ENHANCED ANTIPYRETIC ACTIVITY OF NEW 2,5-SUBSTITUTED 1,3,4-OXADIAZOLES ENCAPSULATED IN ALGINATE/GELATIN PARTICULATED SYSTEMS

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New 1,3,4-oxadiazoles with pharmacological potential, derived from 5-nitroindazole, have been synthesized. Their chemical structure has been established by elemental and spectral analyses (FT-IR and ^{1}H-NMR). The oxadiazoles presented low toxicity, one compound, either in a free form or loaded in polymeric microcapsules, also showing a remarkable antipyretic activity, similar to that of acetylsalicylic acid.

Keywords: 1,3,4-oxadizoles, antipyretic activity, encapsulation, polymeric particles

INTRODUCTION

The interest in oxadiazole derivatives is due to their numerous pharmacological applications. Literature data offer various examples of oxadiazole ring-containing compounds with biological activity, including antimalaric,[1] local anaesthetic,[2] insecticide,[3] antihypertensive,[4] anti-inflammatory, antipyretic,[1,5] analgesic,[6,7] anthelmintic,[8] antibacterial[9,10] or hypoglycemiant effects.[11]

Oxadiazoles are usually synthesised from various 4-substituted acyl-thiosemicarbazides, which suffer intramolecular cyclization by treatment with tosyl chloride and pyridine,[12] polyphosphoric acid[13] or trimethylphosphine and triethylamine in carbon tetrachloride.[14] Another method described in literature for oxadiazole preparation is the cyclization reaction between a hydrazide and a carboxylic acid, in the presence of phosphorus oxychloride and aluminium oxide.[3] Considering the pharmacological potential of substituted oxadiazole heterocycle, our research was focused on synthesizing new 1,3,4-oxadiazoles with antipyretic activity, derived from 5-nitroindazole, through a novel method, and on their encapsulation into microparticulated systems based on sodium alginate and gelatin.

Over the past 30 years, considerable interest has been manifested for the development of polymeric micro/nanoparticulated systems as efficient drug delivery matrices. The naturally occurring polymers are attractive for drug delivery, as due to their biocompatibility, biodegradability and non-toxicity.[15-17] Alginate, an anionic polymer extracted from marine brown algae, is widely used in biomedical fields.[18,19] Monovalent salts, often referred to as alginates, are hydrophilic colloids. Alginate is a linear copolymer composed of 2 monomeric units, D-mannuronic acid and L-guluronic acid (Fig. 1). Calcium alginate hydrogel matrices usually present high water permeability, the hydrosoluble drug release being rarely controlled in an efficient manner.[20] This drawback can be overcome by mixing alginate with other polymers, such as chitosan, pectin or even gelatin.[21]

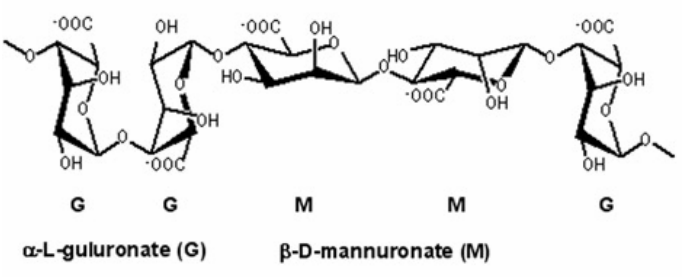


Figure 1: Chemical structure of alginates

EXPERIMENTAL

Materials and method

All reagents were used as purchased (Sigma-Aldrich, Fluka, Merk, S.C. Chemical Company S.A.). FT-IR spectra were recorded using a FT-IR spectrophotometer (ATR) Brucker Tensor-27; ^{1}H-NMR analysis was performed on a Brucker ARX 400 spectrometer (5 mm QNP probe; 1H/13C/31P/19F) and elemental analysis – on an Exeter Analytical CE 440 elemental analyser. The melting points of the obtained compounds were determined with a Mel-Temp melting point module, provided with a digital thermometer. Particle morphology and size were evaluated using a VEGA-3 Tescan Scanning Electron Microscope and a laser light diffractometer (SHIMADZU – SALD 7001), respectively.

Synthesis of 2-substituted 5-aryl-amino-1,3,4-oxadiazoles. General procedure

In a reaction flask provided with a refluxing cooler, 0.02 mol of anhydrous sodium acetate was added to 0.005 mol 5'-nitroindazole-1'-*il*-acetyl-4-R-thiosemicarbazide (I-VI) and 0.0055 mol ethyl chloroacetate, in 50 mL ethanol. The reaction mixture was maintained under reflux on a water bath for 11 h, then filtered under vacuum. The ethanol excess was removed by distillation under vacuum, until reaching a volume of 10-15 mL. The solid product formed upon cooling was filtered under vacuum and then washed several times with ethanol. The final compound was purified by repeated recrystallization from boiling ethanol.

2-[(5'-nitroindazole-1'-methyl)]-5-phenylamino-1,3,4-oxadiazole (VII)

White solid; yield: 64.28% (1.08 g); melting point: 175-177 °C. Anal. calcd. for $C_{16}H_{12}N_6O_3$: 57.14% C; 3.57% H; 25% N. Found: 57.4% C; 3.76% H; 25.35% N. FT-IR (ν cm^{-1}): 2980-3408 (NH); 1586 (NO_2 asymmetrical); 1402, 1495, 1517 (substituted oxadiazole ring); 1653 (C=N); 1170 (C-O-C); 750 (substituted benzene ring). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 5.81 (s, 2H, CH_2); 7.18-7.20 (d, 2H, Ar); 7.30-7.34 (m, 3H, Ar); 7.55 (s, 1H, Ar); 8.08 (s, 1H, Ar); 8.29-8.30 (d, 1H, Ar); 8.53-8.55 (d, 1H, Ar); 8.72 (s, 1H, NH).

2-[(5'-nitroindazole-1'-methyl)]-5-(p-tolyl-amino)-1,3,4-oxadiazole (VIII)

White solid; yield: 72.4% (1.26 g); melting point: 169-171 °C. Anal. calcd. for $C_{17}H_{14}N_6O_3$: 58.29% C; 4% H; 24% N. Found: 58.62% C; 4.02% H; 24.41% N. FT-IR (ν cm^{-1}): 3416 (NH); 1336 (NO_2 symmetrical); 1534 (NO_2 asymmetrical); 1282 (substituted oxadiazole ring); 1623 (C=N); 1188 (C-O-C); 789, 822 (p-disubstituted benzene ring). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 2.26 (s, 3H, CH_3); 6.10-6.11 (s, 2H, CH_2); 7.11-7.13 (d, 2H, Ar); 7.40-7.42 (d, 2H, Ar); 8.01 (s, 1H, Ar); 8.29 (s, 1H, Ar); 8.50-8.51 (d, 1H, Ar); 8.86-8.87 (d, 1H, Ar); 10.29 (s, 1H, NH).

2-[(5'-nitroindazole-1'-methyl)]-5-(p-methoxyphenyl-amino)-1,3,4-oxadiazole (IX)

White solid; yield: 66.66% (1.22 g); melting point: 139-141 °C. Anal. calcd. for $C_{17}H_{14}N_6O_4$: 55.74% C; 3.82% H; 22.95% N. Found: 56.03% C; 4.01% H; 23.26% N. FT-IR (ν cm^{-1}): 3396 (NH); 1332 (NO_2 symmetrical); 1513 (NO_2 asymmetrical); 1299, 1405 (substituted oxadiazole ring); 1659 (C=N); 1175 (C-O-C); 782, 812 (p-disubstituted benzene ring). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 3.70 (s, 3H, CH_3); 6.10 (s, 2H, CH_2); 6.88-6.92 (d, 2H, Ar); 7.43-7.48 (d, 1H, Ar); 8.02-8.05 (d, 1H, Ar); 8.30-8.33 (d, 1H, Ar); 8.47 (s, 1H, Ar); 8.98 (s, 1H, Ar); 10.19 (s, 1H, NH).

2-[(5'-nitroindazole-1'-methyl)]-5-(p-bromophenyl-amino)-1,3,4-oxadiazole (X)

White solid; yield: 62.31% (1.29 g); melting point: 173-175 °C. Anal. calcd. for $C_{16}H_{11}BrN_6O_3$: 46.27% C; 2.65% H; 19.28% Br; 20.24% N. Found: 46.58% C; 2.87% H; 19.67% Br; 20.65% N. FT-IR (ν cm^{-1}): 2947, 3097 (NH); 1397 (NO_2 symmetrical); 1535 (NO_2 asymmetrical); 1136 (substituted oxadiazole ring); 1602 (C=N); 1182 (C-O-C); 898, 948 (p-disubstituted benzene ring); 748, 789 (C-Br). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 6.11 (s, 2H, CH_2); 7.43-7.47 (d, 2H, Ar); 7.53-7.55 (d, 2H, Ar); 8.01 (d, 1H, Ar); 8.49 (d, 1H, Ar); 8.85-8.86 (s, 1H, Ar); 10.59 (s, 1H, NH).

2-[(5'-nitroindazole-1'-methyl)]-5-(p-chlorophenyl-amino)-1,3,4-oxadiazole (XI)

White solid; yield: 62.70% (1.16 g); melting point: 181-183 °C. Anal. calcd. for $C_{16}H_{11}ClN_6O_3$: 51.75% C; 2.96% H; 9.56% Cl; 22.65% N. Found: 51.98% C; 3.32% H; 9.97% Cl; 23.05% N. FT-IR (ν cm^{-1}): 3200 (NH); 1370 (NO_2 symmetrical); 1591 (NO_2 asymmetrical); 1293 (substituted oxadiazole ring); 1625 (C=N); 1180 (C-O-C); 854, 934 (p-disubstituted benzene ring); 766 (C-Cl). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 6.11-6.13 (s, 2H, CH_2); 7.33-7.37 (d, 2H, Ar); 7.56-7.59 (d, 2H, Ar); 8.01-8.03 (d, 1H, Ar); 8.27-8.29 (d, 1H, Ar); 8.86 (s, 1H, Ar); 9.02 (s, 1H, Ar); 10.49 (s, 1H, NH).

2-[(5'-nitroindazole-1'-methyl)]-5-(p-iodophenyl-amino)-1,3,4-oxadiazole (XII)

White solid; yield: 68.39% (1.58 g); melting point: 177-179 °C. Anal. calcd. for $C_{16}H_{11}IN_6O_3$: 41.56% C; 2.38% H; 27.49% I; 18.18% N. Found: 41.83% C; 2.69% H; 27.88% I; 18.43% N. FT-IR (ν cm^{-1}): 3327 (NH); 1357 (NO_2 symmetrical); 1536 (NO_2 asymmetrical); 1272 (substituted oxadiazole ring); 1608 (C=N); 1183 (C-O-C); 869, 912 (p-disubstituted benzene ring); 7682 (C-I). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 6.12 (s, 2H, CH_2); 7.41-7.43 (d, 2H, Ar); 7.63-7.65 (d, 2H, Ar); 8.02 (d, 1H, Ar); 8.29 (d, 1H, Ar); 8.48 (s, 1H, Ar); 8.81 (s, 1H, Ar); 9.02 (s, 1H, Ar); 10.61-10.63 (d, 1H, NH).

Preparation of polymer microcapsules

Gelatin and alginate microcapsules were prepared by crosslinking polymers in an O/W/O emulsion. First, an O/W emulsion was prepared by dropwise dispersion of a mixture of 3 mL drug solution in dimethylsulphoxide (25 mg/mL), 9 mL toluene, 2% (w/v) Span80 and 0.5 mL oleic acid into a 50 mL polymeric aqueous solution (A/G: 3/2; w/w) containing 2% (w/v) Tween80, using a mechanical stirrer (VELP Scientifica), at 1300 rpm for 60 min. Sodium alginate (0.6 g) was left to dissolve in distilled water for 24 h prior to the preparation of the aqueous phase. The formed O/W emulsion was then redispersed into a 200 mL mixture of toluene and 1% (w/v) Span80, under similar conditions. A $CaCl_2$ aqueous solution (2%, w/v) was added dropwise under mechanical stirring, for polymer crosslinking. The microcapsules were left to cure for 24 h and then the formed microcapsules were recovered, washed repeatedly with water and acetone and then dried in a vacuum oven at 30 °C for 24 h. The microcapsules were kept refrigerated.

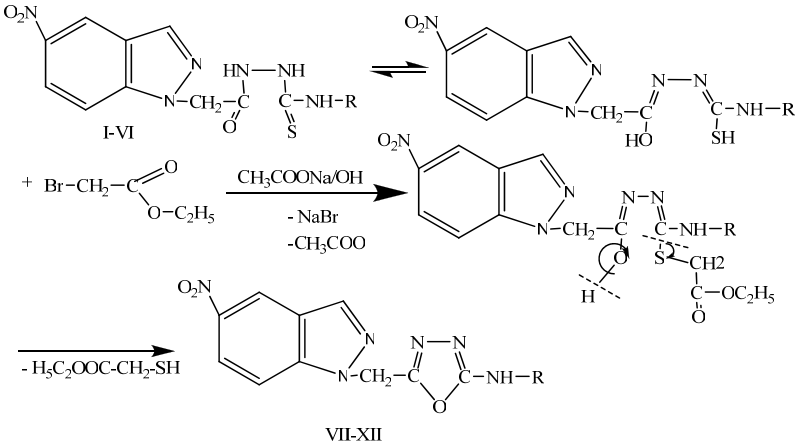
RESULTS AND DISCUSSION

1,3,4-oxadizoles synthesis and characterization

In the present work, oxadiazoles were synthesized by a novel method, using as starting compounds some 4-substituted acyl-thiosemicarbazides obtained by the addition of 5-nitroindazole N-acetyl-hydrazide to various aromatic isothiocyanates.[22]

The substituted acyl-thiosemicarbazides underwent intramolecular cyclization by treatment, under heating, with ethyl bromoacetate (chloroacetate), in an ethanol solution and anhydrous sodium acetate, 2,5-disubstituted oxadiazoles (VII-XII) thus resulting (Fig. 2).

The mechanism of cyclization probably implies, in the first step, the formation of a thioester of the thiosemicarbazide tautomeric form, which, by the nucleophilic attack of the hydroxyl oxygen on the carbon bonded to the thioester residue, followed by the elimination of ethyl mercaptoacetate, forms 2,5-disubstituted oxadiazoles.



I, VII: R = -C_6H_5; II, VIII: R= -C_6H_4-CH_3(p); III, IX: R= -C_6H_4-OCH_3; IV, X: R= -C_6H_4-Br (p); V, XI: R= -C_6H_4-Cl (p); VI, XII: R= - C_6H_4-I (p)

Figure 2: Synthesis of 2-substituted 5-aryl-amino-1,3,4-oxadiazoles

The newly synthesized oxadiazole derivatives (VII-XII), obtained with a yield of 62-72%, are crystalline compounds, purified from ethanol, with fixed melting points. Their chemical structure was confirmed by spectral (FT-IR and ^{1}H-NMR) and elemental analyses.The main modification in the FT-IR spectra of oxadiazoles, compared to those of the corresponding thiosemicarbazides, was the appearance of a new absorption band at 1170-1188 cm^{-1}, corresponding to the newly formed C-O-C bonds from the oxadiazole heterocycle; also, the intense absorption bands at 1234-1292 cm^{-1}, specific to the C=S valence vibration in the thiourea function, no longer appeared in the spectra of oxadiazoles. Moreover, medium intense absorption bands, characteristic of C=N bond vibrations (in the 1,3,4-oxadiazole ring) and intense bands specific to N-H bond vibrations (position 5 in the oxadiazole structure) were recorded at 1602-1659 cm^{-1} and around 3300 cm^{-1}, respectively. In the FT-IR spectra of oxadiazoles (X-XII), absorption peaks specific to C-Br, C-Cl and C-I bond vibrations were registered at 748-789 cm^{-1}.

^{1}H-NMR spectra completed the analysis of the proposed chemical structures. Thus, the aromatic protons presented signals at δ = 7.11-9.02 ppm for all oxadiazoles; CH_2 protons (1' position in the indazole ring) and the NH proton could be detected as singlets at 5.81-6.13 ppm and 10.19-10.63 ppm, respectively. Also, methyl protons from oxadiazoles (VIII) and (IX) presented signals as singlets at 2.26 ppm and 5.70 ppm, respectively.

Microcapsule preparation and characterization

Alginate/gelatin microcapsules were prepared using a polymer coacervation method, in an O/W/O double emulsion, as template phase. Of the various methods of polymer coacervation known, the use of electrolytes (in our case, $CaCl_2$) reduces the solvation of hydrosoluble polymers, thus inducing the formation of a thin insoluble polymer shell. Oxadiazole (VIII), which presented the lowest toxicity, was encapsulated into the microcapsules in the preparation step. Thus, the prepared microcapsules presented an oily core containing the oxadiazole derivatives and an alginate/gelatin shell solidified by crosslinking with calcium chloride.

Polydispersed polymeric microcapsules with a quite smooth surface and sizes between 0.4-10 μm were prepared.

The FT-IR spectrum of alginate/gelatin microcapsules showed the presence of gelatin as characteristic peaks at 3400 cm^{-1} and 1683 cm^{-1}, due to bond stretching of N-H and C=O, respectively. The presence of alginate in microcapsules was confirmed by the absorption bands at 3520 cm^{-1}, specific to O-H stretching, and at 1634 cm^{-1}, respectively, specific to C=O in carboxylate ions.

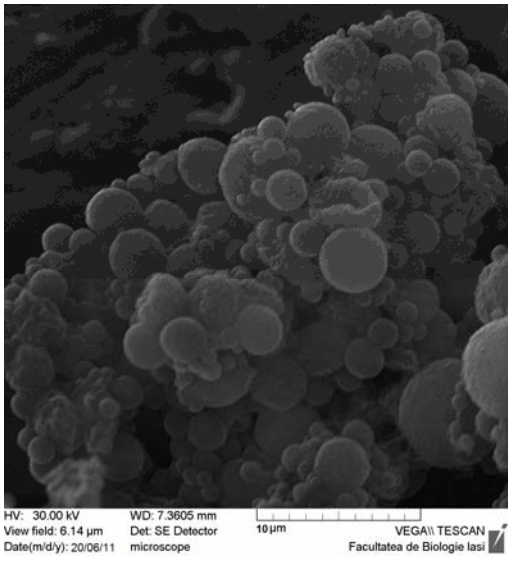


Figure 3: Morphology and size of alginate/gelatin loaded with oxadiazole (VIII)

Effect of crosslinking agent

Calcium chloride, a known crosslinker of alginate, determines the complexation of the carboxylate anions of alginate with calcium ions, thus forming a three-dimensional network. The effect of the $CaCl_2$ amount on the size and size distribution of alginate/gelatin microcapsules has been studied by varying the calcium chloride/alginate weight ratio between 0.05-0.2. Figure 4 indicates that microcapsule size constantly decreases with increasing the amount of ionic crosslinker.

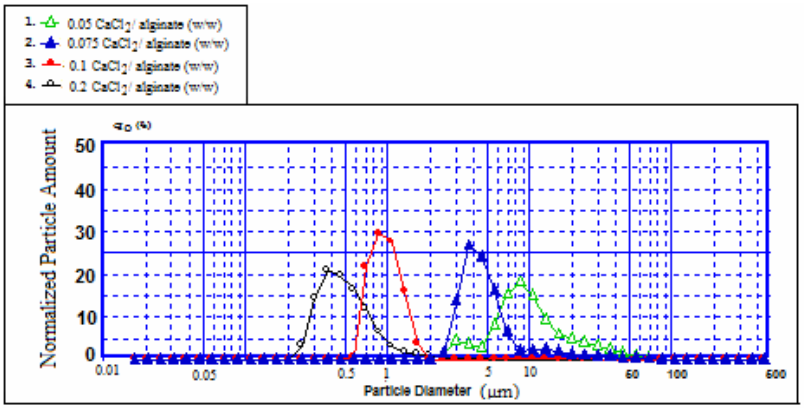


Figure 4: Influence of ionic crosslinker on particle size and size distribution

The increasing number of calcium ions determines the formation of alginate microcapsules with smaller cavities, having a lower water absorption capacity and thus, smaller sizes.

Biological activity

Toxicity evaluation

The determination of acute toxicity involves the evaluation of animal mortality, induced by the administration of a certain drug. The interpretation of the results involves finding of the drug dosage (LD_{50}) that determines the death of 50% of the animals involved in the experiment; this test has become indispensable for testing new therapeutic compounds.[23]

The acute toxicity of the new oxadiazole derivatives (VII-XII) and of the VIII loaded polymeric microcapsules was determined by LD_{50} recording (Table 1), after intraperitoneal administration of drug suspensions in Tween80 to groups of 6 white male mice, 20±2 g each.[24] Before drug administration, the mice were kept under observation for 7 days at constant temperature (22±1 °C), receiving habitual nourishment; the animals were weighed every 48 h, the underweight ones being removed from the experiment. Mortality was recorded at 24 h, 48 h and 7 days after drug administration, and LD_{50} was established by the Spearman-Karber arithmetic method.[25]

Toxicological data showed that oxadiazole derivatives reduced toxicity. 2-[(5'-nitroindazole-1'-methyl)]-5-(p-tolyl)-1,3,4-(oxadiazole) (VIII), whether in a free form or loaded in alginate/gelatin microcapsules, presented the lowest toxicity level, being preferred for further laboratory screening.

Antipyretic activity

The antipyretic activity of 2-[(5'-nitroindazole-1'-methyl)]-5-(p-tolyl)-1,3,4-(oxadiazole) (VIII) was also evaluated by the experimental pyrexia model, induced by sodium nucleinate administered intraperitoneally to rats. Groups of 6 rats of both sexes, weighing 120-125 g, were maintained at room temperature. One day before the test, the rats were allowed only to drink water *ad libitum*. On the test day, the rectal temperature (using special thermometers of veterinary use) was measured and 10 mg/kg body sodium nucleinate was administered i.p. to each rat. 1 hour after the administration, the rectal temperature was measured again and only the animals that showed an increase of at least 1 °C in body temperature were retained for further testing. The rats were then treated perorally with 2-[(5'-nitroindazole-1'-methyl)]-5-(p-tolyl)-1,3,4-(oxadiazole), oxadiazole (VIII) loaded microcapsules or non-steroidal reference drugs (indomethacin, phenylbutazone, acetylsalicylic acid) as suspensions of various concentrations in

CMC (0.5%). The control group was injected only CMC. 1, 2, 3 and 4 h after drug administration, rectal temperature was measured again and compared to that of the control batch (Table 2).

The results of the biological tests showed that indomethacin determined the highest pyrexia inhibition 1 h after drug administration, the antipyretic activity increasing progressively within the following 4 h, which is most likely due to its relatively short biological half-life in rats.[26]

Table 1
Acute toxicity of oxadiazole derivatives (VII-XII) and VIII loaded in microcapsules

Compound	LD_{50} (mg/kg body)			
	24 h	48 h	7 days	Average value
VII	8970	8970	8920	8953
VIII	9685	9685	9625	9665
IX	9715	9715	9425	9618
X	9520	9520	9480	9506
XI	9220	9220	9182	9207
XII	9315	9315	9279	9303
A/G Microcapsules	9842	9840	9725	9802
VIII loaded A/G Microcapsules	9718	9718	9653	9696

Table 2
Antipyretic activity of oxadiazole (VIII) and oxadiazole (VIII) loaded in microcapsules, compared to reference antipyretic non-steroidial drugs

Compound	Dose (mg/kg body)	Body temperature before antipyretic treatment, °C		Body temperature after antipyretic treatment, °C			
		Initial	1 h after pyrexia induction	1 h	2 h	3 h	4 h
Indomethacin	100	36.73±0.15	37.90±0.08	36.93±0.11	36.5±0.14	36.44±0.11	36.40±0.11
Phenylbutazone	100	36.73±0.15	37.82±0.07	37.71±0.11	37.40±0.08	37.36±0.12	37.46±0.13
Acetylsalicylic acid	100	36.71±0.09	37.75±0.12	37.08±0.17	37.06±0.22	36.93±0.16	36.70±0.24
Oxadiazole (VIII)	100	36.68±0.06	37.70±0.11	37.11±0.17	37.08±0.15	36.95±0.11	36.77±0.08
Oxadiazole (VIII) encapsulated in microcapsules	100	36.75±0.1	37.90±0.23	37.20±0.15	37.05±0.14	36.92±0.11	36.74±0.16
Control batch	-	36.72±0.14	37.80±0.07	(control groups received no antipyretic treatment) 38.40±0.11	38.65±0.05	38.95±0.04	39.20±0.12

Oxadiazole (VIII), in a free form or encapsulated in polymeric microcapsules, manifested a remarkable antipyretic activity, similar to that of acetylsalicylic acid, due to an inhibition effect on prostaglandin biosynthesis, specific to oxadiazoles.[27-29] Also, the antipyretic activity could be enhanced by the gradual release of oxadiazole (VIII) from the microcapsule core, an efficient retardation being thus achieved.

CONCLUSIONS

Six new 1,3,4-oxadiazoles (VII-XII) derived from nitroindazole were synthesized by a novel method of cyclization of some 4-substituted thiosemicarbazides (I-VI). The chemical structure of the newly synthesized oxadiazoles (VII-XII) was established by elemental and spectral analyses (FT-IR and ^{1}H-MNR).

Microcapsules were prepared by polymer coacervation, using a double emulsion as a template. Oxadiazole (VIII) was encapsulated into the alginate/gelatin particles in the preparation step.

Oxadiazoles (VII-XII), as well as the polymeric particles encapsulating the oxadiazole (VIII), presented low acute toxicity, within admissible limits for laboratory screening. The antipyretic activity of oxadiazole (VIII), either in

a free form or encapsulated into microcapsules, studied comparatively with that of reference drugs, was remarkable against sodium nucleinate-induced pyrexia, similar to that of acetylsalicylic acid. The obtained data contribute to introducing new compounds with antipyretic activity into human clinical research.

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