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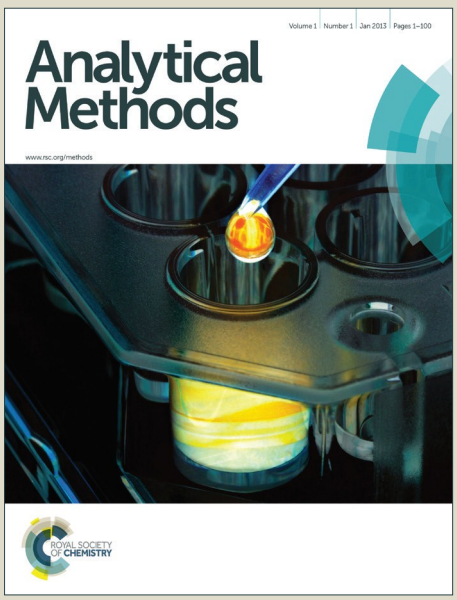

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ARTICLE

Production and Characterization of Diphenyl Ditelluride-loaded Nanocapsules: Validation using an Analytical Method

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

F. D. Silva, [a]* F. R. Ianiski, [a] C. Luchese, [b]** J. S. S. Neto, [c] G. Zeni, [c] L. V. Laporta,[d] A. M. Rubim,[d] and S. C. S. M. Hoelzel[a] A. N.

Organotellurium compounds are important antioxidants, but they are extremely toxic. In order to avoid side effects, nanocarriers can be used to reduce toxicity and increase efficacy in the target cell. Therefore, the aim of this study was to produce and characterize diphenyl ditelluride [$(PhTe)_2$]-loaded nanocapsules. Moreover, an analytical method was proposed to determine $(PhTe)_2$ in nanocapsules. The $(PhTe)_2$-loaded nanocapsules were produced according to the method of interfacial deposition of preformed polymer. Results demonstrated that $(PhTe)^2$-loaded nanocapsules presented a mean particle size of 256 ± 19 nm at 24 hours, 255 ± 13 nm at 7 days and 255 ± 22 nm at 30 days; polydispersity index values were 0.15 ± 0.02, 0.13 ± 0.02 and 0.17 ± 0.03 at 24 hours, 7 and 30 days, respectively; zeta potential was -10.7 ± 0.6 mV, -12 ± 0.3 mV and -9.7 ± 1.6 mV at 24 hours, 7 and 30 days, respectively; pH values were approximately 6 at all times. The analytical method was linear in a range of 25-45 µg/mL-1, with a good correlation coefficient (r = 0.9999). The procedure was specific, linear and precise, therefore, this method can be applied for the quantification of $(PhTe)_2$ in nanocapsule suspensions.

Introduction

In recent years, there has been an increased interest in searching for compounds with antioxidant properties. This is due to the fact that oxidative stress is related to several diseases.[1] Oxidative stress is caused by an imbalance between endogenous antioxidant defences and reactive species production in the organism.[2]

In this context, organoselenium and organotellurium compounds have been synthesized and presented different pharmacological properties, such as antioxidant, anti-inflammatory, anti-ulcer, anticancer, hepatoprotective and neuroprotective.[3] Most pharmacological properties are attributed to their antioxidant effects by the ability to capture reactive oxygen species and reactive nitrogen species. [4]

However, diphenyl ditelluride [$(PhTe)_2$] (Figure 1), an organotellurium compound, presents a superior antioxidant effect compared to to its structural analog, diphenyl diselenide (an organoselenium compound), but it is highly toxic to rodents, with significant neurotoxic effects.[5]

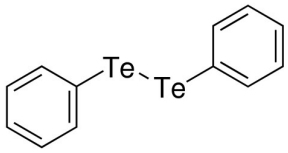

Figure 1. Chemical Structure of $(PhTe)_2$ [$(C_6H_5)_2Te_2$].

Although the specific mechanisms for the toxic effect of organochalcogen have not been fully explained, it is known that selenium and tellurium compounds can interact directly with molecular thiols, oxidizing to disulfides, causing organism toxicity.[6]

Among the alternatives proposed to circumvent the toxicity of these compounds, avoiding side effects and enhancing efficacy in the target cell, it is possible to point out the use of nanocarriers. As a result, the drug can reach, the site of specific action and be released selectively there. The colloidal carriers of drugs are systems that provide vectoring through organs, tissues and cells. The main advantage of these carriers is reduction of drug side effects.[7] Therefore, the use of nanocarriers may be an alternative to reduce $(PhTe)_2$ toxicity, enabling its use for therapeutic purposes.

The development of safe and reliable analytical methods is a very important tool for the quality control of drugs and raw material. In view of this, the present study demonstrated, for the first time, the production and characterization of diphenyl ditelluride loaded nanocapsules for further therapeutic purposes. Moreover, an

[a] *Programa de Pós-Graduação em Nanociências, Centro de Ciências Tecnológicas, Centro Universitário Franciscano, Santa Maria, CEP 97010-032, RS, Brazil.*
[b] *Grupo de pesquisa em Neurobiotecnologia - GPN, Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Pelotas, CEP: 96010-900, RS, Brazil.*
[c] *Departamento de Química, Universidade Federal de Santa Maria, Santa Maria, 97105-900, RS, Brazil.*
[d] *Laboratório de Controle de Qualidade, Centro Universitário Franciscano, Santa Maria, CEP 97010-032, RS, Brazil.*
*Correspondent author e-mail address: fernandadavila.biomedica@gmail.com
** Correspondent author e-mail address: cristiane_luchese@yahoo.com.br

analytical method was developed and validated, using high performance liquid chromatograph (HPLC)- ultraviolet (UV), to quantify and characterize the compound in the nanocapsules. This method was validated according to the official guidelines.[8]

Materials and Methods

Reagents, Solvents and Materials

Sorbitan monooleate, poly (ε-caprolactone) (PCL) molecular weight = 90,000, polysorbate 80 were obtained from Sigma-Aldrich (Saint Louis, MO), canola oil was purchased from Liza®, acetone was obtained from Synth® (Diadema, SP). The HPLC-grade acetonitrile purchased from Merck (Darmstadt, Germany) was used as solvent and ultrafree® membranes - MC Millipore 10,000 Å.

Instrumentation

HPLC Shimadzu YL9100 equipped with a LC-10AD pump model detector with variable wavelength UV / Vis model SPD-10AVP, SLC-10AVP controller, computerized automatic software integrator with Class VP® and autosampler SIL-10-Avp, oven for the column CTO-10Asvp Shimadzu was used. Rotary evaporator 801 (Fisatom®) and pot DM-22 Digimed and Zetasizer Nano-ZS (Malvern Instruments, United Kingdom) were used.

Diphenyl ditelluride

Diphenyl ditelluride was prepared and characterized as previously described.[9] The $(PhTe)_2$ was synthesized in the chemistry laboratory of Federal University of Santa Maria.

Development of nanocapsule suspensions containing $(PhTe)_2$

Suspensions of $(PhTe)_2$-loaded nanocapsules were prepared by the method of interfacial deposition of preformed polymer.[10] The aqueous phase was composed of milli-Q water (125 mL) and polysorbate 80 (Tween 80) (0.2 g). The organic phase was composed of $(PhTe)_2$ (0.037 g), PCL polymer (0.25 g), sorbitan monooleate (Span 80) (0.2 g), canola oil (0.77 g) and acetone (62.5 mL). The organic phase was added under magnetic stirring into the aqueous phase.[11] Lastly, the organic solvents were removed under vacuum, and the suspensions of $(PhTe)_2$-loaded nanocapsules were concentrated to 1.5 mg/mL (w/v) and fixed volume of 25 mL.

Physico-chemical parameters of suspensions

Particle size distribution and polydispersity index

The particle size and polydispersity index (PDI) were measured by photon correlation spectroscopy. Samples were diluted in Milli-Q water and analyses were performed at 25ºC, using a Zetasizer® (Nanoseries, Malvern, UK). Each sample was analyzed in triplicate.

Zeta potential

The zeta potential determination was performed by photon correlation spectroscopy. Samples were diluted in 10 mmol L^{-1} NaCl and analyses were performed at 25ºC, using a Zetasizer® (Nanoseries, Malvern, UK). Each sample was analyzed in triplicate.

Determination of pH

The pH values of the suspensions were determined by direct immersion of the electrode into the suspension, using a calibrated potentiometer (Digimed ®), at room temperature. Each sample was analyzed in triplicate.

Extraction of drug nanocapsules

After preparation, subsequent to the development of suspensions, samples were treated with acetonitrile kept under magnetic stirring for 30 minutes, and sonication for 10 minutes, in order to dissolve the polymer. Afterwards, samples were filtered through polyacrylamide membrane with 0,45µm porosity (Sartorius ®).

Chromatographic parameters

The chromatographic conditions[12] were optimized for the determination of $(PhTe)_2$ in the nanocapsules suspensions (Table 1).

Table 1. Chromatographic conditions used for the quantization $(PhTe)_2$ in suspensions containing nanocapsules.

Characteristics	Description
Column	Synergi™ 4 µm Hydro-RP 80 Å, LC Column 150 x 4.6 mm – Phenomenex
Precolumn	SecurityGuard Guard Cartridge Kit – Phenomenex
Flow	0.6 mL min^{-1}
Injection volumn	10 µL
Detection	248 nm
Mobile phase	acetonitrile / water (80: 20% v/v)

Validation of the analytical method

The technical validation process was carried out according to ANVISA Resolution RE Nº. 899[13] and International Conference on Harmonization (ICH).[14] The parameters evaluated were: linearity, accuracy, selectivity, limit of detection and limit of quantitation.

Evaluated parameters included specificity, linearity, quantification limit, detection limit, accuracy, precision and robustness.

Linearity

Linearity was evaluated with an analytical calibration curve. The mean areas, which correspond to three determinations for each dilution of compound, were plotted on a Cartesian axis, *x* being the concentrations (µg/mL) and *y* the areas. The analytical curve was used at concentrations of 25, 30, 35, 40 and 45 µg/mL (five linear points). The standard curve and its respective straight-line equation were determined by the linear regression study by the least squares method.

Precision

The precision assay was investigated with respect to repeatability (intra-day). The repeatability was evaluated by assaying three determinations at concentrations of 25, 30, 35, 40 and 45 µg/mL during the same day and under the same experimental conditions. Precision was evaluated by estimating the relative standard deviation (RSD), also known as coefficient of variation, obtained from the standard deviation divided by mean areas of analytical curve multiplied by 100.

Selectivity

Selectivity consists of investigating whether the nanocapsules components do not affect the retention time of $(PhTe)_2$. Thus, blank nanocapsules (without active principle) were visualized by chromatogram to compare the nanocapsules components.

Detection limit

The detection limit (DL) was calculated from the division of standard deviation of linear coefficients of three calibration curves by slope and then multiplied by 3.33. According to the equation: DL = SD x 3.33 / IC, SD being the standard deviation and IC the mean inclination of standard curves.

Quantification limit

The quantification limit (QL) was calculated by dividing the standard deviation of three coefficients of linear analytical curves by slope and then multiplying by 10. According to the equation: QL = SD x 10/IC, SD being the standard deviation and IC the mean inclination of the standard curves.

Encapsulation efficiency

Free $(PhTe)_2$ was determined in a liquid fraction obtained by ultrafiltration-centrifugation of the suspensions of the suspensions containing the nanocapsules using ultrafiltration-centrifugation membranes.

Evaluation of the stability parameters

Suspensions were packed in amber vials and stored at 3 ± 2°C, for 30 days. Samples were analyzed at 24 hours, 7 and 30 days, in terms of the physical and chemical characteristics of mean particle size, PDI and pH values. In order to make comparisons, suspensions of blank nanocapsules (no compound) were used under the same storage conditions.

Results and Discussion

Diphenyl ditelluride

Analysis of CG/MS, ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with the structure. Mass spectra and respective table of fragments were described in figure 2.

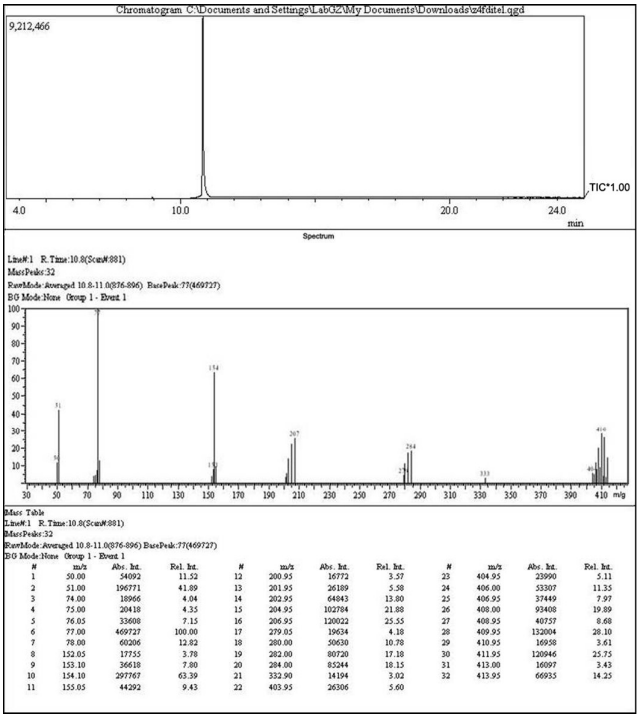

Figure 2. CG/MS spectra of $(PhTe)_2$

Physico-chemical characterization of suspensions

Particle size distribution and PDI

Particle size of nanocapsules containing $(PhTe)_2$ was measured at 24 hours, 7 and 30 days after production. Average particle sizes were 256 ± 19 nm for 24 hours, 255 ± 13 nm for 7 days and 255 ± 22 nm for 30 days. The PDI of suspensions presented values lower than 0.2, demonstrating homogeneous particle size distribution.[15] Particle size obtained for nanocapsules containing $(PhTe)_2$ is compatible with nanoscale structures. In fact, generally nanoparticles present diameters between 5 and 10 nm, with a size limit of ~ 1000nm, although it is usually obtained between 100 to 500nm.[16] Particle size is influenced by several factors, such as chemical nature and concentration of polymer, encapsulated drug, amount of surfactants, amount of oil in organic phase and diffusion velocity of organic phase over aqueous phase. In general, if interfacial tension and oil viscosity were reduced, average particle sizes formed are smaller.

Zeta potential

Values found for zeta potential at 24 hours, 7 and 30 days after the production of suspensions containing $(PhTe)_2$ were -10.7 ± 0.6, -12 ± 0.3 and -9.7 ± 1.6 mV, respectively. These values are typical and indicate the sample stability, since there is no significant alteration in the experimental period. In particular, the nanoparticle surface is negatively charged (zeta potential of -17 mV), which is essential to successful delivery of active principle to the brain and for endocytosis.[17] Major components of formulation, such as

surfactants and polymers, can influence the zeta potential. Several polymers, such as PCL and lecithin, impart a negative charge to the surface, due to the presence of ionized carboxylic groups[18], and consequently, enabling them to keep away, avoiding the aggregation formation and precipitation of nano-structures.[19] Thus, this parameter is used to characterize the surface potential of nanoparticles through the dissociation of functional groups of surface or adsorption of ionic species present in the dispersant medium.[20]

Determination of pH

The results presented pH values of about 6 and showed no significant differences between the suspensions at different times. In general, pH values of nanocapsules vary from 3.0 to 7.5 when prepared according to the nanoprecipitation method. [21]

The methodology used in the present study is standard for similar samples. Several studies presented this methodology for the physicochemical characterization of nanocapsules.[22]

Chromatographic parameters

The methodology used for extraction of $(PhTe)_2$ from the suspension, as well as the chromatographic conditions developed were considered satisfactory for the dosage of compound in nanocapsule suspensions.

Validation of analytical method

Mean areas corresponding to $(PhTe)_2$ concentrations were determined by HPLC (Table 2) at a wavelength of 248 nm.

Table 2. Values the mean areas, SD and RSD for the different concentrations of $(PhTe)^2$

µg/mL	Mean	SD[a]	RSD[b]
25	1016267	19027.709	1.872
30	1264965	25227.904	1.994
35	1502634	7543.8071	0.502
40	1745237	13117.589	0.751
45	1992140	6470.6576	0.324

[a]SD: Standard Deviation [b]RSD: Relative Standard Deviation

In order to ensure a new analytical methodology to generate reliable and interpretable information on a sample, it must undergo a process named validation. The validation method is a continuous process, which starts by planning an analytical and continuous strategy throughout development period. The HPLC method has been highlighted among the techniques for its ability to perform quantitative and qualitative analyzes of environmental, biological and pharmaceutical samples, as well as in food.[23]

"The validation must guarantee, through experimental studies, that the method meets the requirements of the analytical applications, ensuring the reliability of results".[13]

Linearity

From the experimental periods (Table 2), it is possible to calculate the regression coefficients "a" and "b" and correlation coefficient "r". The "r" allows estimating the quality of the obtained curve, since near 1.0 there is a dispersion of a set of experimental data and it reduces the uncertainty of the estimated regression coefficients. [30] The analytical curve of $(PhTe)_2$ showed a significant linear regression ($p \leq 0.01$), with no significant deviation from linearity ($p \geq 0.01$). The line equation for the method was: □*=48640*□ *−198164*, where *x* is concentration in µg/mL and *y* is the area, presenting a correlation coefficient of 0.9999 (Figure 3). The linearity corresponds to the ability of the methodology used to provide results directly proportional to concentration of substance under examination. Linearity was observed within the analyzed interval. The relationship between area and concentration of quantified compound can be verified from a mathematical relationship known as analytical curve.

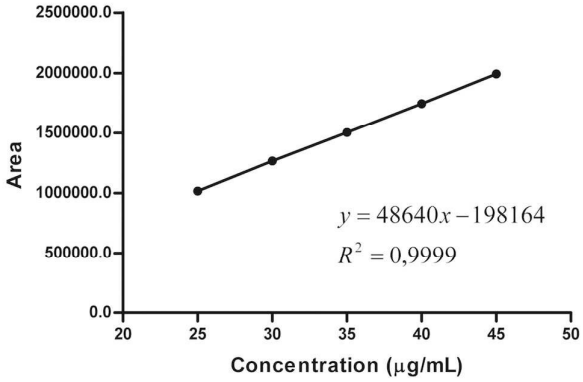

Figure 3. Analytical curve of $(PhTe)_2$

Selectivity

Figure 4a represents the peak obtained after extraction of compound from nanocapsules and figure 4b demonstrates the procedure used for white nanocapsules, highlighting method specificity. The selectivity of a particular method is the ability to verify the substances under examination in the presence of components, which may interfere with its determination in a complex sample. As a result, selectivity ensures that the corresponding peak refers to the compound of interest.[23]

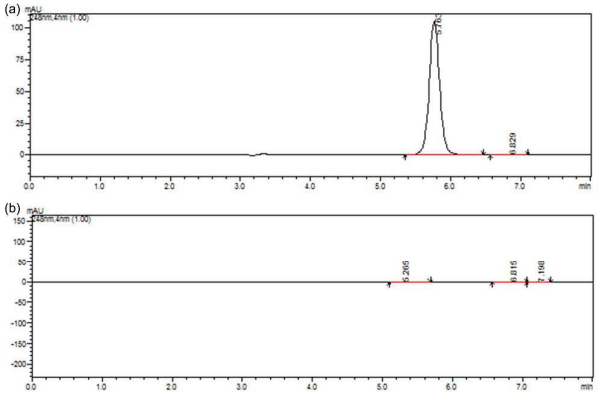

Figure 4. Chromatograms corresponding to: (a) the peak $(PhTe)_2$ viewed by HPLC observed at 5,7 minutes of running after the extraction of the compound of the nanocapsules and (b) blank nanocapsules.

DL and QL

QL is the lowest concentration of compound that can be measured using this methodology.[23] In this study, the QL for $(PhTe)_2$ was 3.28 µg/mL.

The use of an analytical method requires QL values at least 5 % below levels allowed by law for a given compound, in order to have a safety margin in its determination. Ribeiro et al (2008)[24] demonstrated that the safety margin obtained from 5 % below regulatory limits was achieved for all compounds analyzed, such as toluene and ethylbenzene, estimating QL performed with 95 and 99% confidence. The DL values were always lower than QL values, and this is expected. Reported values were considered satisfactory (sufficiently low) in both studies.

DL is the lowest concentration of compound that can be detected, but not necessarily quantitated by using a methodology.[23] The DL for $(PhTe)_2$ was 1.09 µg/mL.

Encapsulation efficiency

In this study, encapsulation efficiency of nanocapsules obtained from suspensions containing $(PhTe)_2$ was 99.97%. This result is in agreement with that found in encapsulation efficiency for $(PhSe)_2$, which presented 99.9% efficacy. These results can be explained as due to high lipophilicity and low water solubility of compounds.[12]

Conclusions

Nanocapsules obtained are consistent with nanoscale, which can be confirmed by physicochemical characterization of particles. Analytical method for the detection and quantification of $(PhTe)_2$ validated according to ANVISA (2003)[13] and *ICH* (2005)[14], showed to be linear, accurate and selective at concentrations of 25 to 45 µg mL^{-1}. The LD and LQ indicated that this method was effective for measuring the minimum concentration of compound (3.28 µg mL^{-1}). Thus, we concluded that the technique was adequate for the development of $(PhTe)_2$-loaded nanocapsule suspensions, as well as the validated method for compound quantification.

Acknowledgements

This study received support by FAPERGS, CAPES, CNPq and Franciscan University Center is gratefully acknowledged.

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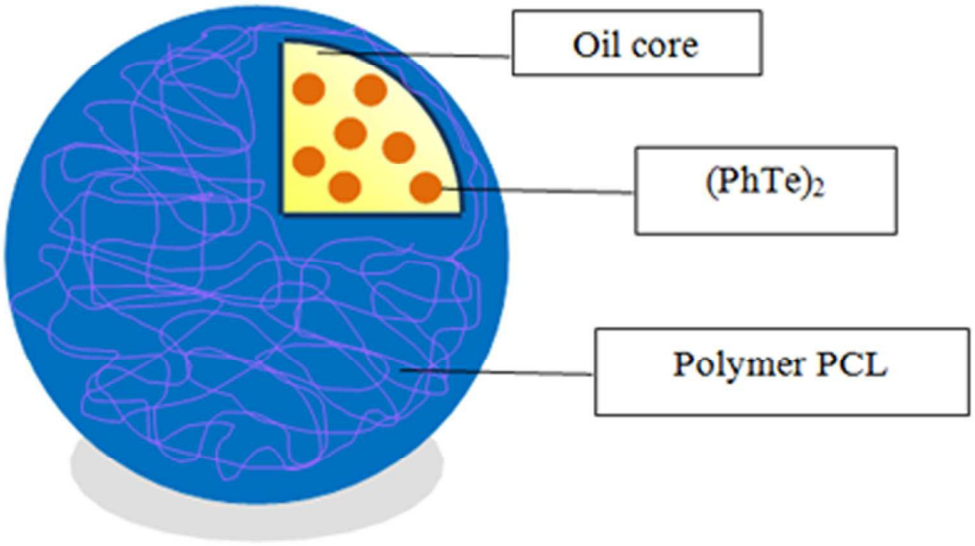

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