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Validation of a spectrophotometric method for quantification of xanthone in biodegradable nanoparticles

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Xanthone has been incorporated for the first time in nanoparticles of poly(D,L-lactide-co-glycolide) (PLGA). For this purpose the estimation of xanthone content in the nanoparticles is a crucial tool for guaranteeing the reliability of the results. Thus, a simple spectrophotometric method was validated according to USP25 and ICH guidelines for its specificity, linearity, accuracy and precision. The method was found to be specific for xanthone in the presence of nanoparticle excipients. The calibration curve was linear over the concentration range of 0.5 to 4.0 μg/mL ($r > 0.999$). Recovery of xanthone from nanoparticles ranged from 86.5 to 95.9%. Repeatability (intra-assay precision) and intermediate precision were found to be acceptable with relative standard deviations values (RSD) ranging from 0.3 to 3.0% and from 1.4 to 3.1%, respectively. The method was found to be suitable for the evaluation of xanthone content in nanoparticles of PLGA.

1. Introduction

Xanthones represent a large group of heterocyclic compounds including natural, semisynthetic and totally synthetic structures (Peres et al. 2000). Among others, antitumoral (Lin et al. 1996a; Kamei et al. 1998), antibacterial (Hnuma et al. 1996), anti-inflammatory (Lin et al. 1996b), hepatoprotective (Fernandes et al. 1995), antimalarial (Ignatushchenko et al. 1997), immunomodulatory (Pinto and Nascimento 1997; Gonzales et al. 1999), as well as inhibitory activities of angiotensine converting enzyme (Chen and Lin 1992) and monoamine oxidase (MAO) (Thull et al. 1993; Gnerre et al. 2001) have been described. Xanthone itself was described as a good MAO-A inhibitor (Thull et al. 1994).

Poor aqueous solubility of xanthone and many of its derivatives is a major obstacle for the assessment of pharmacological activity of these compounds and for their use in therapy. In general water-insolubility is often associated with poor bioavaliability (Speiser 1998). One approach to overcome the difficulty of administration of poorly water-soluble compounds is by incorporation in carrier systems such as polymeric microparticles and nanoparticles.

By incorporating xanthone or its derivatives in nanoparticles, these poorly water-soluble compounds may be administered as nanoparticle aqueous dispersions at concentrations higher than their maximum hidrosolubility. Moreover, incorporation of these compounds in nanoparticles may allow different ways of administration and, simultaneously, may afford their *in vivo* protection and targeting.

This study is part of a broader investigation, which aims the incorporation of xanthone and its derivatives in nanoparticles of poly(DL-lactide-co-glycolide) (PLGA) as well as the *in vitro* and *in vivo* evaluation of the systems. PLGA has been selected since polyesters, including poly(lactic acid), poly(glycolic acid) and their copolymers, have emerged as the most widely studied class of biodegradable polymers for pharmaceutical use due to their biocompatibility and biodegradability (Jain et al. 1998). In the present work, we have used xanthone as a model molecule of this family of compounds for incorporation in PLGA nanoparticles. For this purpose the estimation of xanthone content in the nanoparticles is a crucial step. Thus, the quantification method of incorporated xanthone constitutes a very important tool for guaranteeing the reliability of the results. We report here the validation of a simple and accurate spectrophotometric method for the quantification of xanthone content in nanoparticles of PLGA according to International Conference on Harmonisation (ICH) guidelines (Validation of Analytical Procedures 1998a, b), which are similar to those established by the United States Pharmacopoeia 25 (USP 25). The method was applied to characterize the level of xanthone entrapment in PLGA nanoparticles, which have been prepared for the first time.

2. Investigations, results and discussion

2.1. Validation study

According to the ICH guidelines the specificity of an analytical method is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as degradation products, excipients, etc. (Validation of Analytical Procedures 1998a, b).

In order to evaluate the degradation of xanthone during nanoparticle preparation a TLC was carried out. Two mobile

Table 1: Results of specifity determinations

Xanthone standard solutions		Xanthone standard solutions spiked with empty nanoparticles	$t_{calculated}$*
Theoretical concentration (μg/mL)	Actual mean concentration (μg/mL) (n) [SD]	Mean concentration (μg/mL) (n) [SD]	
1.0	0.9400 (3) [0.017]	0.9003 (3) [0.081]	0.832
2.0	1.983 (3) [0.002]	1.918 (3) [0.142]	0.784
4.0	3.966 (3) [0.021]	3.682 (3) [0.2213]	2.31

* $t_{calculated} = \frac{(\bar{x}_1 - \bar{x}_2)}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$; $s = \sqrt{\frac{(n_1 - 1)\, s_1^2 + (n_2 - 1)\, s_2^2}{n_1 + n_2 - 2}}$

where $\bar{x}_1$ and $\bar{x}_2$ are mean concentrations of the two samples and s_1 and s_2 standard deviation values and t has $n_1 + n_2 - 2$ degrees of freedom.

phases were used to develop TLC pre-coated plates of silica gel $60F_{254}$ (Merck): petrolbenzine (40–60 °C)–Et_2O (5 : 5) and petrolbenzine–EtOAc (5 : 5). Five samples were compared: empty nanoparticles, xanthone, nanoparticles containing xanthone, mixture of xanthone and nanoparticles containing xanthone (1 : 1) and mixture of xanthone and empty nanoparticles (1 : 1). Spots were identified by exposure to the UV light at 254 nm. TLC results showed the absence of xanthone degradation products, demonstrating that xanthone remains stable upon nanoparticle preparation in the referred conditions. Therefore, no degradation products will be present in the medium during xanthone quantification.

In order to evaluate the specificity of the analytical method concerning to the presence of nanoparticle excipients (i.e. the potential interference of the excipients), a comparison of the test results from the analysis of xanthone standard solutions spiked with empty nanoparticles (8 mg) with those obtained from the analysis of xanthone standard solutions alone was carried out (Table 1). Data analysis was done using Student's *t* test ($P = 0.05$). No significant difference was observed between xanthone standard solutions spiked with empty nanoparticles and the correspondent xanthone standard solutions alone, once calculated *t* values were lower than the critical *t* value (2.78, for 4 degrees of freedom and a confidence limit of 95%).

According to ICH guidelines the linearity of an analytical method is its ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in the sample (Validation of Analytical Procedures 1998a, b)

To assess linearity, a calibration curve was constructed at five concentration levels (0.5; 1.0; 2.0; 3.0 and 4.0 μg/mL) using the linear square regression procedure. The absorbance values obtained for three replicate analyses were averaged at each concentration. Linear regression analysis was carried out by plotting mean absorbance at 237 nm (y) versus analyte concentration (x). The calibration curve showed to be linear over the concentration range examined with a correlation coefficient (r) > 0.99926 and a coefficient of determination (R^2) > 0.9985, i.e. over 99.85% of relationship between x and y (Table 2).

According to ICH guidelines the accuracy of an analytical method expresses the closeness of agreement between a value (which is accepted either as a conventional true value or an accepted reference value) and the value found (Validation of Analytical Procedures 1998a, b). Accuracy is often calculated as percent recovery by the assay of known, added amounts of analyte to the sample.

Table 2: Summary of calibration curve results

Xanthone concentration (μg/mL)	Mean absorbance	RSD (%)
0.5	0.1087	0.772
1.0	0.2138	0.446
2.0	0.4678	1.00
3.0	0.6899	3.23
4.0	0.9066	2.03
Y-intercept	-0.0035 ± 0.00496[a]	
Slope	0.23000 ± 0.00221[a]	
Correlation coefficient (r)	0.99926	
Coefficient of determination (R^2)	0.99852	

[a] Confidence limits of Y-intercept and slope ($P = 0.05$)

Accuracy of the assay method was determined by spiking known amounts of xanthone to samples of empty nanoparticles (8 mg) to obtain final xanthone concentrations of 0.5; 1.0; 2.0 and 4.0 μg/mL, corresponding approximately to 13, 26, 52 and 105% of maximum theoretical concentration (MTC) of 3.8 μg/mL (as defined in the Experimental section), respectively. Table 3 summarises the accuracy results, expressed as percent recovery and relative standard deviation (RSD). Values of recovery ranged from 86.5 to 95.9%. These results are clearly in agreement to the criteria proposed by Mehta (1989) for the recovery of an analytical method, which should be preferably higher than 75%.

According to ICH guidelines the precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the conditions prescribed (Validation of Analytical Procedures 1998a, b). Precision may be measured as repeatability, reproducibility and intermediate precision. Repeatability expresses the precision under the same operating conditions over a short interval of time (also termed intra-assay precision). Reproducibility refers to the use of an analytical procedure in different laboratories. Intermediate precision expresses the precision within laboratory variations (different days, analysts, equipment, etc). In this work, we have studied repeatability and intermediate precision for different days.

Repeatability was determined by the analysis of five xanthone standard solutions in the concentration range of 0.5 to 4.0 μg/mL (three replicates each) on the same day. Intermediate precision was determined by the analysis of the same standard solutions on three different days. During this time period, the standard solutions were refrigerated at 4 °C. Table 4 summarises repeatability and intermediate precision results. Obtained RSD values ranged from 0.3 to 3.0% and from 1.4 to 3.1%, respectively, indicating that the proposed method shows acceptable repeatability and intermediate precision. These results are clearly in agreement to the criteria proposed by Mehta (1989) and Calpena et al. (1990) for the precision of an analytical method, whose RSD should be lower than 10%.

Table 3: Results of accuracy determinations

Xanthone concentration		Recovery (%) (n)	RSD (%)
(μg/mL)	(% of MTC)[a]		
0.5	13	86.5 (3)	3.2
1.0	26	90.0 (3)	1.7
2.0	52	95.9 (3)	3.0
4.0	105	92.1 (3)	2.4

[a] % of maximum theoretical concentration; RSD Recovery standard deviation

Table 4: Results of precision determinations

Theoretical concentration (μg/mL)	Mean experimental concentration (μg/mL) (n)	SD	RSD (%)
Repeatability (intra-assay precision)			
0.5	0.467 (3)	0.002	0.3
1.0	0.935 (3)	0.028	3.0
2.0	2.12 (3)	0.021	1.0
3.0	3.02 (3)	0.061	2.0
4.0	3.95 (3)	0.081	2.1
Intermediate precision (different days)			
0.5	0.473 (3)	0.010	2.1
1.0	0.941 (3)	0.013	1.4
2.0	2.09 (3)	0.064	3.1
3.0	3.04 (3)	0.046	1.5
4.0	3.94 (3)	0.067	1.7

2.2. Application of the validated method

Xanthone content of three different batches of PLGA nanoparticles has been determined by the present validated method. Table 5 shows incorporation parameters and particle size of prepared nanoparticles. Incorporation efficiency ranged from 23.9 to 40.8%, with a mean of 32.9%. Mean diameter of xanthone nanoparticles was 117 nm with a narrow particle size distribution (polydispersity index of 0.06).

3. Experimental

3.1. Materials

Xanthone, PLGA 50 : 50 (MW 50000–75000) and Pluronic F-68 were purchased from Sigma. All solvents and reagents were of analytical grade.

3.2. Nanoparticle preparation and characterization

Xanthone nanoparticles of PLGA were prepared according to a modified nanodispersion methodology (Fessi et al. 1989). Briefly, 20 mL of an aqueous medium containing Pluronic F68 (0.25%, w/v) were poured into 20 mL of an acetonic solution containing 125 mg of PLGA polymer and 3 mg of xanthone under moderate stirring, leading to the formation of nanoparticles. Then, acetone was removed under vacuum. In order to separate crystals of nonentrapped xanthone, the nanoparticle dispersion was filtered through a 0.22 μm membrane (Millipore). To separate soluble nonentrapped xanthone, the filtrate was subjected to ultracentrifugation at 110000 × g for 15 min at 20 °C (Beckman UL-80 ultracentrifuge). The supernatant containing free xanthone was discarded and the pellet was freeze-dried (Edwards freeze-drier).
Empty nanoparticles were prepared according to the same procedure but without xanthone in the organic phase.
The mean size and polydispersity index of nanoparticle dispersions were determined by laser light scattering (Malvern Instr. Zetasizer 5000) generating a volume-average distribution for analysed data.

Table 5: Incorporation parameters and particle size of PLGA nanoparticles containing xanthone

Theoretical xanthone loading[a] % (w/w)	Actual xanthone loading[b] % (w/w)	Incorporation efficiency[c] (%)	Diameter (nm)	Polidispersity Index[d]
2.4	0.79 ± 0.20	32.9 ± 8.5	117.1 ± 0.6	0.06 ± 0.02

Values express the mean results ± SD of three different batches (n = 3)

[a] $\frac{\text{Mass of xanthone used in formulation}}{\text{Mass of polymer used in formulation}} \times 100$

[b] $\frac{\text{Mass of incorporated xanthone}}{\text{Mass of freeze - dried nanoparticles}} \times 100$

[c] $\frac{\text{Xanthone actual loading}}{\text{Xanthone theoretical loading}} \times 100$

[d] Varies from 0.0 corresponding to a perfect homogeneous dispersion to 1.0 corresponding to a complete heterogeneous dispersion

3.3. Preparation of sample solution for determination of xanthone in nanoparticles

Freeze-dried nanoparticles (8 mg) were dissolved in 2 mL of methylene chloride, followed by precipitation of the polymer by addition of 23 mL of ethanol. The obtained solution was filtered through a 0.45 μm membrane (Millipore). The filtrate was diluted with ethanol (1 : 2) and assayed by UV spectroscopy at 237 nm (Varian spectrophotometer), which corresponds to the maximum absorption wavelength of xanthone under these conditions. Considering 100% of xanthone entrapment in nanoparticles, the obtained sample solution had a maximum theoretical concentration (MTC) of 3.8 μg/mL. All analyses were performed in triplicate and the mean results are reported.

3.4. Preparation of xanthone standard solutions

Xanthone standard solutions were obtained by dilution of a stock standard solution (50 μg/mL) with ethanol to give five different concentrations over the range of interest (0.5 to 4.0 μg/mL).

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References

Calpena CA, Escribano FE, Fernández LC (1990) Validación de los métodos analíticos. Farm Clín 7: 749–758.
Chen CH, Lin JY (1992) Inhibition of angiotensine-I-converting enzyme by tetrahydroxyxanthones isolated from *Tripterospermum lanceolatum*. J Nat Prod 55: 691–695.
Fernandes ER, Carvalho FD, Remião FG, Bastos ML, Pinto M, Gottlieb OR (1995) Hepatoprotective activity of xanthones and xanthonolignoids against tert-butylhydroperoxide-induced toxicity in isolated rat hepatocytes – comparison with silybin. Pharm Res 12: 1756–1760.
Fessi H, Puisieux F, Devissaguet JPh, Ammoury N, Benita S (1989) Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J Pharm 55: R1–R4.
Gnerre C, Thull U, Gaillard P, Carrupt P-A, Testa B, Fernandes E, Silva F, Pinto M, Pinto MMM, Wolfender J-L-, Hostettmann K, Cruciani G (2001) Natural and synthetic xanthones as monoaminoxidase inhibitors: biological assays and 3D-QSAR Helv Chim Acta 84: 552–570.
Gonzalez MJ, Nascimento MSJ, Cidade H, Pinto MM, Kijjoa A, Anantachoke C, Silva AMS, Herz W (1999) Immunomodulatory activity of xanthones from *Calophyllum teysmannii* var. *inuphylloide*. Planta Med 65: 368–371.
Hnuma M, Tosa H, Asai F, Kobayashi Y, Shimano R, Miyandis KI (1996) Antibacterial activity of xanthones from Guttiferaeous plants against methicillin-resistant *Staphylococcus aureus*. J Pharm Pharmacol 48: 861–865.
Ignatushchenko MV, Winter RW, Bachinger HP, Hinrich DJ, Riscoe MK (1997) Xanthones as antimalarial agents; studies of a possible mode of action. FEBS Lett 409: 67–73.
Jain R, Shah NH, Malick AW, Rhodes C (1998) Controlled drug delivery by biodegradable poly(ester) devices: different preparative approaches. Drug Dev Ind Pharm 24: 703–727.
Kamei H, Koide T, Kojima T, Hashimoto J, Hasegawa Y (1998) Inhibition of cell growth in culture by quinones. Cancer Biother Radiopharm 13: 185–188.
Lin CN, Chung MI, Liou SJ, Lee TH, Wang JP (1996b) Synthesis and anti-inflammatory effects of xanthone derivatives. J Pharm Pharmacol 48: 532–538.
Lin CN, Liou SJ, Lee TH, Chuang YC, Won SJ (1996a) Xanthone derivatives as potential anti-cancer drugs. J Pharm Pharmacol 48: 539–544.
Mehta AC (1989) The validation criteria for analytical methods used in pharmacy practice research. J Clin Pharm Ther 14: 465–473.
Peres V, Nagem TJ, de Oliveira FF (2000) Tetraoxygenated naturally occurring xanthones. Phytochemistry 55: 683–710.
Pinto M, Nascimento MSJ (1997) Anticomplementary activity of hydroxy- and methoxyxanthones. Pharm Pharmacol Lett 7: 125–127.
Speiser P (1981). Poorly soluble drugs, a challenge in drug delivery. In: Müller RH, Benita S, Böhm B (Eds) Emulsions and nanosuspensions for the formulation of poorly soluble drugs, Scientific Pub., Stuttgart, pp. 15–19.
Thull U, Testa B (1994) Screening of unsubstituted cyclic compounds as inhibitors of monoamino oxidases. Biochem Pharmacol 47: 2307–2310.
Thull U, Kneuber S, Testa B, Borges MFM, Pinto MM (1993) Pharm Res 10: 1187–1190.
United States Pharmacopeia 25/NF 20 (2002). United States Pharmacopeial Convention, Rockville, MD, pp. 2256–2259.
Validation of Analytical Procedures: Methodology (1998a) The Rules Governing Medicinal Products in European Union, vol. 3A, 107–117.
Validation of Analytical Procedures: Definition and Terminology (1998b). The Rules Governing Medicinal Products in European Union, vol. 3A, 119–125.