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Preparation and evaluation of carriers for detection of cholinesterase inhibitors

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Key words: **detection tubes; cholinesterase inhibitors; pellets; intraparticular porosity; total tapped porosity; sphericity; pellet regularity; acetylcholinesterase activity**

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Abstract

OBJECTIVES: The aim of the study was to use methods of pharmaceutical technology, and prepare carriers in the form of pellets suitable as a filling of detection tubes for enzymatic detection of cholinesterase inhibitors. The enzymatic detection was based on enzymatic hydrolysis of acetylthiocholine iodide and the subsequent colour reaction of its hydrolysis product with Ellman's reagent. The suitable carriers should be in the form of white, regular and sufficiently mechanically resistant particles of about 1 mm allowing it to capture the enzyme during the impregnation process and ensuring its high activity for enzymatic detection.
METHODS: Carriers consisting of microcrystalline cellulose, lactose, povidone, and sodium carboxymethyl cellulose were prepared using extrusion-spheronization method under three different drying conditions in either a hot air oven or a microwave oven. Subsequently, the carriers were impregnated with acetylcholinesterase and their size, shape, mechanical resistance, bulk, tapped and pycnometric density, Hausner ratio, intraparticular and total tapped porosity, and activity were measured and recorded.
RESULTS: In this procedure, carriers with different physical parameters and different acetylcholinesterase activity were evaluated. It was found that higher acetylcholinesterase activity was associated not only with a higher intraparticular porosity but also with more regular particles characterized by high sphericity and low total tapped porosity.
CONCLUSION: This unique finding is important for the preparation of detection tubes based on enzymatic detection which is still irreplaceable especially in the field of detection and analysis of super-toxic cholinesterase inhibitors.

ORIGINAL ARTICLE

Abbreviations

HR - Hausner ratio
ρ_B - bulk density
ρ_P - pycnometric density
ρ_T - tapped density
P_{TAPPED} - total tapped porosity
P_{INTRA} - intraparticular porosity
S - sphericity

INTRODUCTION

Cholinesterase inhibitors represent an important group of chemical compounds that are of interest to toxicologists, military and security experts (nerve agents as part of the most advanced chemical weapons), experts in agriculture and food (organophosphorus and carbamate pesticides) and elsewhere (Pope *et al.* 2005; Hostovsky *et al.* 2014). In the field of simple devices (chemical sensors), cholinesterase inhibitors can be detected through various chemical processes (amino-peroxide reactions, reaction with oximes, reaction to alkoxy group, etc.), which rely on the extreme toxicity of certain substances, notably military significant organophosphates, completely insufficiently sensitive (Royo *et al.* 2007; Kuneš *et al.* 2014). One of the few successful solutions thus lies in the use of enzymatic (biochemical, cholinesterase) reactions based on the hydrolysis of a suitable substrate and the subsequent colour indication of its hydrolysis product (Holas *et al.* 2012).

Automatic detectors and signalling devices are another means of detecting and analysing cholinesterase inhibitors. They use a variety of physical and physicochemical processes, nowadays ion mobility spectrometry in particular (Armenta & Blanco 2011). While respecting the undeniable quality of these high-tech devices and their wide application in various fields, it is necessary to emphasize their fundamental and structural limits, including in particular the low selectivity and their generally insufficient or borderline sensitivity. A variety of enzymatic biosensors, including those designed as detection tubes thus remain promising in the field of simple technical means available to the widest possible range of users (Hoskovcova & Kobliha 2011). Detection tubes provide an advantage over other implements of this category in the form of user comfort and, as it turns out, also in the complexity of technical solutions, as well.

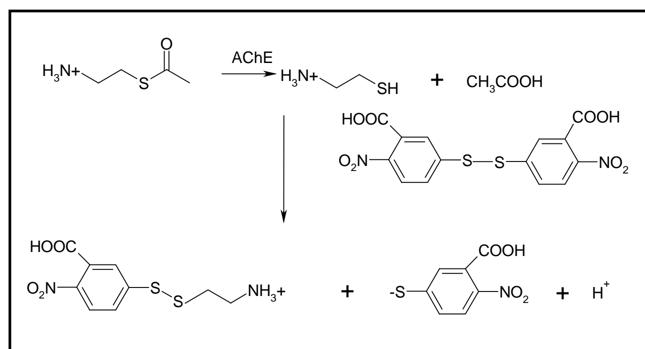

Fig. 1. Ellman's colorimetric reaction (AchE – acetylcholinesterase).

Detection tubes are usually made of glass tubes filled with a carrier on which the analytical reactions with chemical reagents take place. A carrier uses traditional sorbents and inert materials (silica gel, diatomaceous earth, pumice, crushed glass, cellulose) and a variety of modern synthetic and composite materials. Reagents can be immobilized directly on the carrier or used in the form of solutions in sealed ampoules. Controlled air is fed into the tube either manually or via electric pump. The presence (concentration) of obtained agents is indicated through changes in the intensity or duration of coloration of the carrier. Detection tubes are used to inhibit not only contamination from air and soil, as well as various materials, and surfaces, but also contamination from water and other liquids (Pitschmann *et al.* 2011).

The aim of this interdisciplinary research study was to use methods of pharmaceutical technology to prepare carriers in the form of pellets suitable as a filling of detection tubes for the enzymatic detection of cholinesterase inhibitors. The pellets should be in the form of white, regular particles of about 1 mm based on the optimal organoleptic evaluation of Ellman's reaction (Ellman *et al.* 1961), sufficiently mechanically resistant to allow them to fill the tubes, able to capture an enzyme during the impregnation process, and ensure its high activity for enzymatic detection. A similar study, in which methods of pharmaceutical technology were used to influence physical parameters of prepared carriers, especially intraparticular porosity and regularity, essentially improving acetylcholinesterase activity, has not yet been published.

MATERIAL AND METHODS

Materials

As starting materials for the carriers, microcrystalline cellulose Avicel type PH 101 (FMC, United States), microcrystalline cellulose and sodium carboxymethylcellulose Avicel type RC 581 (FMC, United States), and lactose 100 mesh (DMV International, Netherlands) were used. Dissolved polyvinylpyrrolidone Kollidon 90 (BASF, Germany) in purified water was used as the wetting agent. Acetylcholinesterase activity was determined using the following chemicals: sodium tetraborate, boric acid, sodium bicarbonate, ethanol 96%, and isopropanol (Dr. Kulich Pharma, Czech Republic), 5,5'-dithiobis (2-nitrobenzoic acid) (Fluka Sigma-Aldrich, United States), acetyl thiocholine iodide (Fluka Analytical Sigma-Aldrich, United States), and physostigmine (Aldrich Chemico, United States). All materials were of European Pharmacopoeia quality.

Preparation of the carriers

A powdered mixture of Avicel PH 101 (81.0 g), Avicel RC 581 (20.3 g), and lactose 100 mesh (98.7 g) was homogenized in a high-speed Stephan UMC 5 mixer

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(Stephan, Germany) at 1000 rpm for 5 min. In the same device, the mixture was moistened with dissolved polyvinylpyrrolidone (9.8 g) in water (65.8 g). The moistened material was fed into a Pharmex 33 T single-screw axial extruder and spheronizer (Wyss-Probst, Germany) with a 1.00 mm-thick barrier with holes of 1.25 mm in diameter. The extrusion continued for 10 min at 110 rpm. The extruded material was collected in a bowl, and transferred to a running spheronization plate (hatched pattern – grid size 1.0 mm, 2.0 mm apart). The spheronization was carried out at 1,000 rpm for 5 min. The resulting pellets were dried under three different drying conditions in a Horo 038A hot air oven (Hoffman, Germany): sample A30-7: 7 days at 30 °C , sample A40-7: 7 days at 40 °C, and sample A60-1: 1 day at 60 °C, or in a J21MG microwave oven (Zanussi, Italy): sample M385-7: 7 min. at 385 W, sample M540-6: 6 min. at 540 W, and sample M700-4: 4 min. at 700 W. Pellets obtained that ranged in size from 0.8 to 1.25 mm were used for impregnation and further evaluation.

Impregnation of the carriers

Pellets were immobilized using acetylcholinesterase obtained from brain tissue of the nucleus caudatus of *Sus scrofa* f. *domestica*. The tissue was homogenized in a ratio of 1:100 with a phosphate buffer solution (pH 7.6), which contained 5% dextran for infusion and 0.5% Slovasol SF-10 detergent. Immobilization was performed such that 100 g of the pellets were impregnated with 200 ml solution. Pellets were then stripped of excess liquid and dried in an oven at 30–35 °C for 24 hours.

Characterization of the impregnated carriers

The *hardness* (N) of prepared carriers was tested on the C 50 Tablet Hardness Tester (Engineering Systems, Nottingham, UK) equipped with a C5 cell for pellet evaluation. The hardness of 10 randomly selected particles of each sample was evaluated.

Pycnometric density (ρ_P) was determined with a helium pycnometer (Pycnomatic—ATC, Porotec GmbH, Germany) according to European Pharmacopoeia recommendations.

The *Hausner ratio* (HR) was expressed as a ratio of tapped (ρ_T) and bulk (ρ_B) densities measured after 1,250 taps in a settling apparatus (type SVM 102, Erweka GmbH, Germany) using a 100 mL graduated cylinder. Measurement was repeated three times.

The *total tapped porosity* (P_{TAPPED}) was calculated from the tapped density values and the apparent density of particles according to the following formula: $P_{TAPPED} = (1 - \rho_T / \rho_P) * 100$.

Pellet *intraparticular porosity* (P_{INTRA}) was calculated from the pycnometric density values and the true density of responding powder mixtures according to the following formula (El Saleh & Kleinebudde 1998): $P_{INTRA} = 1 - \rho_P / \rho_T$.

Image analysis was employed to evaluate the *sphericity* (S) of the carriers. This analysis was performed on 200 pellets using a NIKON SMZ 1500 stereoscopic microscope (Nikon, Japan) equipped with a 72AUC02 USB camera (The Imaging Source, Germany) linked to a computer operated by NIS-Elements AR 4.0 software (Nicon, Japan). Carrier sphericity was calculated according to the following equation (Sienkiewicz *et al.* 1997): $S = 4\pi * \text{area}/\text{perimeter}^2$.

Activity of the impregnated acetylcholinesterase was evaluated using Ellman's color reaction according to Figure 1.

Glass tubes with a diameter of 0.5 cm were filled with impregnated pellets to a height of about 0.5 cm. Water was added to the pellets in a volume to be wetted. Water could not dwell. After 2 minutes, the same amount of solution for Ellman's reaction was added, and the speed and intensity of the colour change from white to yellow during 2 minutes was evaluated organoleptically.

RESULTS

The physical parameters and enzymatic activities of the carriers are summarized in Table 1.

Pellets dried in a microwave oven exhibited 1.5 to 5.7 times greater intraparticular porosity, lower pycnometric density and total tapped porosity. Pycnometric density values ranged from 1.516 to 1.520 g.cm^{-3} for pellets

Tab. 1. Physical parameters and enzymatic activities of the carriers. (data are expressed as mean ± standard deviation).

Sample	Sphericity	Hardness (N)	Hausner ratio	Pycnometric density (g.cm^{-3})	Total tapped porosity (%)	Intra-particular porosity (%)	Activity
A30-7	0.832±0.082	6.36±3.15	1.109±0.009	1.516±0.005	43.47	0.89	High*
A40-7	0.820±0.069	5.25±1.92	1.107±0.009	1.519±0.010	44.17	0.68	Low*
A60-1	0.799±0.076	6.40±2.44	1.055±0.017	1.520±0.002	46.38	0.67	Low*
M385-7	0.802±0.087	5.53±2.73	1.093±0.007	1.509±0.002	32.75	1.37	Medium*
M540-6	0.819±0.092	6.39±2.04	1.067±0.007	1.494±0.002	32.43	2.31	Medium*
M700-4	0.830±0.084	6.26±2.50	1.044±0.012	1.481±0.001	29.89	3.17	Medium*

*High activity = pellets obtained intensive yellow color during two minutes, Medium activity = pellets obtained yellow color during two minutes, Low activity = pellets obtained slightly yellow color during two minutes

dried in a hot air oven, and from 1.486 to 1.509 $g.cm^{-3}$ for pellets dried in a microwave oven. Total tapped porosity values ranged from 43.47 to 46.38% for pellets dried in the hot air oven, and from 29.89 to 32.75% for pellets dried in the microwave oven. Hausner ratio, pycnometric density and total tapped porosity decreased, and conversely intraparticular porosity increased with increasing drying energy of the pellets dried in the microwave oven. The values of pellet activity were not fully correlated with values of intraparticular porosity.

DISCUSSION

In this study, different drying conditions were used for the preparation of the carriers with different physical parameters leading to different acetylcholinesterase activity. The objective of the experiment was to obtain carriers in the form of white, regular particles of about 1 mm based on optimal organoleptic evaluation via Ellman's reaction that were sufficiently mechanically resistant to fill detection tubes, able to capture an enzyme during the impregnation process, and ensure its high activity for enzymatic detection.

All the obtained carriers were of white colour and ranged from 0.8 to 1.25 mm in size. The regularity of prepared pellets was assessed by total tapped porosity, sphericity, and Hausner ratio. It is known that ideal spheres of uniform size can assume either closest (rhombohedral) or loosest (cubic) packing which corresponds to total tapped porosities of 26 or 48%, respectively (Rodriguez *et al.* 2001). Particle sphericity can assume values from 0 to 1 in which perfect sphericity is equal to 1. Pellets having a sphericity of greater than 0.8 are considered sufficiently spherical (Deasy & Law 1997). The HR could be considered as a measure of interparticle friction. Because frictional forces depend on particle size (i.e., the number of contact points), particle shape, and surface roughness (Gryczova *et al.* 2008), the HR can be considered also as a parameter which evaluates the regularity of the particles. A Hausner ratio value less than 1.20 indicates good particle regularity (Kumar *et al.* 2001). All prepared carriers were of regular size and spherical shape (see Table 1, Figure 2). When comparing the samples dried in the hot air oven or microwave oven, total tapped porosity, sphericity, and Hausner ratio were correlated in most cases. Drying at a lower temperature and longer time in the hot air oven or at higher energy and shorter time in the microwave oven led to higher carrier regularity. When carriers are prepared using excipients able to absorb water, they tend to shrink during drying. Perez & Rabiskova (2002) noticed that microwave drying led to a faster and more uniform process. Almost all water content was evaporated in the first minutes of the process and consequently, the matrix structure remained for the most part intact.

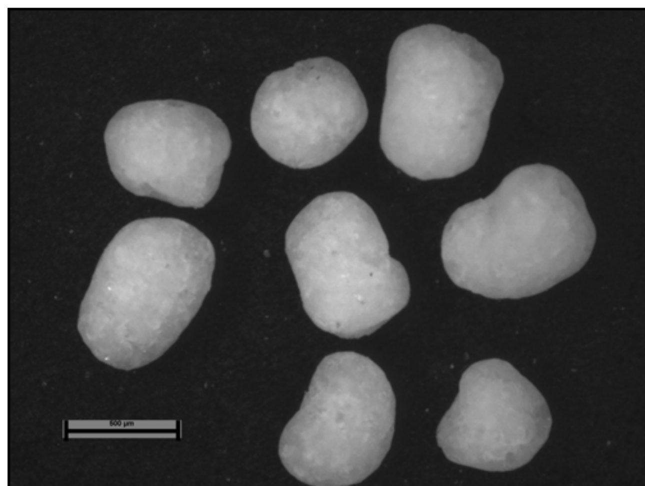

Fig. 2. Pellets of sample A30-7.

In addition to regularity, the drying method used in this study significantly influenced the intraparticular porosity of the carriers, as well. As expected, drying in the microwave oven resulted in particles with a higher intraparticular porosity. It was assumed that the porous particles are able to impregnate more acetylcholinesterase and thus reach higher enzymatic activity. Surprisingly, however, particles showing the highest porosity did not exhibit the highest enzymatic activity. The highest enzymatic activity was observed in sample A30-7, which showed not only higher intraparticular porosity, but also the highest sphericity and a low total tapped porosity. As expected, higher intraparticular porosity correlated with lower pycnometric density.

A hardness greater than 2 N indicates that pellets are sufficiently mechanically resistant (Haring *et al.* 2008). All prepared samples were suitable for filling into detection tubes.

CONCLUSION

The unique finding that the higher acetylcholinesterase activity was associated not only with a higher intraparticular porosity but also with more regular particles characterized by high sphericity and low total tapped porosity is important for the preparation of detection tubes based on enzymatic detection which is still irreplaceable, especially in the field of detection and analysis of super toxic cholinesterase inhibitors.

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