Chapter 6

DISCUSSION
The epilepsies are common central nervous system (CNS) disorders that sometimes require a combined therapy, especially for patients with refractory seizures inadequately controlled with monotherapy. The primary aim of using such combinations is to enhance the efficacy and to minimize the adverse effects. At the preclinical stage, experimental seizure models not only provide an invaluable means of identifying potentially useful anticonvulsant agents but also aid in the bioevaluation of combinations of antiepileptic drugs (AEDs), which is of pivotal importance for their subsequent clinical application. In the present study, the maximal electroshock (MES) test was used since it is one of the standardized and most validated experimental model of generalized tonic-clonic (GTC) seizures (Loscher et al., 1991). While phenytoin (PHT) and sodium valproate (SVP) are the first line AEDs used in the management of GTC seizures (Lowenstein, 1998), we had earlier found a facilitatory action of N-acetylcysteine (NAC), a potent antioxidant, on the anticonvulsant effects of SVP against the electroconvulsive threshold model of seizures in mice (Devi et al., 2006b). The present work was, thus, undertaken to characterize the nature of interaction between standard AEDs (i.e. PHT, SVP) and NAC in the mouse MES test using isobolographic analysis. The latter is considered to be the optimal method to detect drug interactions of AEDs in animal models of epilepsy (Deckers et al., 2000).

PHT, SVP and NAC produced clear-cut anticonvulsant effects against MES induced seizures in mice. The results obtained in the present study with PHT and
SVP are in accordance with the established evidence of the effectiveness of these agents against MES induced seizures (Clark, 1988; Loscher and Schmidt, 1988; Joseph et al., 1998; Bough and Eagles, 2001; Luszczki et al., 2003). However, ours is probably the first experimental evidence of an anticonvulsant activity being reported with NAC against MES induced seizures in mice. Our data further confirms the anticonvulsant properties of NAC that have been reported in other models of seizures viz aminophylline (Gulati et al., 2005) and pentylenetetrazole (PTZ) (Devi et al., 2006a) induced seizures. Even clinically, NAC has been reported to exhibit beneficial effects in refractory epilepsies like progressive myoclonic epilepsies where other classical drugs like clonazepam, valproate and zonisamide have failed to improve the manifestations of the disease (Ben-Menachem et al., 2000).

In the present study, PHT and NAC displayed additive interactions with a slight tendency towards supra-additivity for all the three fixed ratios (i.e. 1:1, 1:3 and 3:1) against MES induced seizures in mice. Isobolographic analysis of the interaction of SVP with NAC at the fixed ratio of 1:1 also revealed an additive interaction. However, SVP appears to act synergistically with NAC at the fixed ratios of 1:3 and 3:1 since the experimentally determined ED$_{50}$ values (ED$_{50}$ exp) were significantly lower than the theoretically calculated ED$_{50}$ values (ED$_{50}$ add). These results reveal beneficial pharmacodynamic interactions between AEDs (i.e. PHT, SVP) and NAC for the prevention of electroshock induced seizures.
PHT, SVP, NAC (ED$_{50}$ doses) and their 3:1 fixed ratio combinations (at the ED$_{50}$ exp values) did not produce any significant changes either in the grip strength or the spontaneous alternation behavior. This reflects that the doses employed were devoid of any adverse effects on the neuromuscular function and spatial memory.

Several lines of evidence including those from a variety of experimental models suggest a possible involvement of oxidative stress in the pathophysiology of epilepsy (Kabuto et al., 1998; Frantseva et al., 2000; Gluck et al., 2000; Patel, 2004; Gulati et al., 2005; Rajasekaran, 2005; Tejada et al., 2006). However, with respect to electrically induced seizures, there are conflicting reports in literature. While Rola and co-workers (2002) had found a significant increase in the levels of malondialdehyde (MDA) in brain tissue of mice immediately after electroconvulsions, Barichello et al. (2004b) reported a significant decrease in the thiobarbituric acid reactive species in the hippocampus with no significant changes in the cortex, striatum and cerebellum of rats immediately after a single electroconvulsive shock. Recently, Nieoczyn et al. (2008) too have reported a significant reduction in the lipid peroxidation intensity in the brain tissue of mice submitted to maximal electroshock and decapitated three minutes after the electroshock. A statistically significant rise of lipid peroxidation intensity was evident only three hours after tonic hindlimb extension. The increase in the glutathione peroxidase (GPx) activity observed immediately after seizures was hypothesized to be responsible for the detoxification of hydrogen peroxide (H$_2$O$_2$) and hence the prevention of lipid peroxidation and oxidative damages. It
is well known that the reduced form of glutathione (GSH) plays a major role in the detoxification reactions catalyzed by GPx. The decrease in the GSH and MDA levels (though statistically non-significant) observed in the present study following electroshock treatment, further supports the recent findings of these workers and indicates the activation of protective endogenous antioxidant defense mechanisms immediately after seizures.

One-week treatment with PHT alone significantly lowered the cortical MDA levels, thus signifying its neuroprotective potential, which is well documented by other workers (Ates et al., 2007). Cui et al. (2007) have reported an increase in the GSH levels in primary cultured rat cerebral cortical cells following one-week treatment with 0.6 mM valproate. In the present study, however, we did not find any significant change in the cortical GSH levels following one-week treatment with SVP alone. The non-significant changes in the cortical MDA levels observed in the present study following treatment with SVP (360 mg/kg) and NAC (320 mg/kg) alone are in agreement with the findings of other investigators (Frey et al., 2006; Kamboj et al., 2006a,b). However, Kamboj et al. (2006b) have reported significant increases in the GSH levels in different brain regions including cerebral cortex of rats following NAC administration for 28 days. In our study, cortical GSH levels were not altered significantly after one-week treatment with NAC alone. The difference in the duration of drug treatment might be an important variable responsible for the observed effects. However, GSH levels in the NAC treated animals subjected to electroshock were almost comparable to the control animals, indicating the ability of NAC to
maintain the glutathione homeostasis during seizures. Combinations of PHT and SVP with NAC (at the fixed ratio of 3:1) lowered the cortical GSH and MDA levels, with the latter combination exerting a statistically significant effect. These results reflect that probably the reduction in MDA levels may be a consequence of GSH utilization. However, compared to the electroshock group, none of the drugs alone or their combinations at their respective ED50 doses against MES induced seizures altered the cortical GSH and MDA levels significantly. Thus none of the drug treatments seem to adversely modulate the body’s endogenous protective mechanisms. While, the results reveal the sensitizing effects of NAC on the anticonvulsant effects of PHT and SVP, the real mechanism(s) for these observed effects are at present not clearly understood.

Electroshock treatment caused an elevation in the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels compared to the control group, however the effect was not statistically significant. These findings are in accordance with our earlier observations in the PTZ induced model of seizures (Devi et al., 2006a) and indicate the ability of seizures to produce mild stress on the liver. Akbas et al. (2005) have reported enhanced oxidative stress in the liver of rats administered a convulsive dose of PTZ. Even clinically, fulminant hepatic failure and hepatomegaly have been considered as rare complications of seizures (Decell et al., 1994; Baxter et al., 2003; Ichai et al., 2003). One-week treatment with PHT (10 mg/kg), SVP (360 mg/kg) and NAC (320 mg/kg) did not modify the serum ALT and AST levels significantly.
Our results with SVP and NAC are in accordance with our earlier observations (Devi et al., 2006a) and those of other investigators (Loscher et al., 1992; Korkmazer et al., 2006; Lee et al., 2008). When PHT and SVP were administered in combination with NAC at doses corresponding to their ED$_{50}$ values for the fixed ratio of 3:1, no significant alterations in serum ALT and AST levels were observed. Even in the groups that were pretreated with either of these drugs or their combinations and subjected to electroshock, no significant alterations in serum ALT and AST levels were evident. These results indicate that at optimal dose levels, the adverse effects of AEDs on liver function are rare. This is similar to the observations in the clinical settings where also only long-term treatment with AEDs is occasionally associated with transient alterations of hepatic enzyme levels (Aiges et al., 1980; Verma and Haidukewych, 1994; Ahmed and Siddiqi, 2006).

The mild though statistically non-significant elevation in serum alkaline phosphatase (ALP) levels following electroshock treatment might be due to the mild stress caused by electroshock on the liver. In the group that was pretreated with the combination of PHT (5 mg/kg) and NAC (54 mg/kg) and subjected to electroshock, a significant elevation in serum ALP activity was observed compared to the normal control group. This biochemical change may well be due to the influence of electroshock on liver since treatment with the combination of PHT and NAC alone was devoid of any significant effects on ALP activity. However, no such significant effects were observed in the group...
that was pretreated with the combination of SVP and NAC and subjected to electroshock, indicating that NAC may be a better adjunct with SVP than PHT.

Body electrolytes play a pivotal role in the development of seizure conditions (Castilla-Guerra et al., 2006). Oladipo et al. (2007) have reported significantly lower levels of serum calcium in epileptic children compared to the controls. However, in the present study, no significant changes were observed in the serum calcium levels following electroshock treatment. This finding bears similarity to the observations of Papavasiliou et al. (1985) and Hamed et al. (2004). While the former had found non-significant alterations in serum calcium levels in patients with severe affective illness who had received electroconvulsive therapy, the observations of the latter were made in untreated epileptics. One-week treatment with SVP (360 mg/kg) alone significantly elevated the serum calcium levels compared to the control group, however no significant differences in the levels of serum calcium were observed in the groups that received either PHT (10 mg/kg) or NAC (320 mg/kg). Our results with SVP, PHT and NAC are in accordance with other clinical and experimental reports (Robinson et al., 1982; Hjortso et al., 1990; Ohta et al., 1995; Onodera et al., 2001). Even in the groups that were pretreated with either of these drugs and subjected to electroshock, no significant alterations in serum calcium were seen. These results reveal that PHT and SVP, when used at optimal dose levels, can protect against seizures without altering calcium homeostasis. Clinically also there are a few reports where no significant differences in serum calcium levels have been found in epileptic patients receiving either PHT or SVP (Ala-Houhala
et al., 1986; Babayigit et al., 2006). Even in the combination groups (at the fixed ratio of 3:1), no significant alterations in serum calcium levels were observed. These results indicate that NAC as an adjunct with either PHT or SVP might not produce any adverse effects on the serum calcium homeostasis, which may be an added advantage.

We found a decrease in the whole brain histamine concentration following MES induced seizures in mice. Consistent with our findings, Jin et al. (2007) have reported a progressive decrease in the histamine levels in the cortex, brainstem, hippocampus and hypothalamus of rats subjected to an intermittent MES procedure, and this decrease was associated with a reduction in the MES seizure severity. However, following the first MES, these investigators reported a significant reduction only in the hippocampus. A seizure-induced activation of protective histaminergic mechanisms and the subsequent increased neuronal histamine release were considered to be responsible for the inhibitory effects on subsequent seizures. Thus, the decrease in brain histamine content, observed in the present study, may be a reflection of an enhanced histamine release.

While pretreatment with both PHT and SVP at their respective ED$_{50}$ doses caused a decrease in the whole brain histamine content, a significant reduction was seen only with the former. Our results with SVP are consistent with those of Vohora et al. (2001), however there seems to be some discrepancy pertaining to our results obtained with PHT and those reported by other investigators (Vohora et al., 2001; Lensu et al., 2002). Probably, the higher dose of PHT (25 and 45
mg/kg, i.p. in mice and rats respectively) employed by these workers could be responsible for the opposite effects on the brain histamine levels. The reduction in the brain histamine levels observed in the current study following NAC treatment could probably be due to the enhanced histamine release caused by this agent. This effect could have probably occurred directly within the brain, since NAC has been reported to cross the blood brain barrier and accumulate in the brain (Farr et al., 2003). A few studies have indicated that NAC is capable of causing histamine release from mast cells (Barrett et al., 1985; Hong et al., 1991). Interestingly, there is now evidence that mast cells may be present in the CNS and they may be one of the sources of brain histamine (Taiwo et al., 2005).

In summary, our results with PHT and NAC reflect the ability of these drugs to modulate the histaminergic mechanisms and thereby facilitate the postictal seizure protection, an endogenous anticonvulsant phenomenon that follows an epileptic seizure and inhibits the induction of further seizures.

In the MES model, both clobenpropit and pyridoxine produced a reduction in the percent incidence of seizures in mice, however statistically significant reduction was evident only with pyridoxine (40 mg/kg, i.p.). While Yokoyama et al. (1994b) have reported a significant reduction in the tonic phase of electrically induced convulsions in mice with 1 mg/kg, i.p. of clobenpropit, Fischer and van der Goot (1998) have reported a significant protection against the maximal electroshock seizure threshold test only at a high dose of 40 mg/kg, i.p. The ability of pyridoxine to decrease the seizure susceptibility reflects its effectiveness against GTC seizures. Our results with pyridoxine are in
confirmation with those of Schlesinger and Lieff (1975) who had reported protection against electroconvulsive seizures following pyridoxine supplementation. The significant inhibition of seizures seen after the simultaneous use of clobenpropit (at its sub-effective doses) and pyridoxine signifies a beneficial interaction between these drugs.

H₃ receptors are known to regulate the release and synthesis of histamine (Arrang et al., 1983, 1987). Therefore, it appears that clobenpropit, a H₃ antagonist stimulates histamine release from the histaminergic presynaptic terminals, which might be responsible for the observed decrease in the brain histamine levels, a finding consistent with that of other investigators (Yokoyama et al., 1994b; Kakinoki et al., 1998). However, clobenpropit has also been reported to dose-dependently increase the activity of histidine decarboxylase (HDC) (Yokoyama et al., 1994b), which might probably be the other mechanism of action of clobenpropit that influences the brain histamine levels.

H₃ receptors are now considered as heteroreceptors and regulate the release of a wide variety of transmitters including gamma-aminobutyric acid (GABA), which is the principal inhibitory transmitter in the CNS (Schlicker et al., 1988, 1989, 1993; Dai et al., 2007). Several scientific reports indicate the existence of a close relationship between the histaminergic and the GABAergic systems. While Ericson et al. (1991) suggested that neurons of the histaminergic tuberomammillary nucleus contain the neurotransmitter GABA, Sakai et al. (1995) found a decrease in the brain GABA content of mice after treatment with
alpha-fluoromethylhistidine (a HDC inhibitor). Moreover, bicuculline has been reported to antagonize the inhibition of amygdaloid kindled seizures produced by clobenpropit (Ishizawa et al., 2000). Recently, Dai et al. (2007) have suggested the protective effects of clobenpropit against N-methyl-D-aspartate (NMDA) induced excitotoxicity to be mediated in part by the GABAergic system.

Pyridoxal phosphate is a coenzyme for various decarboxylases involved in the formation of neurotransmitters (Bender and Mayes, 2003). In the present study, pretreatment with pyridoxine did not change the brain histamine levels significantly suggesting its differential affinity for different decarboxylases involved in the turnover of various neurotransmitters. In vitro studies have reported activation of glutamate but not histidine decarboxylase by pyridoxal phosphate (Tapia and Pasantes, 1971). The decrease in GABA content and glutamic acid decarboxylase activity caused by convulsants like DL-penicillamine and thiosemicarbazide is reversed by treatment with pyridoxine (Abe and Matsuda, 1979). Ishizawa et al. (2000) have reported potentiation in the protective action of clobenpropit against amygdaloid kindled seizures by GABAergic drugs like diazepam, SVP and muscimol. Therefore, it is likely that the beneficial pharmacodynamic interaction between clobenpropit and pyridoxine observed in the present study may in part be mediated by the GABAergic system, since no significant effects were evident on the brain histamine levels. However, further studies are needed to substantiate the role of
GABAergic mechanisms in the effects of these drugs against MES induced seizures.

In view of a critical role of calcium ions in various physiological and pathological states, the determination of intracellular calcium \([\text{Ca}^{2+}]_i\), within the synapse is of special interest. The synapse is a major regulatory site that has been implicated in modulating neuronal excitability and seizure discharge (DeLorenzo, 1986). Sohn and Ferrendelli (1973) reported the inhibition of \(\text{Ca}^{2+}\) uptake into rat brain synaptosomes by PHT. However, in the present study using fura 2-AM, we provide evidence for the ability of PHT to decrease the resting \([\text{Ca}^{2+}]_i\) levels in the mouse brain synaptosomes. In the current study, we also found a dose-dependent reduction in the \([\text{Ca}^{2+}]_i\) levels following SVP treatment. At a concentration of 400 \(\mu\text{M}\), SVP did not modulate the \([\text{Ca}^{2+}]_i\) levels significantly, a finding consistent with that of Shao et al. (2005). These investigators have reported no effect of valproate treatment (0.6 mM) on basal \([\text{Ca}^{2+}]_i\), concentration in primary cultured rat cerebral cortical cells. However, in the present study, at a concentration of 800 \(\mu\text{M}\), we found a significant reduction in the \([\text{Ca}^{2+}]_i\) levels in the mouse brain synaptosomes. Supplementation with 50 \(\mu\text{M}\) NAC has been reported to inhibit the \(\text{Ca}^{2+}\) influx induced by hypoxia in primary hippocampal culture (Jayalakshmi et al., 2005), however in the present study, this concentration (and also higher) did not significantly effect the basal \([\text{Ca}^{2+}]_i\) levels in the mouse brain synaptosomes. Similarly, neither \(\text{H}_3\) receptor ligands (i.e. clobenpropit and imetit) nor pyridoxine had any appreciable effect on the basal \([\text{Ca}^{2+}]_i\) levels. Our results in mouse brain synaptosomes with
clobenpropit and pyridoxine are in agreement with the earlier reports of other investigators (Dakshinamurti et al., 2003; Dai et al., 2007) in primary cultured neurons. Imetit, a H₃ receptor agonist did not produce any significant effect on the basal [Ca²⁺]ᵢ levels in the mouse brain synaptosomes. This is in agreement with the findings of Vohora et al. (2007), who have reported a non-significant effect of another H₃ agonist namely R-alpha-methylhistamine on the mouse brain synaptosomal Ca²⁺ levels. In summary, our results reveal a role of PHT and SVP in modulating the brain neuronal calcium homeostasis beneficially, thereby highlighting their potential in the clinical management of acquired epilepsy.