Annals of Phytomedicine 5(1): 73-79, 2016

Journal homepage: www.ukaazpublications.com

ANNALS OF PHYTOMEDICINE
An International Journal

ISSN: 2393-9885

In vivo activity and pharmacokinetic analysis of combination of piperine and subtherapeutic dose of sodium valproate in refractory epilepsy

Bushra Parveen, Sayeed Ahmad* and Krishna Kolappa Pillai**

Department of Pharmaceutical Medicine, Faculty of Pharmacy, Hamdard University, New Delhi-110062, India
**Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Hamdard University, New Delhi-110062, India*
***Department of Pharmacology, Faculty of Pharmacy, Hamdard University, New Delhi-110062, India*

Received May 20, 2016: Revised May 30, 2016: Accepted June 5, 2016: Published online June 30, 2016

Abstract

Sodium valproate (SVP), a broad spectrum anticonvulsant, is associated with liver toxicity. The long term treatment may result in adverse consequences. Piperine (PIP), the major constituent obtained from the peppercorns of black pepper is used in anticonvulsant formulations in traditional Chinese medicine. A combination of subtherapeutic dose of SVP and PIP was evaluated for their activity in refractory epilepsy, using 6-Hz psychomotor seizure model (6 Hz, 44 mA for 3s duration) in mice. Further, the mechanism of seizure control by PIP was evaluated through reserpinisation, which was used as pathology inducer. A gas chromatography-mass spectrometry method was developed and validated for simultaneous analysis of SVP and PIP to evaluate their pharmacokinetic interaction. SVP in presence of PIP produced highest protection against 6 Hz induced seizures. However, the protection afforded by SVP and PIP was abolished in reserpinised mice, which was attributed to the depletion of monoamines by reserpine. The plasma estimation indicated 11-fold increase in the concentration of SVP in presence of PIP. Plasma analysis of SVP and PIP suggests that the doses can further be reduced to obtain therapeutic plasma concentrations. Attenuation of monoamine lowering effect of reserpine by PIP and SVP indicates that the mechanism is related to the levels of monoamine neurotransmitters in brain. Further, studies are warranted measuring the levels of neurotransmitters to provide clear insights on the role of monoamines in seizure control.

Key words: Piperine, sodium valproate, reserpine, 6 Hz, refractory epilepsy, gas chromatography mass-spectrometry

1. Introduction

Epilepsy accounts for 50 million sufferers worldwide, affecting 4-10 per 1000 people (Kwan and Brodie, 2002). The first line of treatment for epilepsy is antiepileptic drug (AED) therapy and sometimes surgery (Ryvlin, 2003), through which seizures are controlled in about 60% of patients. The remaining 30%, that fails to respond to AED's or their combination at a befitted dose for 1-2 years, are demarcated as pharmacoresistant, thus, constituting refractory epilepsy (Kwan and Brodie, 2000).

The pathomechanisms underlying refractory epilepsy are diverse, as upregulation of transporters (Mody, 1998) to modification of target (Pati and Alexopoulos, 2010) or abnormality in the neuronal networks (Poolos *et al*., 2012). Refractory epilepsy is synonymous to syndrome, with manifestations as intractable seizures, psychosocial dysfunction, neurochemical plastic changes, drug burden, cognitive decline and comorbidities like depression and psychosis (Kwan and Brodie, 2002). Hence, it is recommended to depend on polytherapy for better management of patients with refractoriness. The combination therapy is supposed to yield superior efficacy by widening the spectrum of action, as compared to monotherapy (Chateauvieux *et al*., 2010). Sodium valproate (SVP) is a well established and clinically effective anticonvulsant drug, having a broad spectrum of action (Wang *et al*., 1993). The anticonvulsant action of piperine (PIP), obtained from the peppercorn of the plant *Piper longum* (Piperaceae), is well supported from several pharmacological studies (Pei, 1983; Barton *et al*., 2001).

The present study focuses on developing refractory epilepsy, using 6-Hz psychomotor seizure model and evaluating the efficacy of combination of PIP and subtherapeutic dose of SVP. Reserpine was also used as a pathology inducer, in this study, as monoamine depleter. The role of monoamines in the anticonvulsant action of PIP has been advocated in maximal electroshock seizure (MES) model in mice (Pei, 1983). The response of reserpinised mice was evaluated in a refractory model of epilepsy, *i.e*., 6-Hz psychomotor seizure model. Since, SVP is a drug with narrow therapeutic index; its drug monitoring becomes inevitable. While coadministering PIP with SVP, pharmacokinetic interaction needs to be evaluated, as PIP is a potent bioenhancer. Several analytical methods are available for the plasma and serum quantification of SVP and PIP, individually. However, gas chromatography (GC) is considered to be the method of choice, owing to the volatile nature of SVP (Kang *et al*., 2011).

Author for correspondence: Ms. Bushra Parveen
Department of Pharmaceutical Medicine, Faculty of Pharmacy, Hamdard University, New Delhi-110062, India
E-mail: bushra8904@gmail.com
Tel.: +91-9971984966; Fax: + 91-11-26059663

In the present work, an effort has been made to develop and validate a rapid and sensitive GC-MS method in order to determine the effect of combination of SVP and PIP on plasma concentrations.

2. Materials and Methods

2.1 Reagents and chemicals

Sodium valproate and piperine were procured from Sigma Aldrich (St. Louis, MO, USA), reserpine was procured from Inverni Della Beffa (Milano, Itlay). The MS grade solvents (methanol, inert helium gas) used in GC-MS method was procured from Sigma Aldrich (St. Louis, MO, USA). Other chemicals used were of analytical grade obtained from commercial sources.

2.2 Animals

The proposed study protocol was approved by Jamia Hamdard, Institutional Animal Ethics Committee (Registration No. 173/ CPCSEA, 912), New Delhi. Swiss albino male mice weighing 18-25 g, were procured from the Central Animal House Facility, Hamdard University, New Delhi, India and were maintained under standard laboratory conditions of temperature (25±2°C) and relative humidity (55±5 % RH) with a 12 h light/dark cycle. The animals were housed in propylene cages with free access to food (Lipton feed, Mumbai, India) and water *ad libitum*. The animals were randomly divided into seven groups, five groups with six animals each and two groups with ten animals each and were dosed as below:

Ist group : Normal saline (10 ml/kg)
2nd group : SVP (150 mg/kg)
3rd group : PIP suspension 5.0 mg/kg
4th group : PIP suspension 10 mg/kg,
5th group : PIP suspension (5.0 mg/kg) + SVP (150 mg/kg)
6th group : Reserpine (1.0 mg/kg) + Normal saline (10 ml/kg)
7th group : Reserpine (1.0 mg/kg) + PIP suspension (5.0 mg/kg) + SVP (150 mg/kg)

All the drugs were given orally once in a day for three days except reserpine which was given as a single dose intraperitonealy, 24 h. before treatment.

2.3 Psychomotor seizures

Psychomotor seizures were induced *via* corneal stimulation (6-Hz, 44 mA for 3s duration). Saline was applied to the corneas of the animals before the stimulation. Animals were restrained during the stimulation and were released immediately following it. The psychomotor seizures are characterized by immobility or stun, forelimb or jaw clonus, twitching of the vibrissae and an elevated tail (straub tail). The animals were observed for duration of 120 s (Wojda *et al*., 2009). Protection against the seizures was regarded as the endpoint of the study. The animal was said to be protected if it resumes the normal behavior within 10 s after the stimulation (da Cruz *et al*., 2013).

2.4 GC-MS method for simultaneous estimation of SVP and PIP in plasma samples

2.4.1 *Instrumentation*

GS-MS analysis of the standard and plasma samples were carried out on a GC-MS system [Agilent 7890A series (Germany)] equipped with split-splitless injector and CTC-PAL auto sampler. The sampler was attached to a polar HP-5MS (5% phenyl polymethyl siloxane) capillary column (30 m x 0.25 mm i.d. and 0.25 µm film thickness) and fitted to a mass detector. Carrier gas flow rate (Helium) was 1.5 ml/min for first 7 min followed by 2.5 ml/min for next 6 min, splitless, injector temperature was 260°C, detector temperature at 300°C, while column temperature was kept at 70°C for 3 min, followed by 180°C for 3-6 min (at a rate of 35°C/ min), then at 280°C for 6-8 min (at a rate of 50°C) and then kept at hold for 4 min. Transfer line was heated at 280°C. Mass spectra were acquired in EI mode (70 eV); in m/z range 30-600. The amount of 0.1 µl of sample solution in methanol was injected. The components of the sample and standard were identified by comparison of their mass spectra to those from NIST/NBS libraries, using different search engines.

2.4.2 *Calibration curves of standard* SVP *and* PIP

Standard stock solution of SVP and PIP were prepared by dissolving them in LCMS grade methanol to produce a final concentration of 1.0 mg/ml, respectively. Working standard solutions for SVP were prepared in methanol producing 5, 10, 25, 50, 85 and 100 µg/ml. Concentration of standard PIP at 10, 25, 50, 75, 85 and 100 µg/ml, prepared in methanol were used to develop a standard plot taking total ion chromatogram (TIC) of each sample.

2.4.3 *Sample preparation*

The plasma (250 µl) was transferred to a centrifuge tube and equal volume of 2 M HCl (250 µl) was added and vortex mixed for 15 s. Extraction was performed using 2.0 ml ethyl acetate and then mixed for 10 min. It was then centrifuged for about 10 min at 3000 rpm. The organic layer was decanted and transferred to another eppendorf tube. It was dried under the stream of nitrogen and reconstituted into 250 µL LCMS grade methanol. Finally, a volume of 0.1 µl was injected into the chromatographic system.

2.4.4 *Method validation*

The method was validated as per the requirement of ICH guidelines for linearity, precision, accuracy, specificity, robustness, limit of quantification (LOQ) and limit of detection (LOD).

2.4.5 *Quantification of* SVP *and* PIP *in plasma samples*

The method was applied for the study of pharmacokinetic analysis of plasma samples of group 2nd (SVP, 150 mg/kg, p.o.) and group 5th (PIP, 5.0 mg/kg + SVP, 150 mg/kg, p.o.) in order to determine the effect of PIP on the plasma level concentration of SVP.

2.5 Statistical analysis

Protection of animals of various groups was analyzed by Chi-square test. Group means were compared by ANOVA followed by Dunnet t-test, to identify the differences between groups.

Statistical evaluation of pharmacokinetic data was performed, using one-way analysis of variance (ANOVA). All values are expressed as their Mean ± SEM. Statistically significant differences were assumed when $p<0.05$.

3. Results and Discussion

3.1 Effect of PIP, SVP and their combination on psychomotor seizures induced in mice

3.1.1 *Effect on protection against psychomotor seizures*

The normal saline did not show any overall protection against 6-Hz seizures and protection in all the behavioral categories. The SVP group (150 mg/kg, p.o.) and high dose PIP group (10 mg/kg, p.o.)

produced protection in half of the animals, thereby, exhibiting 50% protection. The behavioral percent protection shown by SVP group against stun/stupor, twitching of vibrissae and straub's tail was significant ($p<0.001$). Whereas, the protection afforded by low dose piperine group was not significant. The high dose PIP group showed similar overall protection to SVP group but slightly reduced protection (66.6%) against straub's tail (significant), ($p<0.001$). However, when low dose of PIP (5 mg/kg, p.o.) was coadministered with SVP (150 mg/kg, p.o.), the number of animals protected as well as inhibition of stun/stupor, twitching of vibrissae and straub's tail was increased significantly, showing improved protection at 83.3%. ($p<0.001$), as illustrated in Figures 1(a) and 1(b).

The normal saline group exhibited stun/stupor for 34.33 s, twitching of vibrissae for 110.833s and straub's tail for 73.166s. The SVP group and high dose PIP group showed significant decrease in the duration of all the behavioral categories. Low dose PIP showed long duration of stun/stupor for 64s (not significant), but the duration of other parameters was significantly reduced as compared to the normal saline group. In the combination group of SVP and PIP (5.0 mg/kg, p.o.), very significant decrease was observed in the duration of stun and straub's tail, however twitching of vibrissae was only significantly reduced ($p<0.01$), as illustrated in Figure 1 (c).

The psychomotor seizures elicited by the normal saline group were characterized by stunned posture, twitching of vibrissae and straub's tail in 6-Hz seizure model induced *via* corneal stimulation (44 mA for 3s duration), resulting in development of resistant epilepsy. The appearance of the above said behavioral parameters indicates establishment of therapy- resistant epilepsy, as 6-Hz psychomotor seizure test is one of the models of pharmacoresistant epilepsy (Barton *et al*., 2001).

In the present investigation, subtherapeutic dose of SVP, 150 mg/kg, p.o. exhibited 50% protection against 6-Hz-induced psychomotor seizures in mice. Significant protection was observed against stun/stupor and straub's tail but not with twitching of vibrissae. In the previous studies, sodium valproate at a dose range of 258-335 mg/kg is reported to be effective against psychomotor seizures (Wojda *et al*., 2009). Previous recordings at our lab demonstrated significant protection against 6-Hz-induced psychomotor seizures in mice with equivalent clinical dose of SVP.

Piperine exhibited protection against 6-Hz psychomotor seizures at doses of 5.0 mg/kg and 10 mg/kg, p.o. The protection observed in 10 mg/kg PIP was, however, comparable to that produced by 150 mg/kg SVP. The proposed study, therefore, indicates the presence of anticonvulsant activity of PIP in refractory model of epilepsy. The present study conforms to a study by da Cruz *et al.* (2013) where PIP was shown to have anticonvulsant activity in pilocarpine-induced seizures at doses of 2.5, 5, 10 and 20 mg/kg (da Cruz *et al*., 2013). In another study, PIP blocks the convulsions induced by intra-cerebroventricular injection of threshold doses of kainite (D'Hooge *et al*., 1996).

In the present study, a combination of PIP 5.0 mg/kg and SVP 150 mg/kg, p.o. improved the efficacy of SVP against therapy-resistant seizures, as evident by enhanced percentage protection against psychomotor seizures induced *via* 6-Hz psychomotor seizure model. In a study by Sarogi and others, the isobolographic analysis of combination of piperine and phenytoin displayed additive interaction in the mouse maximal electroshock model (Sarogi *et al*., 2013).

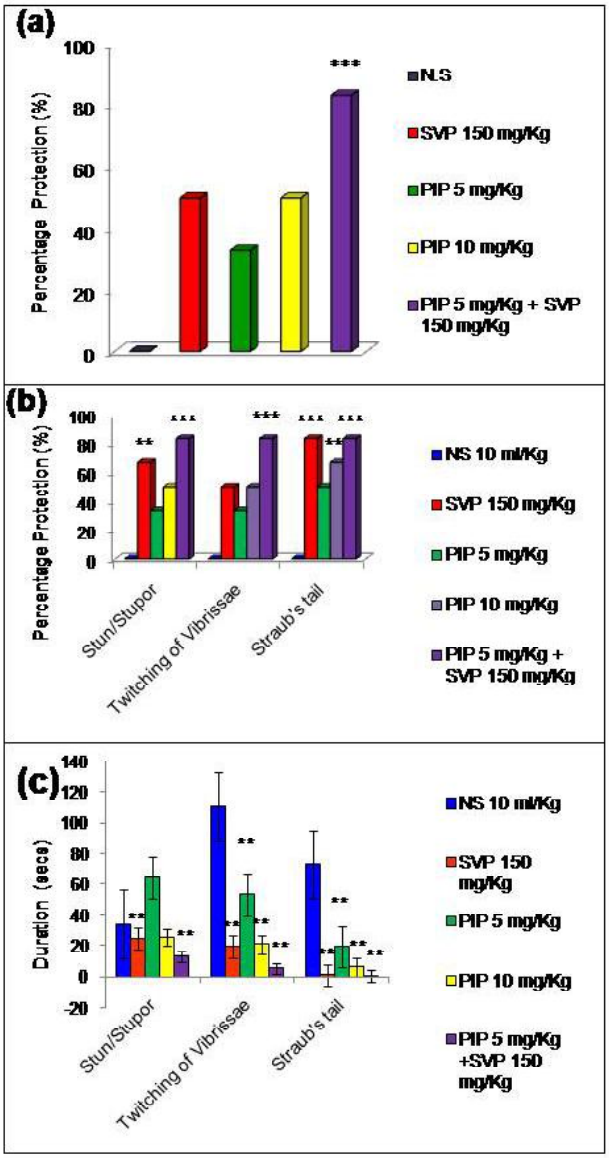


Figure 1: Effect of SVP, PIP and their combination on percentage protection and duration of stun/stupor, twitching of vibrissae and straub tail in 6 Hz psychomotor seizure model in mice. N.S. = Normal Saline, SVP = Sodium Valproate, PIP = Piperine. (a) Overall percentage protection by SVP, PIP and their combination. (b) Percentage protection against stun/stupor, twitching of vibrissae and straub's tail. (c) Duartion of stun/stupor, twitching of vibrissae and straub's tail. Values are expressed as Mean ± SEM.

3.2 Effect of PIP, SVP and their combination on psychomotor seizures induced in reserpinised mice

3.2.1 *Effect on protection against psychomotor seizures*

The administration of reserpine (RES) (1.0 mg/kg, i.p.) ,followed by NS (10 ml/kg, p.o.) resulted in survival of two animals out of ten, with percentage survival accounting to 20%, whereas the group that received piperine (5.0 mg/kg, p.o.) and SVP (150 mg/kg, p.o.) in reserpinised animals resulted in 80% percentage survival, showing

mortality in four animals. Therefore, piperine and SVP treatment has enhanced the percentage survival in the reserpinised animals as given in Figure 2(a).

Reserpinisation has sensitized the animals to convulsive stimuli. The protection exhibited by piperine and SVP combination against 6 Hz seizures was abolished by reserpinisation. Twitching of vibrissae was not observed in any animal. Clonus was, however, observed in one animal, in the reserpinised normal saline group, as shown in Figure 2(b).

The reserpinised normal saline group (RES 1.0 mg/kg, i.p. + N.S 10 ml/kg, p.o.) showed stupor/stun for 62.5 s, clonus for 9 s and straub's tail for 48.5 s. In the reserpinised combination group (RES 1.0 mg/kg, i.p. + PIP 5.0 mg/kg + SVP 150 mg/kg, p.o.), stun/stupor was observed for 32.33 s, clonus for 8 s and straub's tail for 97.831 s, as shown in Figure 2(c).

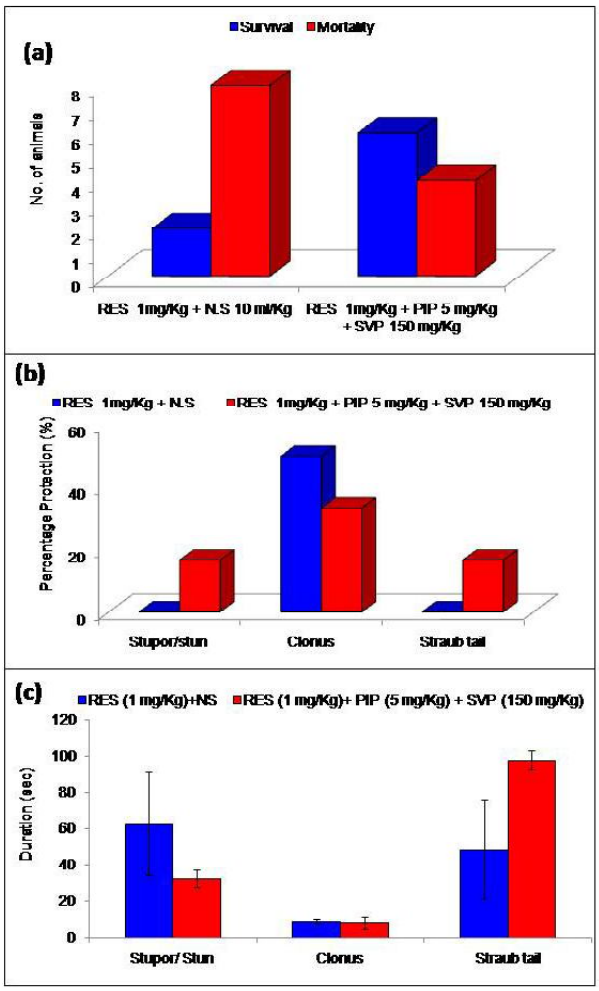


Figure 2: Effect of SVP, PIP and their combination on percentage protection and duration of stun/stupor, twitching of vibrissae and straub's tail in 6 Hz psychomotor seizure model in reserpinised mice. RES = Reserpine, N.S. = Normal saline, PIP = Piperine, SVP = Sodium valproate (a) Survival status of reserpinised mice in normal and treatment group. (b) Percentage protection against stun/stupor, clonus and straub tail in reserpinised mice. (c) Duartion of stun/stupor, twitching of vibrissae and straub's tail in reserpinised mice. Values are expressed as Mean ± SEM.

The treatment of reserpinised mice with PIP and SVP combination resulted in decreased mortality, indicating that PIP and SVP attenuated the monoamine lowering effects of reserpine. The reserpinisation of the mice abolished the protection exhibited by the combination of SVP and PIP, again affirming that the action of piperine is related to the levels of monoamines in brain. The reserpinisation of the mice results in depletion of the monoamine neurotransmitters including 5-hydroxytryptamine (5-HT) and norepinephrine (NE) and increases the susceptibility to seizures in MES. The slight increase in protection of different behavioral changes and a slight decrease in the duration of behavioral changes in reserpinised combination group can be attributed to the 5-HT and NE elevating action of piperine and sodium valproate (Pei, 1983; Vriend and Alexiuk, 1996; Baf *et al.*, 1994).

The depression associated with epilepsy has been showed to be related to the alteration in monoaminergic pathways and GABAergic pathways. The antidepressant effects of PIP in epilepsy associated depression are due to MOA-inhibitor activity of PIP (Pal *et al.*, 2011).

3.3 Method development

SVP and PIP are the low molecular weight compounds with favourable sensitivity in electric ionization mode detection. The mass spectrum of SVP showed SRM (selected reaction monitoring) transition from *m/z* 101.9 to *m/z* 72.9 (Figure 3a) and from *m/z* 280.9 to *m/z* 206.9 in PIP (Figure 3b). The retention times (RT) for SVP and PIP were 5.3 min and 11.2 min, respectively (Figure 4).The chromatograms showed a stable baseline and good resolution between drugs and endogenous materials of plasma. The peaks are completely separated and the shapes are symmetrical.

3.3.1 *Calibration curves of* SVP *and* PIP

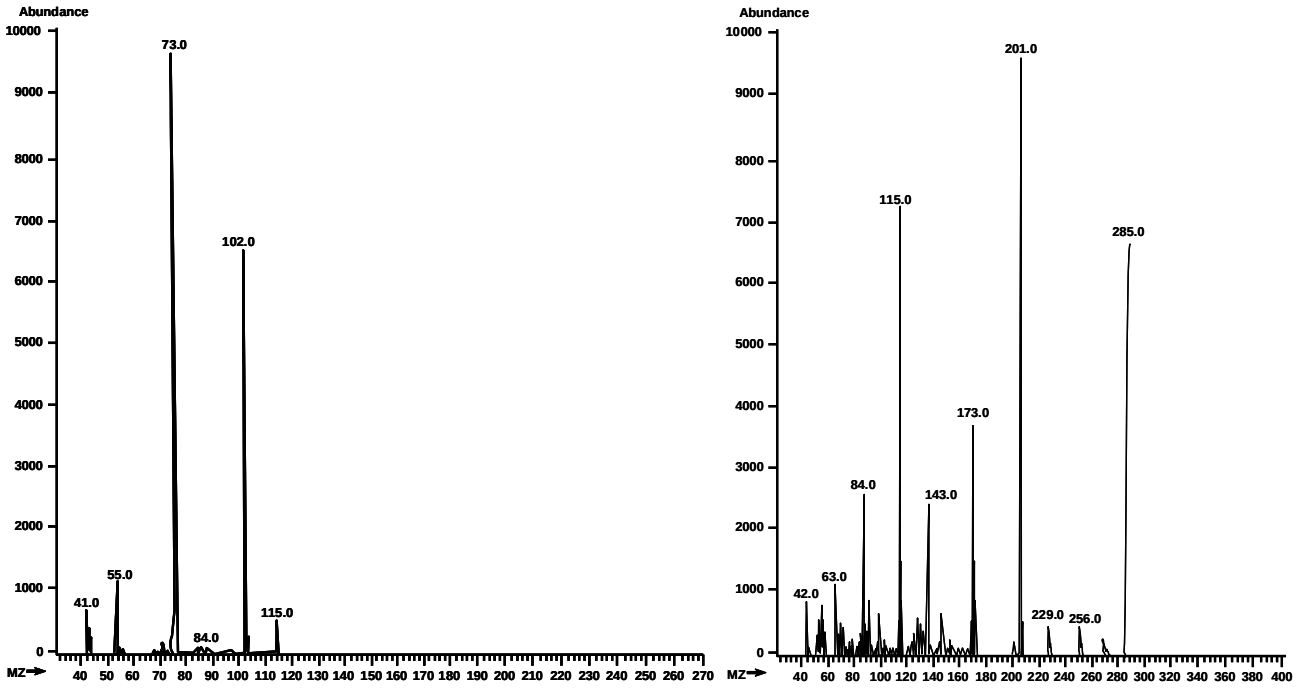
Calibration curve was plotted between abundance and concentration of drugs. For SVP, the linearity was obtained in the range of 5-100 µg/ml with regression coefficient $r^2 = 0.9922$. The regression equation for straight line was y = 17285x + 31524. The regression coefficient confirmed that the calibration curve was linear over the wide concentration range for SVP. The typical calibration curve had a slope of 17285 ± 11.6 and an intercept of 31526 ± 36.04.

For PIP, the linearity was obtained in the range of 10-100 µg/ml with regression coefficient $r^2 = 0.993$. The regression equation for straight line was y=10874x - 26909, with a slope of 10873 ± 2.51 and an intercept of 26894 ± 106.29.

3.4 Method validation

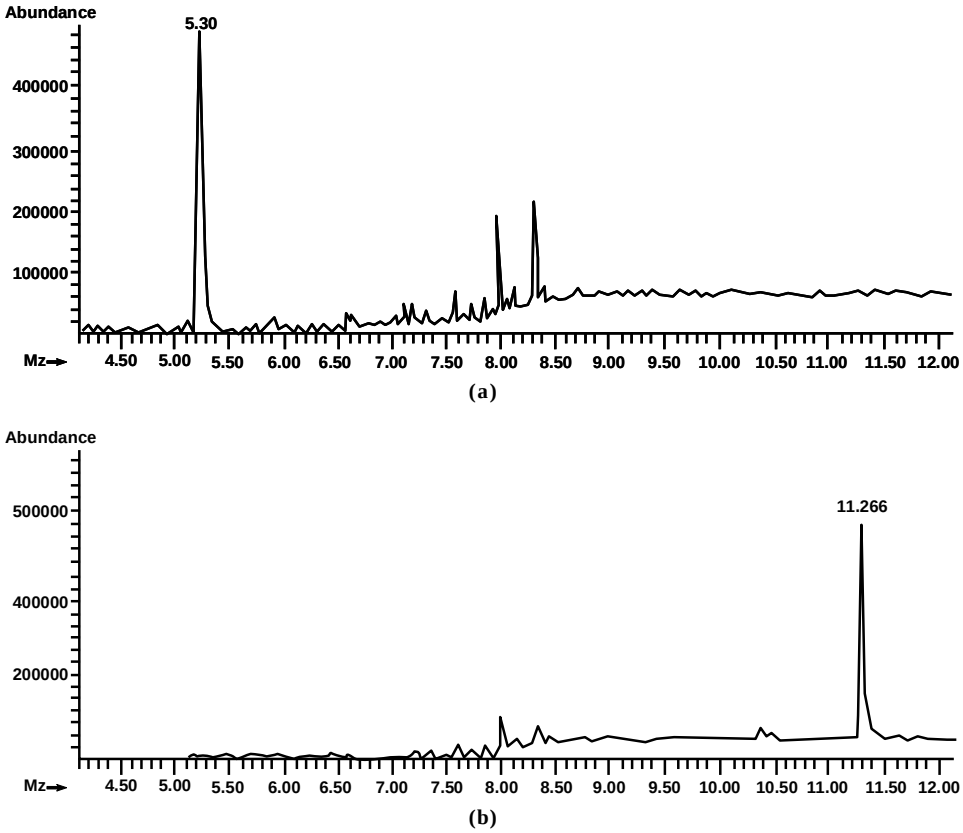
The precision of the method was calculated in terms of repeatability and intermediate precision at three concentration levels (25, 50, and 100 µg/ml). The %RSD values of TIC (total ion chromatogram) and RT were calculated, to determine repeatability. For intermediate precision, three samples of SVP and PIP, respectively were injected in triplicate on three consecutive days (inter-day precision) and three times on the same day (intra-day precision). The %RSD values were within accepted variable limit of ± 2% (Table 1).

The accuracy of the method was determined by spiking SVP and PIP three times at three concentrations (10%, 25%, and 50%) to sample containing 50 µg/ml of SVP and PIP each, respectively. The %RSD values after spiking were found in the range of 0.83-1.25% (Table 1).



(a) **(b)**

Figure 3: Mass spectrum of (a) sodium valproate and (b) piperine, showing SRM (selected reaction monitoring) transition from m/z 101.92 to m/z 72.9 and m/2 280.9 to m/z 206.9, respectively



(a)

(b)

Figure 4: Total ion chromatogram of 100 μg/ml of (a) sodium valproate and (b) piperine, showing RT at 5.3 min and 11.2 min, respectively

Robustness of the method was carried out by introducing very small but deliberate changes in the analytical methodology. For the newly proposed method, robustness studies were carried out by varying gas chromatographic conditions-deviation of ± 10% on the carrier gas flow, ± 2°C on the initial oven temperature, and ± 1°C/ min on the ramp rate. The RT and TIC of method were almost the same, with low values of % RSD (<0.82) indicating the robustness of the method (Table 1).

The LOQ and LOD determined on the basis of signal-to-noise ratio were found to be 5.0 µg/ml and 1.8 µg/ml, respectively for SVP and 10 µg/ml and 3.0 µg/ml for piperine, respectively.

Table 1: Validation parameters of the proposed GC-MS method

Validation parameters	Observed values
Precision (n = 3), as RSD range (%)	
Inter-day	0.31-1.89
Intra-day	1.24-3.10
Accuracy (n = 3), as recovery range (%)	0.83-1.25
Robustness (n = 3), as RSD range (%)	
Carrier gas flow	0.47-0.63
Initial oven temperature	0.23-0.47
Lamp temperature	0.33-0.59
Repeatability, as RSD range (%)	
RT	0.27-2.01
Total ion chromatogram	1.25-3.06

RSD-Relative Standard Deviation, RT-Retention time, n-no. of animals

3.5 Estimation of SVP and PIP simultaneously in plasma samples using GC-MS

The plasma sample of group 2nd (SVP 150 mg/kg, p.o.) showed 23.33 µg/ml of plasma concentration of SVP, whereas, group 5th (PIP 5.0 mg/kg+ SVP 150 mg/kg, p.o.) showed 323.69 µg/ml and 126.49 µg/ml of plasma concentration of SVP and PIP, respectively (Figure 5 and Table 2).

Table 2: Plasma concentration of SVP and PIP using GC-MS method

Groups	Treatment	Concentration of SVP in plasma (µg/ml)	Concentration of PIP in plasma (µg/ml)
V	PIP 5 mg/kg +SVP 150 mg/kg, p.o.	323.69	126.49
II	SVP 150 mg/kg, p.o.	23.33	-

SVP- Sodium valproate; PIP- Piperine

A sensitive and specific method was developed and validated for simultaneous analysis of SVP and PIP. The analysis of the plasma samples indicated 11-fold increase in the C_{max} of SVP 150 mg/kg + PIP 5.0 mg/kg, p.o. group, as compared to SVP alone, resulting in higher plasma levels, than the earlier reported therapeutic range of SVP, *i.e.*, 40-100 µg/ml. Piperine is a bioenhancer and results in increased plasma concentration of the drugs administered with it,

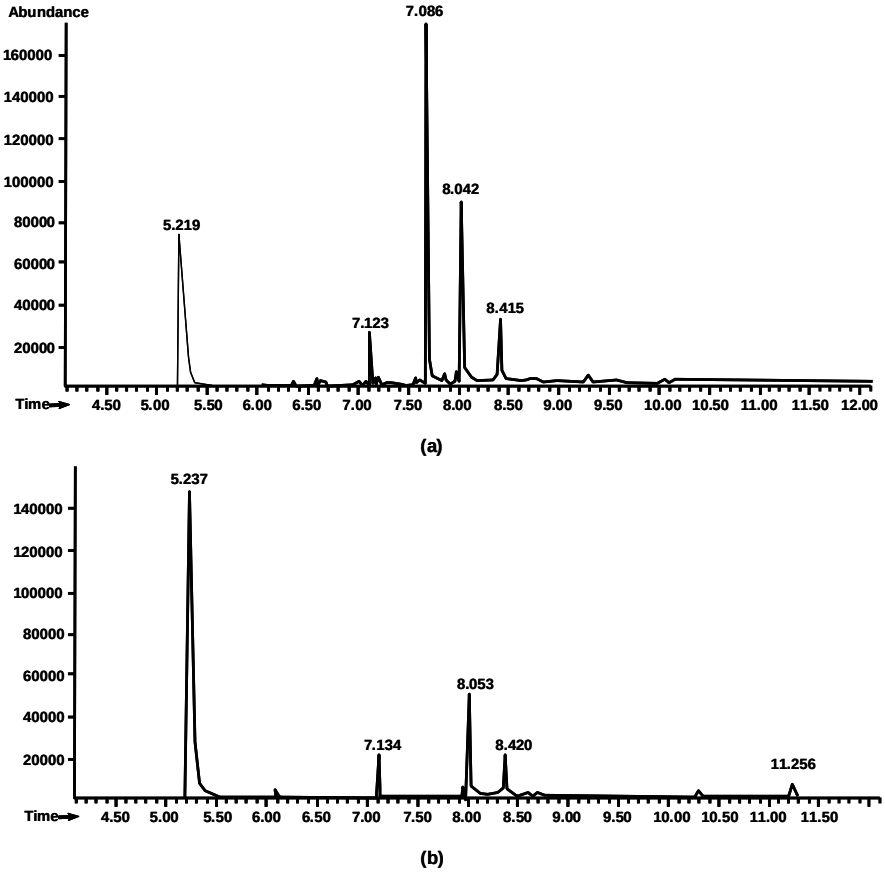


Figure 5: Total ion chromatogram of plasma samples of (a) Group 2nd (SVP,150 mg/kg) (b) Group 5th (PIP, 5 mg/kg + SVP, 150 mg/kg)

whereas, sodium valproate is known to increase the plasma concentration of the co-administered drug, like lamotrigine. However, this drug interaction does not limit the coadministration of SVP and lamotrigine. In a study by Poolos *et al.* (2012) out of 32 most frequent combinations, only SVP and lamotrigine had superior efficacy (Poolos *et al*., 2012). The clinical studies evaluating the effect of piperine on steady-state pharmacokinetics of carbamazepine (Pattanaik *et al*., 2009) and phenytoin (Pattanaik *et al*., 2006) reported enhanced bioavailability of carbamazepine and phenytoin, respectively.

The present study, therefore, suggests that PIP enhances the efficacy of SVP in resistant epilepsy. However, the higher plasma levels of SVP indicate the possibility of further reducing the dose of SVP. The administration of subtherapeutic dose of SVP may, however, require thorough monitoring to explore any possibility of drug resistance.

4. Conclusion

The combination of PIP with SVP facilitated the protection against refractory epilepsy. However, the dose of either PIP or SVP needs to be adjusted to keep the levels within the therapeutic range. A combination of SVP and PIP attenuated the monoamines lowering effect of reserpine as they enhanced the survival in reserpinised mice suggesting that the mechanism of action of PIP or both PIP and SVP is related to the levels of monoamine neurotransmitter in brain. The bioanalytical method was developed and validated in accordance with the ICH criteria. The assay achieved good sensitivity and speciûcity for the determination of SVP in presence of PIP in mice plasma after oral administration. Our results, thus, hold a great promise for the use of PIP as an adjunct to SVP therapy in refractory epilepsy. The role of monoamines in refractory epilepsy strongly deserves to be explored further.

Conflict of interest

We declare that we have no conflict of interest.

References

Baf, M.H.; Subhash, M.N.; Lakshmana, K.M. and Rao, B.S. (1994). Sodium valproate induced alterations in monoamine levels in different regions of the rat brain. Neurochem. Int., **24**:67-72.

Barton, M.E.; Klein, B.D.; Wolf, H.H and White, H.S. (2001). Pharmacological characterization of the 6 Hz psychomotor seizure model of partial epilepsy. Epilepsy Res., **47**:217-27.

Chateauvieux, S.; Morceau, F.; Dicato, M. and Diederich, M. (2010). Molecular and therapeutic potential and toxicity of valproic acid. J. Biomed. Biotechnol., pp:1-18.

D'Hooge, R.; Pei, Y.Q.; Raes, A.; Lebrun, P.; van Bogaert, P.P. and de Deyn, P.P. (1996). Anticonvulsant activity of piperine on seizures induced by excitatory amino acid receptor agonists. Arzneimittelforschung, **46**:557-60.

da Cruz, G.M.; Felipe, C.F.; Scorza, F.A.; da Costa, M.A.; Tavares, A.F.; Menezes, M.L.; de Andrade, G.M.; Leal, L.K.; Brito, G.A.; da Graça Naffah-Mazzacoratti, M.; Cavalheiro, E.A. and de Barros Viana, G.S. (2013). Piperine decreases pilocarpine-induced convulsions by GABAergic mechanisms. Pharmacol. Biochem. Behav., **104**:144-53.

Kang, J.; Park, Y.S.; Kim, S.H.; Kim, S.H. and Jun, M.Y. (2011). Modern methods for analysis of antiepileptic drugs in the biological fluids for pharmacokinetics, bioequivalence and therapeutic drug monitoring. Korean J Physiol. Pharmacol., **15**:67-81.

Kwan, P and Brodie, M.J. (2000). Early identification of refractory epilepsy. N. Engl. J. Med., **342**:314-9

Kwan, P. and Brodie, M.J. (2002). Refractory epilepsy: A progressive, intractable but preventable condition. Seizure., **11**:77-84.

Mody, I. (1998). Ion channels in epilepsy. Int. Rev. Neurobiol., **42**:199-226.

Pal, A.; Nayak, S.; Sahu, P.K and Swain, T. (2011). Piperine protects epilepsy associated depression: A study on role of monoamines. Eur. Rev. Med. Pharmacol. Sci., **15**:1288-1295.

Pati, S. and Alexopoulos, A.V. (2010) Pharmacoresistant epilepsy: From pathogenesis to current and emerging therapies. Cleve. Clin. J. Med., **77**:457-67.

Pattanaik, S.; Hota, D.; Prabhakar, S.; Kharbanda, P. and Pandhi, P. (2006). Effect of piperine on the steady-state pharmacokinetics of phenytoin in patients with epilepsy. Phytother. Res., **20**:683-686.

Pattanaik, S.; Hota, D.; Prabhakar, S.; Kharbanda, P and Pandhi, P. (2009). Pharmacokinetic interaction of single dose of piperine with steady-state carbamazepine in epilepsy patients. Phytother. Res., **23**: 1281-1286.

Pei, Y.Q. (1983). A review of pharmacology and clinical use of piperine and its derivatives. Epilepsia, **24**:177-182.

Poolos, N.P., Warner, L.N., Humphreys, S.Z and Williams, S. (2012). Comparative efficacy of combination drug therapy in refractory epilepsy. Neurology. **78**:62-68.

Ryvlin, P. (2003). Beyond Pharmacotherapy: Surgical Management. Epilepsia, **44**:23-28.

Sarogi, P.; Vohora, D.; Khanam, R. and Pillai, K.K. (2013). Combination therapy of piperine and phenytoin in maximal electroshock induced seizures in mice: Isobolographic and biochemical analysis. Drug Res. (Stuttg), **63**:311-318.

Vriend, J.P and Alexiuk, N.A.M. (1996). Effects of valproate on amino acid and monoamine concentrations in striatum of audiogenic seizure-prone balb/c mice. Mol. Chem. Neuropathol., **27**:307-324.

Wang, L.; Veith, R.; Landes, R.C.; Suzuki, Y. and Walson, P.D. (1993). Antiepileptic effect of antiepilepserine in pentylenetetrazole and amygdal kindled rats. Epilepsy Res., **15**:1-5.

Wojda, E.; Wlaz, A.; Patsalos, P.N and Luszczki, J.J. (2009). Isobolographic characterization of interactions of levetiracetam with the various antiepileptic drugs in the mouse 6Hz psychomotor seizure model. Epilepsy Res., **86**:163-174.