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ISOBOLOGRAPHIC ANALYSIS OF THE INTERACTION OF TRAMADOL WITH ANTICONVULSANT DRUGS IN MICE

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ABSTRACT. Experimental research followed by clinical studies have demonstrated the existence of several types of pain, thus the pain classification according to the mediation has widely extended. In addition, the study of the interactions with pharmacodynamic mechanism expanded very much during the last years; therefore in the new theories appear significant changes concerning synergism, addition and subadditivity in binary combinations. The investigations in this paper were aimed the demonstration of the antinociceptive of some drugs with anticonvulsant action and the analysis of their binary combinations with tramadol, using isobolar analysis. As model of nociception has been used the test of abdominal constrictive response in mouse induced by Zymosan A. the test substances were administered orally alone or in fixed proportion combinations. The data obtained were subjected to isobolar analysis. According to the statistical analysis the following have been observed: the binary combination tramadol-VA has proven to be synergistic (Zmix < Zadd, f = 0,5, p1 = 677, Tc =3.936, Tt = 3,529, c = 12.78, Ft = 4.46, $p < 0.05$), while the binary combination tramadol-CBZ has proven to be borderline additive (Z mix < Zadd).

Key words: Isobolographic analysis; Valproic acid; Tramadol; Carbamazepine; Antinociceptive effect.

REZUMAT. Analiza izobolară a interacţiunii dintre tramadol şi medicamente anticonvulsivante la şoarece. Cercetări experimentale, urmate de studii clinice, au demonstrat, în timp, existenţa mai multor tipuri de durere, astfel clasificarea durerii după mediaţia pusă în evidenţă s-a extins foarte mult. De asemenea, studiul interacţiunilor cu mecanism farmacodinamic a luat amploare

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în ultimii ani, astfel încât în teoriile noi apar modificări substanţiale în ceea ce priveşte sinergismul, adiţia şi subaditivitatea în combinaţiile binare. Cercetările efectuate au avut drept scop demonstrarea acţiunii antinociceptive a unor medicamente cu acţiune anticonvulsivantă şi analiza combinaţiilor binare dintre acestea şi tramadol prin analiza izobolară. S-a utilizat ca model de nocicepţie testul răspunsului constrictiv abdominal la şoarece, indus prin Zymosan A. Substanţele de studiat au fost administrate pe cale orală, singure sau în asociere, în proporţie fixă. Datele obţinute au fost supuse analizei izobolare. În urma analizei statistice, s-au constatat următoarele: combinaţia binară tramadol-VA s-a dovedit a fi sinergică (Zmix < Zadd, f = 0,5, p1 = 677, Tc =3.936, Tt = 3.529, c = 12.78, Ft = 4.46, $p < 0.05$), în timp ce combinaţia binară tramadol-CBZ s-a dovedit a fi la limita adiţiei (Z mix < Zadd).

Cuvinte cheie: analiza izobolară; acid valproic; tramadol; carbamazepină; efect antinociceptiv.

INTRODUCTION

Analgesic therapy is largely based on two main groups of drugs, which include painkillers (opioid and non-opioid) and non-steroidal anti-inflammatories (NSAIDs). During the last decade the study of pain became more and more important, pain itself changing from a mere symptom to a disease in itself.

Experimental investigations, followed by clinical studies have demonstrated with time the existence of several types of pain, extending the classification of pain according to its mediation. In the same time, under the name of analgesics have been classified many drugs from other groups, these being called co-analgesics or para-analgesics (Mungiu, 2002). Some of these have their own analgesic activity, very useful in some types of pain (like carbamazepine in the trigeminal neuralgia) (Zakrzewska and Linskey, 2014), while others do not have it, but associated with analgesics might increase their antinociceptive action (like glucosamine) (Tallarida *et al.,* 2003). Also, as during the last years the study of the interactions with pharmacodynamic mechanism became more important, in the newly emerging theories appear substantial changes concerning the synergism, addition and subadditivity of binary combinations.

On the other hand, a principle formulated concerning the pharmacology of analgesic combinations as a rational way of improving pain treatment states that by associating drugs with different action mechanisms we can obtain a multimodal coverage of a wider spectrum of types of pain. Thus it is created the potential for an interaction which might be higher than an additive one (Raffa, 2001).

The research presented in this paper had as purpose the demonstration of the antinociceptive action of some drugs with anticonvulsant action and the analysis of their binary combinations with tramadol.

MATERIALS AND METHODS

These studies used adult male Swiss mice weighing 20-30 grams (source, Cantacuzino Institute, Bucharest, Romania). The tests were performed in the Laboratory of experimental pharmacodynamics of the Department of Pharmacodynamics and Clinical Pharmacy of the Faculty of Pharmacy within the University of Medicine and Pharmacy "Gr.T. Popa", Iaşi, Romania.

Habitation conditions were insured in a room with controlled temperature (21 ± 2°C) with a 12/12 hours light / dark cycle. In each Plexiglas cage were placed 6-10 animals. The mice received standard fodder (Băneasa biological base, Romania) and water *ad libitum*, while their behavior has been followed 10 days before beginning the experiment; 3 hours before the administrations access to food and water has been discontinued. Testing began at 10 AM for each group.

All experimental procedures have been executed in agreement with the regulations of the bioethical committee of the University of Medicine and Pharmacy "Gr.T. Popa", Iaşi, and with the European legislation concerning experiments performed using live animals (Zimmermann, 1986). In this paper, the substances used were drugs with anticonvulsant and potential antinociceptive activity [Valproic acid sodium salt (VA) - Sigma, Carbamazepine (CBZ) - Sigma, a known opiate analgesic (Tramadol) and Zymosan A].

The drugs have been suspended in sodium-carboxy-methyl-cellulose (CMC-Na 0.1%) gel and the dosage was administered in geometrical progression either alone or in combination with tramadol. As a nociception model, we used the test of the abdominal constrictive response (writhing test) (Le Bars *et al.,* 2001a; Le Bars *et al.,* 2001b) induced by intraperitoneal injection of a saline suspension of Zymosan A 40 mg/kg/bw.

The recording of the abdominal constrictive responses was made during 12 minutes from the moment of the injection and the interpretation of the response was quantal, depending on its presence or absence. An antinociceptive effect has been considered the significant inhibition of the pain response or its absence. Nociceptive inhibition percentage has been calculated following the formula: % antinociception = no. non-responders/total no. of animals x 100.

The data obtained were subjected to isobolar analysis and statistical analysis (Anova, t-test). A statistical significance has been considered for a value of $p < 0.05$.

The study method is based on a recent concept of the composite additive curve that extends isobolar analysis to further levels of effect. In contrast with the isobologram, which uses sets of combinations of equal active doses for a single specific effect, isobolar analysis examines drug combinations on a larger effect range, offering information that is more complete. Through such an experiment an experimental regression curve is obtained which is further compared with the composite additive regression curve, in a variance analysis on the relation log dose - effect (Tallarida, 2001). In addition, the final data obtained constitute elements of graphical representation of the isobole (Tallarida *et al.,* 1997a; Tallarida *et al.,* 1997b).

The doses in combination are set as fractions of the ED_{50} of each substance such as their sum equals 1. When these units are expressed as mass units, the total amount of the combination is a sum, Zadd: $Zadd = fA + (1-f)B$, where A and B are fractions of the drugs **A** and **B.**

By administering the combination in several doses, the Zmix value is determined, necessary for obtaining the required effect. From the derived and calculated values Zadd/Zmix a test of statistical significance is performed. A statistically significant difference indicates a non-additive combination thus:

Synergism: Zmix < Zadd
Addition: Zmix ~ Zadd
Sub-addition: Zmix > Zadd

The "interaction index" or the ratio between the combination potency and the additive potency indicates the size and nature of the interaction. When this number is smaller than 1, we are talking about synergism (super-addition); when the index is higher than 1 we are calling it sub-addition (when synergism is detected *Zmix*< *Zadd*; therefore *Zmix* = *αZadd* for values of α smaller than 1).

It is important to notice that a potency value (like the ED_{50}) is a value of the dose or of the concentration for a certain specific effect (like a 50% effect level). Hence the interaction index, which is calculated from the values of the potency, may differ according to the level of effect (Vucković *et al.,* 2006).

RESULTS

By orally administering tramadol in doses comprised between 3.75 - 30.00 mg/kg/bw an ED_{50} value of 10.77 mg/kg/bw has been obtained (*Table 1*).

By orally administering carbamazepine in doses comprised between 7.5-60.00 mg/kg/bw an ED_{50} value of 41.14 mg/kg/bw has been obtained (*Table 1*).

By orally administering valproic acid in doses comprised between 5.00 – 40.00 mg/kg/bw an ED_{50} value of 21.77 mg/kg/bw has been obtained (*Table 1*).

Table 1 - ED50 for the drugs in the study, administered alone

	Tramadol	Carbamazepine (CBZ)	Valproic acid sodium salt (AV)
ED50 (SEM)[1] mg/kg/p.o.	10.77 (2.54) Y = 2.17 +2.77*X R = 0.908	41.14 (12.20) Y = 0.59 +2.72*X R = 0.976	21.77 (6,68) Y = 1.94 +2.28*X R = 0.926

[1]SEM: Standard error of the mean

From *Table 1* can be observed that each substance demonstrated an antinociceptive action on the model of nociception taken in study. The values of ED_{50} are necessary for plotting the composite additive curve (*Fig. 1a, Fig. 2 a*). From the analysis of these curves the Zadd value can be established and the proportions of each substance for obtaining the binary combination (*Table 2*).

In *Table 2*, the Zadd values for each combination can be obtained, the proportions of the drugs taken in study and the maximum possible effect (MPE%) resulted from their administration.

Table 2 - ED50 for the combinations of the drugs taken in the study

	Total dose mg/kg/p.o.	Maximal possible effect (MPE) %	ED50 (SEM)	
			Zadd (SEM)	Zmix (SEM)
Valproic acid / Tramadol (0.677/0.323)	2.00	16.66	16.00 (3.08) Y = 1.97 +2.51*X R = 0.918	4.39 (1.06)[1] Y = 2.84 +3.11*X R = 0.956 Interaction index=0.274
	4.00	33.33		
	8.00	66.66		
	16.00	100.00		
Carbamazepine/ Tramadol (0.799/0.201)	3.22	0.00	25.77 (4.74) Y = 1.11 +2.75*X R = 0.940	22.90 (10.02)[2] Y = 2.09 +2.13*X R = 0.953
	6.44	16.66		
	12.88	33.33		
	25.77	50.00		

[1]Synergistic combination; [2]Sub-additvity/additivity

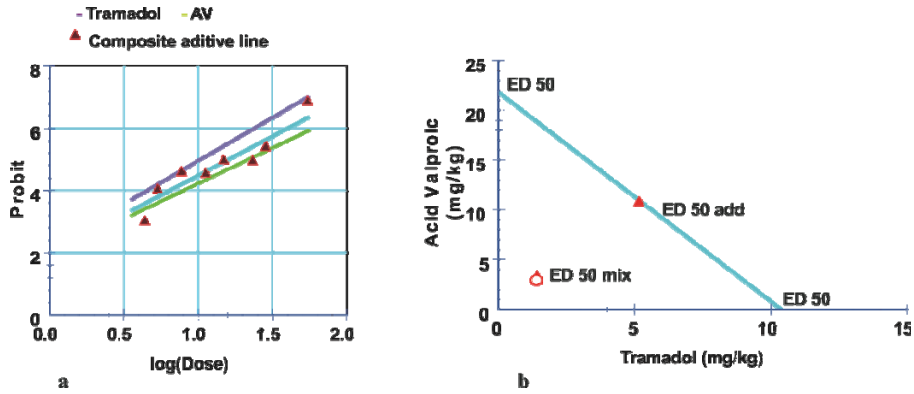

Figure 1 - Isobolographic analysis for the combination tramadol/valproic acid (AV) (a - analysis for the composite additive line; b - isobole)

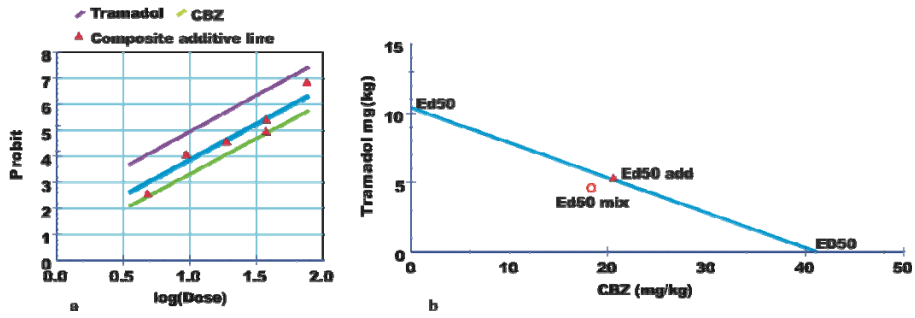

Figure 2 - Isobolographic analysis for the combination tramadol/carbamazepine (a - analysis for the composite additive line; b - isobole)

After the statistical analysis, the following have been ascertained: the binary combination tramadol-VA has proven to be synergistic (Zmix < Zadd, f = 0,5, p1 = 677, Tc =3.936, Tt = 3,529, Fc = 12.78, Ft = 4.46, $p < 0.05$) (*Fig. 1b*), while the binary combination CBZ has proven to be at the additive limit Zmix ~Zadd (Tc<Tt, Fc<Ft, $p > 0.05$) (*Fig 2b*).

DISCUSSION

Tramadol is a drug belonging from the opiate group, with a complex action mechanism, some authors considering it an atypical analgesic (Rojas-Corrales *et al.,* 2005). It is used in clinic for the treatment for moderate or severe pain, alone or combined with other analgesic drugs. In the literature, there are hypothesized two important action mechanisms: agonist of the μ-opioid receptors and inhibitor of norepinephrine and serotonin reuptake (Hsu *et al.,* 2012).

Some studies demonstrate the antinociceptive action of tramadol in mice using the model of abdominal constrictive response induced with acetylcholine (Raffa *et al.,* 1992). Thus, it has been demonstrated that beside the direct opioidergic mechanism, the tramadol might act indirectly by modulating the GABA or NMDA receptors (Hara *et al.,* 2005; Yajima *et al.,* 2000). Other studies demonstrate an anticonvulsant effect of tramadol, mediated by the κ-opioid receptors using an electroshock convulsive model, which strengthens the idea of an atypical opioid (Raffa *et al.,* 1992; Scott and Perry, 2000). Also it has been made the hypothesis of the anticonvulsant action of tramadol by the direct action on the κ-opioid receptors and indirectly by modulating GABA receptors (Manocha *et al.,* 2005).

Valproic acid is a drug with anticonvulsant activity used in clinic in various types of epilepsy. Its anticonvulsant action is explained by the inhibition of the GABA transaminase, the enzyme responsible for the degradation of GABA. It also increases the activity of the glutamic acid decarboxylase acting indirectly on the GABA (Ximenes *et al.,* 2013).

The valproic acid attenuates the excitatory mediation through NMDA and blocks the voltage dependent Na and Ca channels (L, C, D, N, F, T types) and also voltage dependent K channels (Diederich and Koch, 2005; Rosenberg, 2007; Willmore, 2003). Recently valproic acid has begun to be used in combination with analgesic drugs in the treatment of various types of pain.

In experimental conditions, valproic acid has demonstrated its antinociceptive action in models of peritonitis with carrageenan, inflammatory paw edema with carrageenan, second phase of the formalin test etc. Valproic acid reduces the leukocyte infiltration and the release of myeloperoxidase in the peritoneal exudate in the carrageenan peritonitis model. In this model the carrageenan produces an inflammatory response with

neutrophil infiltrate, plasma exudation, cellular migration and mediator release like NO, PGE2, IL-1 β, IL-6, TNF-α (Salvemini *et al.,* 1996; Ximenes *et al.,* 2013).

The carbamazepine is an anticonvulsant drug, useful in various types of epilepsy and lately employed frequently in various types of pain, like the trigeminal neuralgia (Flickinger *et al.,* 2012). The action of the drug is explained by the blockade of certain voltage-gated sodium channels. There are several subtypes of voltage-gated sodium channels noted with Na_v, from 1.1 to 1.9. According to the literature, of a major importance is the subtype $Na_v1.1$, which is expressed in the GABA-ergic interneurons, suggesting an involvement in convulsing phenomena produced by the decrease in their inhibition, while $Na_v1.2$, 1.3 and 1.6 are rather involved in the propagation of the impulse at the level of the axons and dendrites (Qiao *et al.,* 2013). $Na_v1.4$ is not significantly expressed in the dorsal root ganglion (DRG), but mostly in the skeletal muscle and does not seems to be involved in generating and transmitting pain. $Na_v1.5$ has been identified within the myocardium while the $Na_v1.7$, 1.8 and 1.9 are expressed only on the peripheral sensory neurons and are considered to be involved both in the initiation of the acute pain and in maintaining the post-lesion inflammatory hyperalgesia. Channels $Na_v1.4$, 1.6 and 1.7 are sensitive to tetrodotoxin (TTX), while $Na_v1.5$, 1.9 and 1.9 are TTX-resistant (Dib-Hajj *et al.,* 2009a; Dib-Hajj *et al.,* 2009b).

The $Na_v1.7$ subtype is significantly expressed on the neurons from the DRG, in the sympathetic and myenteric neurons, while $Na_v1.8$ is preferentially expressed in the DRG and in the Gasser ganglion. Its involvement in the inflammatory pain and in the cold stimuli-induced pain is well documented (Cummins and Rush, 2007). It is also known that subtype $Na_v1.7$ is sensitive to carbamazepine blockade and its mutations are involved in a series of hereditary pain syndromes (Lee *et al.,* 2005).

$Na_v1.9$ is expressed in the sensory neurons and in the enteral nervous system, mainly in small size neurons with nociceptive properties. According to these data, $Na_v1.7$, 1.8 and 1.9 might be important target for analgesic drugs (Dib-Hajj *et al.,* 2002).

In recent papers, several authors have shown that by the intraperitoneal zymosan administration in mice appears an inflammatory response, characterized by adbomina constrictive response, plasma exsudation, leukocyte infiltration and eicosanoid biosynthesis (Doherty cit Pettipher *et al.,* 1997).

Lately it has been demonstrated that inflammatory agents do not stimulate directly the release of hypernociceptive primary mediators, but their release is preceded by a cytokine cascade (Poole, 1999). It is known that in mice, the zymosan and acetic acid-induced abdominal constrictive response is mediated by

TNF-α, IL1-β and IL8, which act simultaneously and synergistically (Ribierro cit Cunha *et al.,* 2005).

In the light of this information, the synergism between tramadol and the valproic acid can be explained by the action mechanisms that are characteristic and different for the two substances:

- The direct opioidergic action mechanism of tramadol;
- The indirect action mechanism of tramadol through the modulation of the GABA and NMDA receptors;
- Direct GABA-ergic action mechanism of the valproic acid by GABA transaminase inhibition and indirect GABA-ergic action mechanism by the stimulation of the glutamic acid decarboxylase;
- Valproic acid blocks voltage dependent cation channels and attenuates excitatory mediation through NMDA.
- Valproic acid inhibits a series of nociceptive mediators (NO, PGE2, IL-1β, IL-6, TNF-α), which appear during inflammatory pain.
- The additive antinociceptive action obtained by the combination between CBZ and tramadol might be explained by:
- Blockade of TTX sensitive Na channels ($Na_v1.7$) and TTX resistant Na channels ($Na_v1.8$) and Na channel blockade in the GABA-ergic neurons;
- Intervention of CBZ on the phospholipase A2 and implicitly on the prostaglandin biosynthesis. (Matoth *et al.*, 2000)

CONCLUSIONS

The difference in the effect level of these two combinations could be explained by the fact that, unlike the CBZ, valproic acid, besides their common action mechanisms, inhibits the GABA transaminase, while the CBZ has no action on the enzyme.

The demonstration of a synergic effect between tramadol ant the valproic acid might be one of the first steps towards obtaining analgesic drugs that are more efficient and better tolerated, keeping in mind an eventual possibility of reducing the doses of active substances.

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