.M.) for the formalin-induced licking or biting after oral administration of the anticonvulsants eslicarbazepine acetate, carbamazepine and oxcarbazepine and the opioid morphine are presented in Table 1.

Table 1: The effects of different doses of eslicarbazepine acetate and single doses of carbamazepine, oxcarbazepine and morphine in the late phase of the formalin paw test (evaluated between 15 and 30 minutes after injection of formalin) in mouse.

Treatment (mg/kg) p.o. 60 min before the test (i.e. 45 min before formalin)	Licking / bitting time (sec) (15 to 30 min after formalin) mean	S.E.M.	*p* value	% Protection
Vehicle	87.6	12.3	-	0.0
Eslicarbazepine acetate (1)	84.2	4.3	NS	3.9
Eslicarbazepine acetate (10)	66.5	8.4	NS	24.1
Eslicarbazepine acetate (30)	48.6	9.2	< 0.05	44.5
Eslicarbazepine acetate (100)	29.5	11.4	< 0.01	66.3
Eslicarbazepine acetate (200)	10.1	3.2	< 0.001	88.5
Eslicarbazepine acetate (300)	0.0	0.0	< 0.001	100.0
Carbamazepine (100)	5.3	5.2	< 0.001	93.9
Oxcarbazepine (100)	13.6	7.6	< 0.001	84.5
Morphine (64)	0.0	0.0	< 0.001	100.0

Each group represents the mean ± S.E.M. for the formalin-induced licking or biting (in seconds) for 10 animals. Significant differences between vehicle and eslicarbazepine acetate, carbamazepine, oxcarbazepine and morphine treated groups calculated by means of one-way ANOVA followed by Dunnett's test. *P* values less than 0.05 were regarded as statistically significant.

Oral administration of eslicarbazepine acetate (1, 10, 30, 100, 200 and 300 mg/kg) caused a dose-dependent reduction in the time that the animals spent licking or biting the injected paw, as compared with control. This dose-dependent reduction was significant at all tested doses except at the lower tested doses 1 and 10 mg/kg (Figure 17).

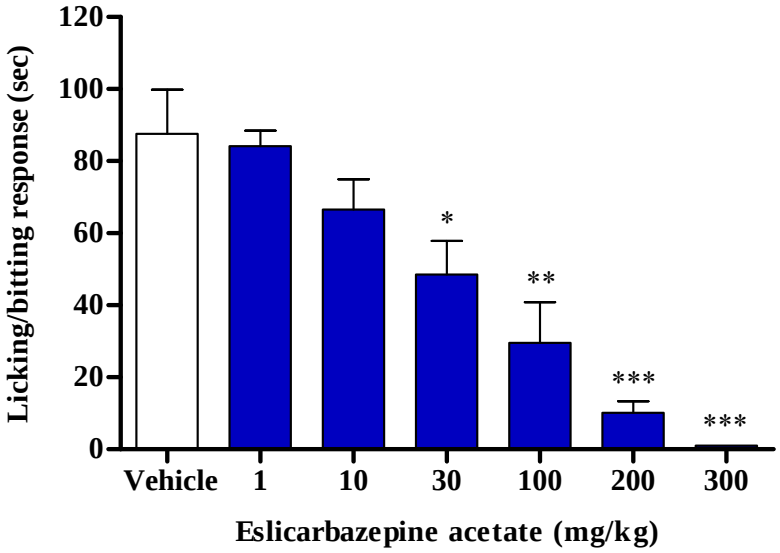

Figure 17: Effect of eslicarbazepine acetate orally administered in the late phase of the formalin paw test (15 – 30 min). Pain behaviour was scored as the amount of time spent licking or biting the injected paw in seconds. Each bar represents the mean ± S.E.M. for 10 animals. Statistical analysis of the data was evaluated by means of one-way ANOVA followed by Dunnett's test between vehicle and eslicarbazepine acetate treated groups. Significances are indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

Observing the plot in Figure 18, although there is no significant difference in the inhibition of pain when eslicarbazepine acetate is administered as compared with the other tested anticonvulsants or morphine, it is clear that eslicarbazepine acetate has a more prominent protection against pain as compared with carbamazepine and oxcarbazepine. The protection was at the morphine protection level, completely abolishing the licking or biting pain-evoked behaviour (Table 1).

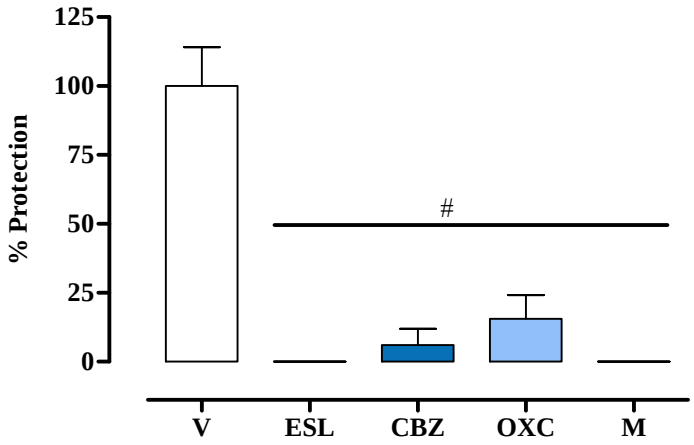

Figure 18: Protective effect of oral administration of vehicle (V), eslicarbazepine acetate (ESL) 300 mg/kg, carbamazepine (CBZ) 100 mg/kg, oxcarbazepine (OXC) 100 mg/kg and morphine (M) 64 mg/kg on the nociceptive reaction to subcutaneous injection of formalin in the hindpaw of mice. Each bar represents the mean ± S.E.M. for 10 animals. Statistical analysis of the data was evaluated by means of one-way ANOVA followed by Bonferroni's multiple comparison test between all groups with significances indicated by # $p < 0.001$ for all groups compared to vehicle.

The antinociceptive activity of eslicarbazepine acetate in the formalin paw test was evaluated by the ED_{50} value. It was calculated by orally administering 1 - 300 mg/kg of the referred anticonvulsant to mice. The ED_{50} value obtained for eslicarbazepine acetate 60 minutes after treatment was 41.4 mg/kg (Figure 19).

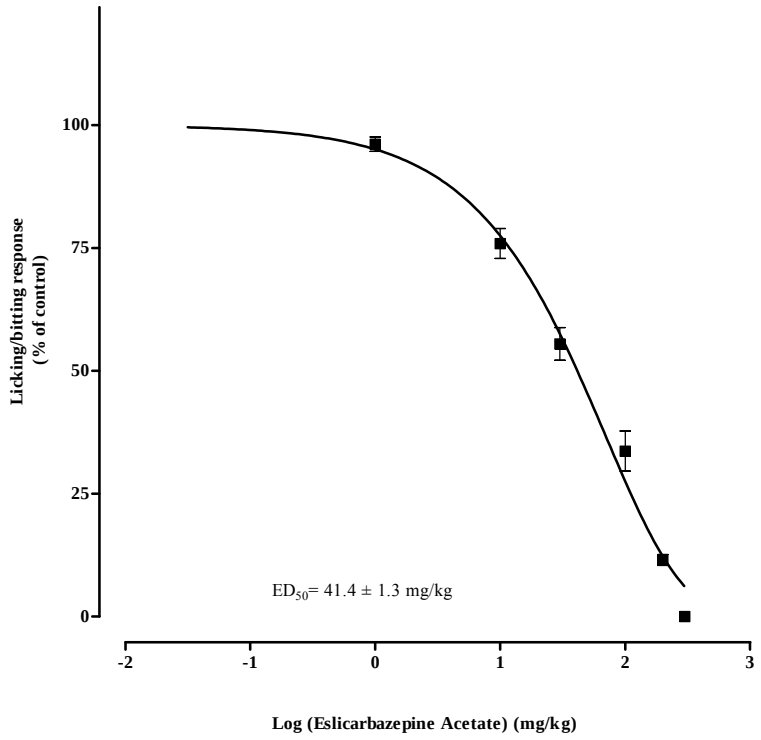

Figure 19: ED_{50} value for eslicarbazepine acetate licking/biting response in the late phase of the formalin paw test. Eslicarbazepine acetate was orally administered 60 minutes before the formalin paw test. Data was fitted using nonlinear regression with a goodness of fit (R^2) of 0.988. The Gompertz equation was used to derive the ED_{50} value (41.4 ± 1.3 mg/kg).

In the view of the data (Table 1) indicating that at 100 mg/kg carbamazepine is more protective against formalin pain-evoked behaviour than oxcarbazepine at the same administered dose and also because the literature lacks the information, the ED_{50} value for carbamazepine in the formalin paw test was determined. The ED_{50} value obtained for carbamazepine 60 minutes after oral administration was 40.3 mg/kg (Figure 20).

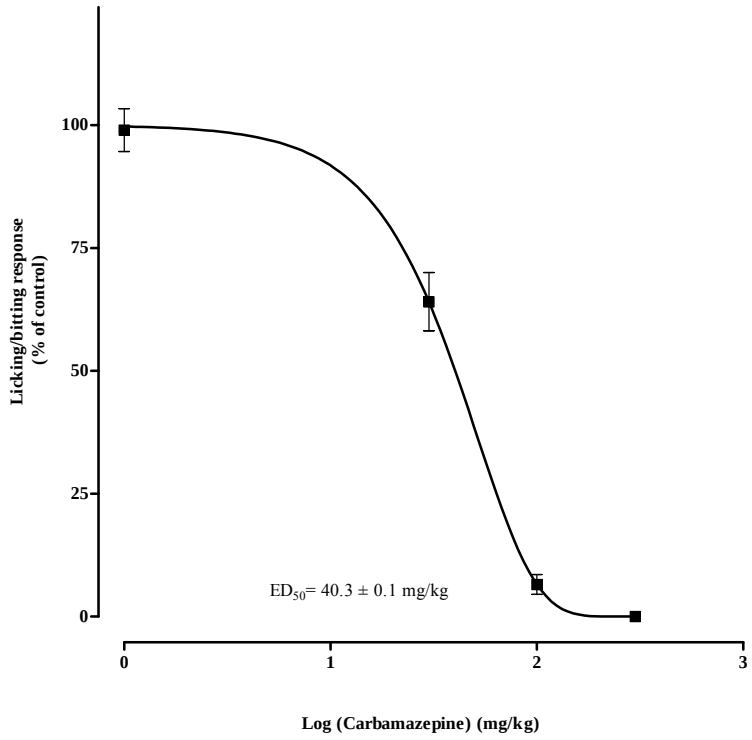

Figure 20: ED_{50} value for carbamazepine licking/biting response in the late phase of the formalin paw test. Carbamazepine was orally administered 60 minutes before the formalin paw test. Data was fitted using nonlinear regression with a goodness of fit (R^2) of 1.000. The Gompertz equation was used to derive the ED_{50} value (40.3 ± 0.1 mg/kg).

3 Writhing Test

The writhing test is also, as the formalin paw test, a classical model of nociception in mice, being used by many researchers to evaluate the potential analgesic properties of several different drugs. The behavioural scoring in mice consisted of writhing production shortly after the intraperitoneal injection of acetic acid. A writhe was defined as stretching of both hind limbs accompanied by a contraction of the abdominal muscles.

3.1 The Effect of Eslicarbazepine Acetate, Carbamazepine and Oxcarbazepine in the Writhing Test in Mice

In the experiments done in this experimental work to develop and implement the writhing test, morphine was used to validate the model and the anticonvulsants carbamazepine and oxcarbazepine were tested as comparison drugs to the analgesic effect of eslicarbazepine acetate.

The effects of eslicarbazepine acetate, carbamazepine, oxcarbazepine and morphine on acetic acid-induced writhing behaviour in mice are shown in Table 2. The per cent protection values produced by eslicarbazepine acetate (300 mg/kg), carbamazepine (100 mg/kg) and oxcarbazepine (100 mg/kg) were 91.3 %, 62.1 % and 68.3 % respectively. Morphine (64 mg/kg) completely abolished the number of writhes induced by acetic acid in mice, conferring a complete protection against pain.

Table 2: The effects of different doses of eslicarbazepine acetate and single doses of carbamazepine, oxcarbazepine and morphine in the writhing test (evaluated for 10 minutes, starting 5 minutes after injection of acetic acid) in mouse.

Treatment (mg/kg) p.o. 60 min before the test	Number of writhes (s) (5 min after acetic acid for 10 min) mean	S.E.M.	*p* value	% Protection
Vehicle	35.6	2.3	-	0.0
Eslicarbazepine acetate (1)	33.2	1.8	NS	6.7
Eslicarbazepine acetate (10)	31.3	2.3	NS	12.1
Eslicarbazepine acetate (30)	25.5	2.8	< 0.01	28.4
Eslicarbazepine acetate (100)	22.7	1.4	< 0.001	36.2
Eslicarbazepine acetate (200)	12.1	2.3	< 0.001	66.0
Eslicarbazepine acetate (300)	3.1	0.7	< 0.001	91.3
Carbamazepine (100)	13.5	1.4	< 0.001	62.1
Oxcarbazepine (100)	11.3	1.7	< 0.001	68.3
Morphine (64)	0.0	0.0	< 0.001	100.0

Each group represents the mean ± S.E.M. for the acetic acid- induced writhing (in seconds) for 10 animals. Significant differences between vehicle and eslicarbazepine acetate, carbamazepine, oxcarbazepine and morphine treated groups calculated by means of one-way ANOVA followed by Dunnett's test. *P* values less than 0.05 were regarded as statistically significant.

Eslicarbazepine acetate (30, 100, 200 and 300 mg/kg) when administered orally, dose-dependently and significantly reduced the number of writhes induced by acetic acid in mice. The lower doses 1 and 10 mg/kg did not interfere with the acetic acid pain-evoked effect as compared with vehicle (Figure 21).

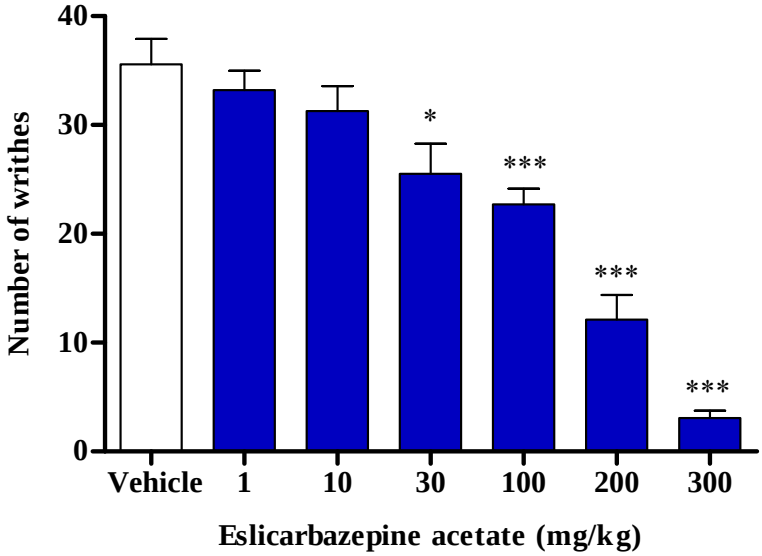

Figure 21: Effect of eslicarbazepine acetate in the writhing test, expressed as number of writhes induced by p.o. injection of acetic acid in mice. Each point represents mean ± S.E.M. for 10 animals. Significant differences between vehicle and drug administered groups are indicated by * ($p < 0.05$) and *** ($p < 0.001$) (Dunnett's test subsequent to one-way ANOVA).

After statistical analysis of the pain inhibitory effect of eslicarbazepine acetate, the other anticonvulsants tested and morphine, many differences were observed (Figure 22). Morphine as expected completely abolished the writhes provoked by acetic acid intraperitoneal injection conferring a 100 % inhibition of the pain-evoked behaviour. Eslicarbazepine acetate conferred a decrease in the number of writhes at the same level as morphine, with no statistical significant difference between the two referred results. When comparing the inhibitory effect of eslicarbazepine acetate with the pain-evoked inhibition conferred by carbamazepine and oxcarbazepine, eslicarbazepine acetate was more analgesic, with statistically significant differences between eslicarbazepine acetate and the other anticonvulsants.

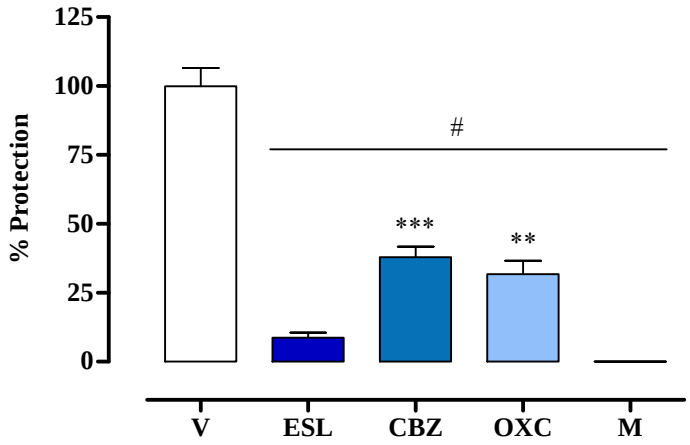

Figure 22: Protective effect of oral administration of vehicle (V), eslicarbazepine acetate (ESL) 300 mg/kg, carbamazepine (CBZ) 100 mg/kg, oxcarbazepine (OXC) 100 mg/kg and morphine (M) 64 mg/kg on the nociceptive reaction to intraperitoneal injection of acetic acid in mice. Each bar represents the mean ± S.E.M. for 10 animals. Statistical analysis of the data was evaluated by means of one-way ANOVA followed by Bonferroni's multiple comparison test between all treated groups. Significances indicated by * ($p < 0.05$) and *** ($p < 0.001$) for all treated groups compared to eslicarbazepine acetate. Significances indicated by # $p < 0.001$ for all treated groups compared to vehicle.

To assess the antinociception activity of eslicarbazepine acetate in the writhing test, the ED_{50} value was calculated. The ED_{50} value obtained for eslicarbazepine acetate 60 minutes after oral administration was 108.1 mg/kg (Figure 23).

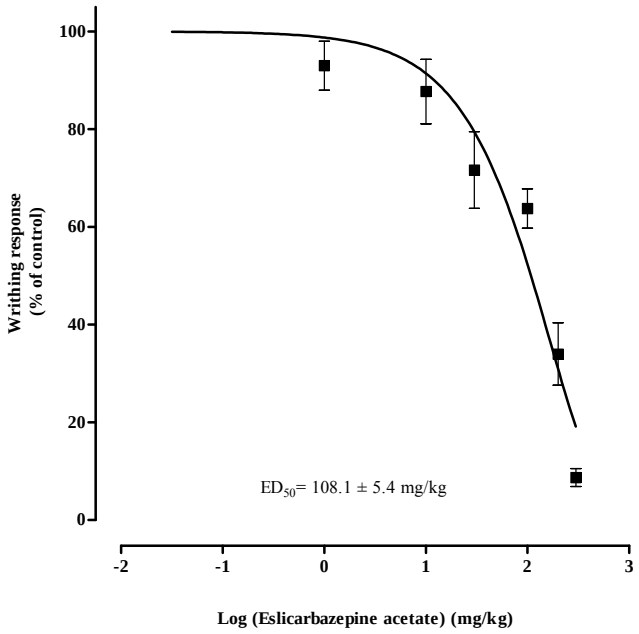

Figure 23: ED_{50} value for eslicarbazepine acetate acetic acid-pain evoked response in the writhing test. Eslicarbazepine acetate was orally administered 60 minutes before the test. Data was fitted using nonlinear regression with a goodness of fit (R^2) of 0.934. The Gompertz equation was used to derive the ED_{50} value (108.1 ± 5.4 mg/kg).

Spanovic-Petrovic et al. (2008) published ED_{50} values for carbamazepine and oxcarbazepine nociception in the writhing test as being 38.9 mg/kg and 15.8 mg/kg, respectively. These values will be considered in the next section for evaluation of the antinociceptive activity of the referred drugs in the writhing test.

4 Determination of the Protective Index of Eslicarbazepine Acetate, Carbamazepine and Oxcarbazepine

The potential analgesic activity of eslicarbazepine acetate in mice was evaluated in this experimental work using two different animal models of nociception: (i) the formalin paw test associated with the rotarod test and the open-field test (FRO); (ii) and the writhing test associated with the rotarod test and the open-field test (WRO). In order to ensure that the antinociceptive activity observed for a certain tested drug was due to its analgesic effect and not caused by drug induced neurotoxicity or drug induced motor impairment, sedation or stimulation, prior to the formalin paw test or to the writhing test, the rotarod and open-field tests were performed.

Carbamazepine and oxcarbazepine are anticonvulsants that have been reported recently as having antinociceptive activity, so they were used here as comparison substances in the models developed in this experimental work (Kiguchi et al., 2004). Opiate drugs such as morphine have been widely used in the management of pain. Morphine was used here as a reference drug for development and implementation of the formalin paw and writhing tests (Miranda et al., 1992).

It is clear that the animals treated with eslicarbazepine acetate up to 300 mg/kg, carbamazepine 100 mg/kg or oxcarbazepine 100 mg/kg presented no signs of neurotoxicity or motor impairment (observing the rotarod test results). Observing the open-field test result values, one can say that the animal's ability to elevate its paw following formalin injection or the animal's pain perception induced by the intraperitoneal injection of acetic acid were not compromised by the administered drugs at the former referred doses. An interesting observation was that oxcarbazepine 300 mg/kg, when administered orally 60 minutes before the test, displayed gross deficits in motor coordination (rotarod performance) but did not have pronounced effects on the open-field parameters.

The ED_{50} values for antinociception in the formalin paw test and in the writhing test were determined using the doses 1 to 300 mg/kg of eslicarbazepine acetate, and the TD_{50} value for motor impairment in mice in the rotarod test was determined using the doses 1 to 450 mg/kg. The observed absence of effect upon exploratory behaviour or motor system on animals administered with the doses 1 to 300 mg/kg of eslicarbazepine acetate 60 minutes before the formalin paw and writhing tests, allowed the statement that the observed effect upon pain-evoked behaviour was

undoubtly antinociceptive. For carbamazepine the doses administered for ED_{50} value determination were 30 to 300 mg/kg.

The dose that produced antinociceptive effect in 50 % of the mice tested in the formalin paw test was 41.4 mg/kg for eslicarbazepine acetate and 40.3 mg/kg for carbamazepine. The calculated protective index for eslicarbazepine acetate was 8.3 and for carbamazepine was 2.3. The oxcarbazepine ED_{50} and TD_{50} were obtained from the literature (Tomic et al., 2010; Stepanovic-Petrovic et al., 2008) and the protective index presented was calculated from the former referred values. The protective index, calculated as TD_{50} / ED_{50}, is a measure of therapeutic tolerability (Learmonth et al., 2001). The values for antinociception in the formalin paw test, motor impairment and protective index in mice are shown in Table 3.

Table 3: ED_{50}, TD_{50} values and the corresponding Protective index (PI), with 95% confidence limits (95% CL) and correlation coefficient (R^2) in inducing antinociception in the formalin paw test and motor impairment in mice.

	Antinociception		Motor Impairment		
Substance	ED_{50} mg/kg (95% CL)	R^2	TD_{50} mg/kg (95% CL)	R^2	PI
Eslicarbazepine Acetate	41.4 (38.9-44.0)	0.988	342.5 (329.8-355.3)	0.980	8.3
Carbamazepine	40.3 (40.1-40.5)	1.000	91.0 (22.2-373.3) *	-	2.3
Oxcarbazepine	92.7 (-) **	-	239.4 (172.5-332.2) *	-	2.6

* value obtained from (Stepanovic-Petrovic et al., 2008);

** value obtained from (Tomic et al., 2010).

The ED_{50} value for eslicarbazepine acetate antinociception in the writhing test was 108.1 mg/kg, which corresponds to a therapeutic tolerability of 3.2. The carbamazepine and oxcarbazepine ED_{50} and TD_{50} and protective index values were obtained from the literature (Tomic et al., 2010; Stepanovic-Petrovic et al., 2008). The ED_{50} and TD_{50} values for antinociception in the writhing test and motor impairment in mice in the rotarod test are shown in Table 4.

Table 4: ED_{50}, TD_{50} values and the corresponding Protective index (PI), with 95% confidence limits (95% CL) and correlation coefficient (R^2) in inducing antinociception in the writhing test and motor impairment in mice.

	Antinociception		Motor Impairment		
Substance	ED_{50} mg/kg (95% CL)	R^2	TD_{50} mg/kg (95% CL)	R^2	PI
Eslicarbazepine Acetate	108.1 (97.4-118.9)	0.934	342.5 (329.8-355.3)	0.980	3.2
Carbamazepine	38.9 (31.2-48.2)*	-	91.0 (22.2-373.3) *	-	2.3
Oxcarbazepine	15.8 (11.1-22.3)*	-	239.4 (172.5-332.2) *	-	15.2

* value obtained from (Stepanovic-Petrovic et al., 2008)

The present results show that eslicarbazepine acetate has greater therapeutic tolerability in the formalin paw test than in the writhing test, suggesting that eslicarbazepine acetate is a better tolerated antinociceptive drug in the formalin paw test than in the writhing test. Carbamazepine and oxcarbazepine-induced antinociception in the formalin paw and in the writhing tests are in accordance with previously published information (Tomic et al., 2010; Aoki et al., 2006; Stepanovic-Petrovic et al., 2008). By comparison, eslicarbazepine acetate is about 3-fold more protective against formalin induced-pain than carbamazepine and oxcarbazepine. In the writhing test, eslicarbazepine acetate has a therapeutic tolerability between carbamazepine and oxcarbazepine.

CHAPTER IV

DISCUSSION AND CONCLUSION

In the present work, two *in vivo* models for evaluation of drugs with potential analgesic activity were developed and fully implemented. One model is constituted by the formalin paw test associated with the rotarod test and the open-field test (FRO). The other model is constituted by the writhing test, also associated with the rotarod test and the open-field test (WRO). Both models are performed in mice. In both models, the potential analgesic activity of the tested drugs was assessed by quantification of the protection that the referred drugs conferred against the insult pain-evoked behaviour, as compared to vehicle administered animals. The formalin paw test and the writhing test provide a measure of drug protection against pain. The rotarod test and the open-field tests provide means to assess motor toxicity and sedative, stimulant or motor behaviour impairment effects on drug treated mice, respectively. The protocol for the first referred model - FRO - consists of three tests occurring in two different days: on day 1, using the same group of mice for each dosing drug, the rotarod test is performed for 60 seconds immediately followed by the open-field test for 15 minutes; on day 2, using a new group of mice for each dosing drug, the formalin paw test is performed for 15 minutes, with the formalin being injected 15 minutes before the beginning of the test. The protocol for the second referred model - WRO - also consists of three tests occurring in two different days: on day 1 the rotarod test and the open-field test are performed as described for the FRO model; on day 2, using a new group of mice for each dosing drug, the writhing test is performed during 10 minutes, with the acetic acid being injected 5 minutes before the beginning of the test.

Many authors have been neglecting the importance of the assessment of drug induced neurotoxicity or drug induced motor impairment, sedation or stimulation, prior to the nociception tests. For instance, if a drug administered to an animal induces motor impairment it will compromise the animal's ability to elevate its paw following formalin injection or if a drug induces neurotoxicity, this can eventually disrupt or even kill neurons, and compromise the transmission and processing of pain signals in the brain or other parts of the nervous system, causing the absence of pain-evoked behaviour with this having no correlation with the antinociceptive effect of the administered drug. Observing the rotarod test results, it is clear that the animals treated 60 minutes before the test with eslicarbazepine acetate up to 300 mg/kg, carbamazepine 100 mg/kg or oxcarbazepine 100 mg/kg, presented no signs of neurotoxicity. The administered drugs at the former referred doses did not have any significant effect on the open-field test parameters (ambulation and rearing). Ambulation and rearing are parameters that allow the determination of the locomotor activity and exploratory activity, respectively. The

open-field test results show that eslicarbazepine acetate up to 300 mg/kg, carbamazepine 100 mg/kg and oxcarbazepine 100 mg/kg have no effect on locomotor and exploratory activity of mice. Oxcarbazepine 300 mg/kg, when administered orally 60 minutes before the test, displayed gross deficits in motor coordination (rotarod performance) but did not have pronounced effects in the open-field parameters. So, oxcarbazepine 300 mg/kg results for the rotarod and the open-field tests emphasize the importance of assessing both drug induced neurotoxicity and drug induced motor impairment, sedation or stimulation, prior to the nociception tests.

Opiate drugs such as morphine have been widely used in the management of pain. Morphine was used here as a reference drug for development and implementation of the formalin paw and writhing tests (Miranda et al., 1992). Morphine completely abolished the formalin-induced licking or biting as well as the number of writhes induced by acetic acid in mice, conferring a complete protection against pain. These results support the statement that the formalin paw and the writhing tests developed during this experimental work were implemented with success.

Carbamazepine and oxcarbazepine are anticonvulsants that have been reported recently as having antinociceptive activity, so they were used here as comparison substances in the development and implementation of the models in this experimental work (Kiguchi et al., 2004). In the present studies, the putative sodium channel blockers carbamazepine (100 mg/kg) and oxcarbazepine (100 mg/kg) all produced statistically significant analgesic effects in the formalin paw test and writhing test which is consistent with previously published data (Shannon et al., 2005; Stepanovic-Petrovic et al., 2008). The doses of the sodium channel blockers that produced analgesia in the formalin paw and writhing tests were smaller in magnitude than the doses required to produce effects in both the rotarod and open-field tests.

The results obtained in the present study demonstrated that eslicarbazepine acetate exhibited antinociceptive activity in the two models tested (FRO and WRO). This effect was significant against chemically (acetic acid and formalin) induced nociception. The formalin paw test, characterized by two phases, is used to evaluate the mechanism by which an animal responds to moderate, continuous pain generated by the injured tissue (Abbott et al., 1995). The early phase (immediately after formalin injection) seems to be caused by C-fiber activation induced by the peripheral *stimulus* (Abbott et al., 1995). The late phase (starting approximately 15 min after

injection) appears to depend on the combination of an inflammatory reaction, activation of *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors, and the nitric oxide cascade (Davidson and Carlton, 1998) in the peripheral tissue and functional changes in the dorsal horn of the spinal cord (Abbott et al., 1995). The formalin is the most common substance to be used as noxious *stimulus* for intradermal injections (Wheeler-Aceto and Cowan, 1991). A range of 0.02 to 5 % of formalin solution concentration have been reported to be injected in mice, with the most frequently injected volume ranging from 20 to 25 µl (Tjolsen et al., 1992). In the formalin paw test developed and implemented in this experimental work 25 µl of formalin solution were injected subcutaneously in the hindpaw of the mouse at a concentration of 5 %, with the intent of inducing the late phase. The reason why only the late phase of the formalin paw test was assessed here is because it is described as being related to a peripheral inflammatory pain as is the writhing test (Collier et al., 1968; Hunskaar and Hole, 1987). In the writhing test, acetic acid activates directly peripheral nociceptors on the sensory nerve fibers by inducing capillary permeability and releasing endogenous substances that excite pain nerve endings (pro-inflammatory substances) producing inflammation of visceral (subdiaphragmatic) and subcutaneous (muscle wall) tissues (Cervero, 1995). This produces the characteristic 'writhing response': lengthwise stretches of the torso with a concomitant concave arching of the back and extensions of both hind limbs (Collier et al., 1968; Bentley et al., 1981). The acetic acid concentration most frequently used in the writhing test ranges from 0.6 to 0.8 % (v/v). Here a 0.8 % (v/v) acetic acid solution was chosen to be used for comparison purposes with the data published by Stepanovic-Petrovic (2008). In both the formalin paw and writhing tests, oral administration of eslicarbazepine acetate at doses 10 to 300 mg/kg caused a statistically significant dose-dependent reduction of the pain evoked-behaviour caused by the insult injection (formalin and acetic acid, respectively) as compared with the vehicle treated mice. Morphine completely abolished the pain evoked-behaviour in both the formalin paw and in the writhing tests, allowing the statement that the formalin paw test and writhing test were successfully developed and implemented. In the formalin paw test, when comparing the inhibition of pain induced by eslicarbazepine acetate as compared to carbamazepine, oxcarbazepine and morphine, eslicarbazepine acetate showed a more prominent protection against pain than the other tested anticonvulsants, although this effect was not statistically significant. The pain protection conferred by eslicarbazepine acetate was at the morphine protection level, abolishing completely the formalin pain-evoked behaviour. In the writhing test, eslicarbazepine acetate conferred an inhibition of the writhes at the same level as

morphine with no statistical significant difference between the two results. When comparing the protective effect of eslicarbazepine acetate with the protection conferred by carbamazepine and oxcarbazepine, eslicarbazepine acetate was more analgesic, with statistically significant differences between itself and the other two tested anticonvulsants. Eslicarbazepine acetate showed remarkable activity in the late phase of the formalin paw test and showed significant effect in the writhing test induced by acetic acid.

The proof of concept for these *in vivo* models for evaluation of drugs with potential analgesic activity is dependent upon the establishment of a parallelism between the analgesic effect of the tested drug eslicarbazepine acetate, and the presence of the parent drug or its metabolites in mouse. Eslicarbazepine acetate is a sodium channel blocker, currently being used as an adjunct in the treatment of epilepsy, and shares with carbamazepine and oxcarbazepine the dibenzodiazepine nucleous bearing the 5-carboxamide substituent, but is structurally different at the 10, 11-positions (Benes et al., 1999; Hainzl et al., 2001). It is thought that these differences are on the basis of their different metabolic profiles. In humans carbamazepine undergoes oxidative metabolism leading to the formation of toxic epoxide carbamazepine metabolites and oxcarbazepine is bio transformed to S- and R- licarbazepine in a proportion of 4:1 (Almeida and Soares-da-Silva, 2003; Alves et al., 2008). Eslicarbazepine acetate in humans is quickly and extensively metabolized to S-licarbazepine to an extent of 95 - 98 % and to R-licarbazepine and oxcarbazepine to a very small extent (Almeida and Soares-da-Silva, 2003; Almeida and Soares-da-Silva, 2004; Almeida et al., 2005). Eslicarbazepine acetate was specially designed to circumvent its further transformation to toxic epoxides and unnecessary production of enantiomers of its metabolites (Benes et al., 1999; Hainzl et al., 2001). Metabolic studies in mice showed that as in humans, eslicarbazepine acetate is rapidly hydrolysed to S-licarbazepine, being the mouse amongst small laboratory animals, the best whole animal model to better understand the eslicarbazepine acetate disposition in humans (Hainzl et al., 2001; Almeida and Soares-da-Silva, 2007). Alves et al. (2008) studied the plasma and brain disposition of eslicarbazepine acetate and its metabolites in mouse following a single oral administration of eslicarbazepine acetate of 350 mg/kg, determining the concentrations of eslicarbazepine and its metabolites. These authors observed that eslicarbazepine acetate in mice is rapidly and extensively metabolized, lacking the detection of the parent drug (eslicarbazepine acetate) at the first sampling point (withdrawn 0.25 hours after administration) and finding at this time-point large

amounts of S-licarbazepine in plasma and brain. Alves et al. (2008) determined that S-licarbazepine represents 85 % of the total brain drug exposure and oxcarbazepine represents 15 %, with a t_{max} (the time that it takes to reach the maximum concentration of drug) of 1 hour for both drugs. The present study demonstrates that eslicarbazepine acetate orally administered 1 hour before the formalin paw test as well as 1 hour before the writhing test showed great efficacy on protecting mice from pain. As described before, eslicarbazepine acetate transforms rapidly into its major metabolite S-licarbazepine and at 1 hour post oral administration one can find the maximum concentration of S-licarbazepine in the brain.

The primary mechanism of action of S-licarbazepine is blockade of voltage-dependent sodium channels (Benes et al., 1999; Almeida and Soares-da-Silva, 2007). The affinity of S-licarbazepine for the inactivated state of the sodium channel is similar to that of carbamazepine, but its affinity for the resting state of the sodium channel is 3-fold lower (Bonifacio et al., 2001). S-licarbazepine inhibits the sodium-dependent release of neurotransmitters, with a potency comparable to that of carbamazepine and oxcarbazepine (Parada and Soares-da-Silva, 2002). Voltage-activated sodium channels induces the initial rapid membrane depolarization during an action potential in neural cells and are responsible for the propagation of the action potential, allowing the transmission of pain. The pain function of sodium channels can be up-regulated in the inflammatory conditions (Sahebgharani et al., 2006), the conditions present in both the late phase of the formalin paw test and in the writhing test. Because carbamazepine and oxcarbazepine are sodium channel blockers as is eslicarbazepine acetate active metabolite S-licarbazepine, because these anticonvulsants showed analgesic activity in the pain models developed in this experimental work, although with different protective indices, and because sodium channel blockers are involved in the process of transmission of pain, the most likely explanation for the analgesic activity of eslicarbazepine acetate is that part of the antinociceptive response of S-licarbazepine is at the voltage-gated sodium channels level. The results obtained for protective index of eslicarbazepine acetate, when compared with the protective indices for carbamazepine and oxcarbazepine show that eslicarbazepine acetate is just about 3-fold more protective against formalin induced-pain than carbamazepine and oxcarbazepine. In the writhing test, eslicarbazepine acetate showed a protective index between carbamazepine and oxcarbazepine. The protective index is considered a measure of therapeutic tolerability, as being the relative safety of the drug for a particular

treatment (Learmonth et al., 2001).

In conclusion, one can state that the two models developed here FRO and WRO are able to evaluate the analgesic activity of drugs in mice, with sensitivity to different doses of an analgesic drug. The rotarod and the open-field tests together play an important role in the developed models and many authors have been neglecting the importance of drug induced neurotoxicity or drug induced motor impairment, sedation or stimulation, as conditions that can mask the true analgesic effect of a drug. The data supports the statement that both models FRO and WRO are tools of great importance for the evaluation of the analgesic activity of both old and new drugs.

CHAPTER V

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