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Synthesis and application of doxorubicin-loaded silica gels as solid materials for spectral analysis

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Abstract

A new approach to synthesis and the possibility of application of solid gel matrices containing the target analyte, a cancer drug—doxorubicin for the calibration of UV–vis spectrometers is presented. The doxorubicin-loaded gels were prepared by the acid-catalyzed sol–gel pre-doping method. The efficiency of doxorubicin encapsulation in gels by the sol–gel process was $100 \pm 1.2\%$ (w/w). The amount of doxorubicin encapsulated per unit mass of the gel (1 g) was found to be independent of the mass of the gel taken for analysis, which demonstrates the homogeneity of encapsulation of the drug in the gel matrix. The gelation process of doxorubicin in the sol was found to be both repeatable (R.S.D. = 11.2% for $n = 80$, confidence level $P = 95\%$) and reproducible (largest value of R.S.D. = 4.8% for $n = 27$, $P = 95\%$).

The prepared doxorubicin-loaded gel matrices are characterized primarily by their lack of toxicity as compared to the toxic free form of doxorubicin as well as by high stability over a long-time span.

Keywords: Encapsulation; Sol–gel methods; Doxorubicin; Gel matrices; Reference materials

1. Introduction

One of the trends in the area of preparation of reference materials is to develop methods that would allow making standards with a very wide and controlled concentration range of the analyte(s). The calibration of an instrument over a wide measuring range using such reference materials would result in the reduction of time and tediousness as well as elimination of errors caused by the lack of traceability. According to International Organization for Standardization—*ISO Guide 30, 1992*, all reference materials should meet the basic requirements of representativeness, homogeneity, and stability [1,2]. A search of the literature available reveals a number of new procedures for the preparation of reference materials, but they deal primarily with the generation of gaseous standard mixtures [3–13].

The main problem encountered by the analyst when preparing standard solutions of doxorubicin is its high toxicity. Doxorubicin is a cytotoxic and cytostatic drug with a low-therapeutic index, used in chemotherapy of cancers [14–17]. Another problem is its sensitivity to many physical and chemical factors, which has been confirmed by our preliminary investigations as well as by other authors [15–17]. The stability of doxorubicin in aqueous solution can be affected by many factors such as pH (doxorubicin is stable in acidic solutions in the pH range 3.0–6.5, but rapid decomposition occurs at higher pH (6.5–12), buffer concentrations, temperature, light, and metal ions [15,16]. We also found that doxorubicin is gradually destabilized in the presence of visible light and with an increase in temperature from 21 to 37 °C the rate of degradation process increases [17]. Doxorubicin has also been found to adsorb onto various materials such as polytetrafluoroethylene (PTFE), glass, polyethylene, and plastic containers, but not to silanized glass or polypropylene [16].

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At the calibration stage of an analytical device, the preparation of a series of standard liquid mixtures of doxorubicin is limited by the time it takes for the drug to be analyzed. Otherwise, this time of preparation can be the source of a statistically significant uncertainty in the result associated with the change in true value of concentration of doxorubicin in standard solutions resulting from its photodegradation.

This paper presents a new approach to the preparation of reference materials with doxorubicin based on using the sol–gel pre-doping method for the synthesis of stable solid and monolithic materials containing the drug. The principle of the sol–gel method is based on reactions of hydrolysis and polycondensation of alkoxides taking place in solution during formation of a silica gel [18,19]. Admixtures of an active substance are introduced into the polymer during the sol formation step and the molecules are permanently trapped in the gel matrix as a result of polycondensation reaction. Introducing an acid or base catalyst, which also determine the pore size and the shape of polymeric structures, reduces the gelation time of solution. This also provides a number of different applications for gel polymers. The products of acid condensation are weakly linked gels-yielding structures with small pore, but of improved mechanical strength as compared to the products of basic catalysis. Basic catalysis favors the formation of agglomerate consisting of branched colloidal particles, which yields a porous material with high-adsorption capacity [20–22].

This paper describes the preparation of amorphous silica gels in the form of monoliths whose important properties are their high purity and homogeneity, which allows spectrophotometric investigation of thus prepared materials. The use of solid, silicate gel matrices with encapsulated doxorubicin as reference materials is also encouraged by the trends in preparation of new solid forms of the drug, including tablets, pellets, microspheres, nanospheres as well as implants [16,17,23,24]. The authors of this paper already attempted to prepare a new form of the drug—doxorubicin using gel matrices loaded with the active ingredient synthesized by the base-catalyzed sol–gel pre-doping method facilitating the diffusion of the drug through the pores [17]. Every new form of a drug requires closely controlled investigations in vitro related to the determination of both efficiency and reproducibility of the drug preparation process, the kinetics of drug release, the stability of the active ingredient under different measurement conditions, etc. An additional important reason for search for such reference materials is the fact that typical silicone implants unmodified with organic components and containing an active substance are not biodegradable either in vivo or in vitro; consequently, in the course of laboratory studies in vivo and using animals a continuous monitoring of the implant during and after its biomedical function is recommended.

The search for materials, which could be used in the future as reference materials that would improve the stability of the analyte and limit both the effect of environmental conditions and the toxicity of the compound itself, is therefore the objective of this research.

2. Experimental

2.1. Materials

- Reference substance: Doxorubicin hydrochloride 200 mg, 99.0% $C_{27}H_{29}NO_{11}HCl$; $M = 580.0\,g\,mol^{-1}$ from Promochem Standard Supplies, Poland;
- 0.05 M Tris buffer (pH 7.4), tetraethoxysilane, hydrochloric acid (36%), ethanol (99%) (Analytical Reagent Grade) were all from Merck, Poland.

2.2. Apparatus

Analytical balance RADWAG, Poland. The manufacturer's calibration uncertainty was ±0.0001 g. It included the analyst's repeatability (at a confidence level 95%) and the buoyancy correction. The system of supercritical fluid drying consist of an autoclave, a supercritical fluid-pumping module, and a pump to deliver the rinse solvents. The process of supercritical fluid drying was computer-controlled to facilitate reproducibility.

An HP 8452A Diode-Array UV–vis spectrophotometer equipped with an accessory for solid samples (Hewlett Packard, Palo Alto, CA) and connected to an IBM Pentium 100 computer were used in the investigations. For statistical calculations, version 4.2 of Matlab program (Math Works Inc.) was used.

2.3. Preparation of standard solutions of doxorubicin by a static (volumetric) method

A weighed sample of doxorubicin (DOX HCl) 10 ± 0.1 mg was dissolved in 50 mL of Tris buffer solution (pH 7.4) in a silanized polypropylene volumetric flask in the absence of light. Various standard solutions of doxorubicin (from $0.01\,\mu g\,mL^{-1}$ to $200.0\,\mu g\,mL^{-1}$) were then prepared from this stock solution after adequate dilution with a buffer solution. The solutions were prepared quickly to limit the exposure to light and carefully due to a high toxicity of doxorubicin.

2.4. Synthesis of doxorubicin-loaded gels using the sol–gel pre-doping method

The first step in the preparation of the gel with a mass of 1 g was the reaction of partial hydrolysis and condensation of the precursor—tetraethoxysilane with deionized water: 3.2 g (±0.0001 g) of tetraethoxysilane and 15 mL of ethanol were slowly stirred for 15 min in a polyethylene flask (25 mL volume). Next, 1 mL of deionized water with a catalyst (one drop of HCl) was added, and the mixture was stirred for further 30 min (during this time an aqueous sol containing hy-

droxylated water-soluble species—polysilanols was obtained through polycondensation [18]).

In the second step, the sol was mixed with 1 mL of a 200 μg standard, buffered solution of doxorubicin and gelation was initiated (at this time hydrolysis is complete [18]). From this time on, the solation process was carried out in the dark. The solution was initially red because of the color of doxorubicin. Afterwards, the sol solution of doxorubicin was poured into polypropylene capsules 15 mm in diameter and with variable thickness from 15 mm to 35 mm.

In the next step, a doxorubicin-doped hydrogels were obtained through polycondensation (polycondensation leads to the formation of soluble macromers and then colloids, which coalesce and raise the solution viscosity to the sol–gel transition, at which bulk gelation occurs [18]). In the last step, the doxorubicin-loaded hydrogels were aged in a closed system (further polycondensation leads to formation of the aged hydrogel [18]). Then, the aged doxorubicin-doped hydrogels were washed and conditioned with Tris buffer (pH 7.4) and subjected to controlled drying (the room temperature drying of the hydrogels at 25 ± 2 °C for 1 day), which results in pore collapse. Afterwards, the products were carefully put into an autoclave and covered with ethanol to obtain monolithic gels without cracks. Under constant cooling to 15 °C, the autoclave was filled with liquid CO_2 and pressure increased to 55 bar. The exhange process lasted 1–3 days at 15 °C depending on the thickness (in the range from 10 mm to 30 mm) of the gel samples. The average flow of liquid CO_2 through the autoclave was adjusted to about 250 mL min^{-1}. After the exchange, a heating rate of 0.5 °C min^{-1} from 10 °C to 35 °C was chosen. The pressure was pumped to 85 bar during this operation. After holding for 2 h at maximum temperature and pressure, the CO_2 was slowly released over 24 h and the dried gels are removed from the autoclave. The color of the aged gel containing doxorubicin was red.

As a result of the synthesis, CO_2—drying and shrinkage of doxorubicin-loaded gel matrices, some of the cylindrical pellets (which are monolithic, crack-free and transparent) were obtained with masses ranging from 0.01 g (±0.0001 g) to 1 g (±0.0001 g). Thus, selected prepared gels containing doxorubicin were kept in a desiccator for 2–6 days prior to spectral studies. About a 100 syntheses of doxorubicin-loaded gel matrices were carried out using the procedure described above.

About 10 doxorubicin-loaded gel pellets prepared by means of the sol–gel method were stored for a period of 6 and 12 months in a desiccator at room temperature in order to check their stability. On the other hand, to investigate the reproducibility of doxorubicin encapsulation by the sol–gel process, successive batches of doxorubicin-loaded gels were prepared under the same conditions and in the same number of samples but over different and independent time periods of 1, 6, 12, 18 and 24 months.

2.5. Formulation of pure gel without doxorubicin using the sol–gel method

Control samples (pure gels) were analyzed at the same time as investigated samples in order to check both the transmission of UV–vis light and the purity of gel matrices obtained by the sol–gel process.

To that end, in the second step of formulation of the gels described above the sol was mixed with water without an addition of doxorubicin and the rest of the procedure was exactly the same.

Colorless, monolithic gels (without doxorubicin) with masses ranging from 0.01 g (±0.0001 g) to 1 g (±0.0001 g) were chosen. Fig. 1 represents a UV–vis (in the region from 300 nm to 800 nm) transmission spectrum of thus obtained pure gel (mass 0.5 ± 0.0001 g; thickness 25 mm).

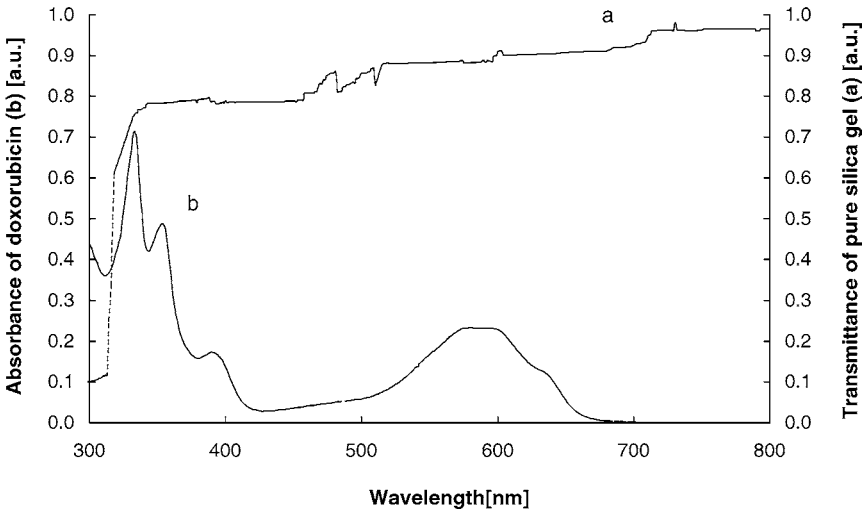

Fig. 1. UV–vis transmission spectra of pure gel (blank probe) of mass 0.5 g with thickness 25 mm (a). UV–vis absorption spectra of doxorubicin-loaded gel of mass 0.5 g with thickness 25 mm (b).

2.6. Spectral characteristics of doxorubicin encapsulated in gel matrices and in buffer solutions

Spectral characteristics in the UV–vis region (from 300 nm to 800 nm ± 0.1 nm, measured quantity—absorbance A ± 0.005 AU) of doxorubicin (100 μg) encapsulated in gel matrices with masses 0.5 g (±0.0001 g) (thickness 25 mm) were determined (Fig. 1). The pure gel (0.5 g; thickness 25 mm) was used as a blank reference. Spectral analysis of doxorubicin in liquid samples containing the same amount of the analyte (100 μg) was carried out in 10-mm cuvettes using the buffer as a reference solution under the same conditions. The two sets of spectra obtained in the analyzed wavelength range were not statistically different either qualitatively or quantitatively. Consequently, spectral characterization of the synthesized doxorubicin-loaded gels can be carried out on the basis of pre-calibration of a spectrometer using standard liquid solutions of doxorubicin.

The absorption spectra from 300 nm to 800 nm of doxorubicin in liquid samples exhibit six maxima: two strong: $\lambda = 333.4$ nm and 352.0 nm, and four weak: 388.0 nm, 375.0 nm, 595.0 nm and 630.0 nm. Quantitative determination of the analyte in liquid samples at the six wavelengths corresponding to these maxima revealed that in the concentration range of the analyte:

- from 0.1 μg mL^{-1} to 100 μg mL^{-1} linear calibration curves following the Lambert–Beer law were obtained at the analytical wavelength $\lambda = 333.4$ nm corresponding to the absorbance maximum;
- from 10 μg mL^{-1} to 160 μg mL^{-1} at the wavelength $\lambda = 352.0$ nm;
- from 20 μg mL^{-1} to 200 μg mL^{-1} at the wavelength $\lambda = 595.0$ nm.

For the remaining three wavelengths: $\lambda = 388.0$ nm, 575.0 nm and 630.0 nm small deviations from the Lambert–Beer law were observed in the investigated range of doxorubicin concentrations in solution.

2.7. Evaluation of doxorubicin encapsulation method using the sol–gel process

Characterization of doxorubicin encapsulation by the sol–gel process required the following steps:

- examination of transparency (purity) in the region from 300 to 800 nm of pure gel matrices;
- investigation of the amount and efficiency of doxorubicin encapsulation during sol gelation by the sol–gel method as well as checking whether the drug was uniformly trapped in the bulk of the gel during gelation of the sol with the doxorubicin solution (investigation of the degree of homogeneity of doxorubicin encapsulation);
- examination of reproducibility of the sol–gel method of doxorubicin encapsulation;
- determination of stability of doxorubicin-loaded gel matrices during storage in a desiccator at room temperature;
- determination of the effect of UV–vis light on the transparency of doxorubicin-loaded gel and pure gel samples over time;
- determination of the dependence of readout of a UV–vis spectrophotometer on the mass of pellets of doxorubicin-loaded gels.

To this end, the weighed pellets of doxorubicin-loaded gel (10 samples for each mass of the gel) were analyzed spectrophotometrically in the selected wavelength region using pure gels having the same masses as the analyzed doxorubicin-loaded gels as a reference. This procedure was used in all stages of the investigation.

The quantitative determination of an analyte in the weighted pellets of doxorubicin-loaded gel was carried out on the basis of pre-calibration of a spectrometer using standard liquid solutions of doxorubicin.

2.8. Investigation of the efficiency of sol–gel method for encapsulation of doxorubicin in gels

The analysis of spectra of pure gel matrices revealed a general transparency and purity, which is a function of wavelength in the region from 320 nm to 800 nm (Fig. 1), which demonstrates that the selected conditions of preparation and drying of gels were appropriate, thus allowing spectrophotometric investigation. The characteristics of doxorubicin-loaded gels (expected and determined values for the amounts of doxorubicin in doxorubicin-loaded gels as well as the determined value of the amount of doxorubicin per unit mass of the gel) are listed in Table 1. Acceptance of the sol–gel method for encapsulation of doxorubicin in terms of its accuracy and precision (expressed as R.S.D.%) was based upon the confidence interval values of the average content of doxorubicin per unit mass of the gel calculated at the 95% confidence level (1 g). The average amount of encapsulated doxorubicin was expressed as the average value of 80 determinations of the amount of encapsulated doxorubicin per unit mass of the gel (w/w). The loading efficiency was calculated as the percent ratio of the actual amount of doxorubicin incorporated into the gels (per 1 g) to the initial amount used (expected value).

2.9. Determination of degree of homogeneity of doxorubicin encapsulation in the bulk of gel

The investigation of degree of homogeneity of doxorubicin encapsulation in the bulk of the gel involved statistical evaluation whether there was dependence between the amount of drug released per unit mass of the gel (1 g) and the mass of gel taken for analysis over the mass range from 0.01 g to 1 g (±0.0001 g). Fig. 2 shows the functional dependence ($y = a + bx$) of the amount of doxorubicin encapsulated per unit mass of the gel (1 g) (y) on the mass of a sample of

Table 1
Statistical evaluation of the dependence of the amount of doxorubicin encapsulated in gel on the mass of doxorubicin-loaded gel

Mass of doxorubicin-loaded gel (g)		Expected amount of doxorubicin in gel (μg)	Determined amount of doxorubicin in gel (μg)		Confidence limits for the average (R.S.D.%)	Amount of doxorubicin per unit mass of gel (μg g^{-1})	
0.001	0.001	0.2	ND	ND		ND	
0.005	0.005	1	ND	ND		ND	
0.01	0.01	2	1.9	2.0		190	180
0.01	0.01		2.1	2.2	2.1 ± 0.10 (5.8)	210	220
0.01	0.01		2.2	2.1		220	210
0.01	0.01		1.9	2.0		190	200
0.02	0.02	4	3.9	4.0		195	200
0.02	0.02		3.9	4.1	3.9 ± 0.13 (3.9)	195	205
0.02	0.02		4.3	3.8		215	190
0.02	0.02		4.0	3.9		200	195
0.05	0.05	10	11.0	11.0		220	220
0.05	0.05		10.3	9.8	10.4 ± 0.41 (4.7)	206	196
0.05	0.05		9.9	10.8		198	216
0.05	0.05		10.5	10.0		210	200
0.08	0.08	16	15.7	15.4		196	193
0.08	0.08		15.5	16.0	15.9 ± 0.45 (3.4)	194	200
0.08	0.08		16.3	16.3		204	204
0.08	0.08		17.0	15.6		213	195
0.10	0.10	20	21.2	22.0		212	220
0.10	0.10		20.4	21.0	20.7 ± 0.62 (3.6)	204	210
0.10	0.10		21.0	19.9		210	199
0.10	0.10		19.7	20.6		197	206
0.2	0.2	40	42.9	40.5		215	203
0.2	0.2		41.0	43.2	41 ± 1.6 (4.1)	205	216
0.2	0.2		38.9	38.2		195	191
0.2	0.2		42.5	39.0		213	195
0.35	0.35	70	71.1	72.2		203	206
0.35	0.35		74.5	70.5	71 ± 1.8 (2.9)	213	201
0.35	0.35		70.9	68.9		203	197
0.35	0.35		69.0	67.8		197	194
0.5	0.5	100	100.6	99.8		201	200
0.5	0.5		105.2	106.0	102 ± 2.2 (2.6)	210	212
0.5	0.5		99.0	101.1		198	202
0.5	0.5		102.1	104.8		204	210
0.75	0.75	150	152.2	144.6		203	193
0.75	0.75		163.4	151.0	153 ± 5.2 (4.1)	218	201
0.75	0.75		150.0	147.8		200	197
0.75	0.75		155.5	160.0		207	213
1	1	200	201.4	213.2		201	213
1	1		205.3	200.8	205 ± 4.5 (2.6)	205	200
1	1		199.2	205.3		199	205
1	1		199.5	211.4		199	211
						201 ± 5.0; n = 80; S.D. = 22.5; R.S.D. = 11.2%	

ND—not detected; $\bar{x} \pm \Delta\bar{x}$—confidence limits of the average value; $\bar{x}$—the average value; $\Delta\bar{x} = t_{crit} \times S_{n-1}/\sqrt{n}$—the standard error; $S_{n-1}/\bar{x}$—the relative standard deviation (R.S.D.); S_{n-1}—the standard deviation (S.D.); n—the number of measurements; $t_{crit}(\alpha, n-1)$—critical value found in statistics tables; α—significance level.

doxorubicin-loaded gel (x). Ideally, when the drug encapsulation is homogeneous in the bulk of the gel, there should be no relationship between the two variables and the function should have the form $y = a$, where a is the average mass of doxorubicin encapsulated per unit mass of the gel.

2.10. Statistical analysis of reproducibility of the sol–gel method of doxorubicin encapsulation

Statistical analysis of reproducibility of the sol–gel method of doxorubicin encapsulation involved estimating

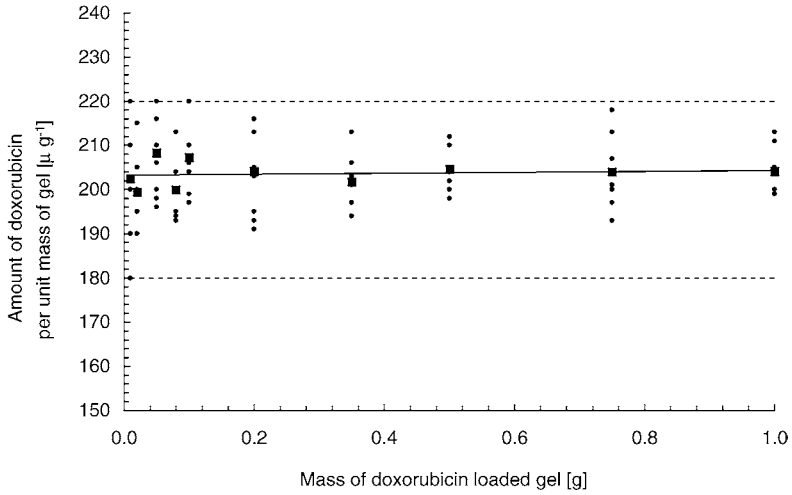

Fig. 2. Dependence of the amount of doxorubicin encapsulated per unit mass of gel (y) on the total mass of doxorubicin-loaded gel used (x). Linear regression equation for the average value: $y = 203.3 + 0.98x$; $r_{calc} = 0.12$ standard deviation of the slope, $S_b = 5.43$; ($t_{calc} = 0.18$); standard deviation of the intercept, $S_a = 2.42$; ($t_{calc} = 0.20$).

the parameters characterizing precision and accuracy of determinations obtained for all series (see Section 2.3) of preparations being compared (R.S.D. and 95% limits of the confidence interval of the average value). The average amounts of doxorubicin encapsulated per unit mass of the gel prepared in different independent time intervals using the sol–gel method and 95% limits of their confidence intervals are shown in Table 2. Hartley's F_{max} test was used to examine the equality of variances in all the measurement series being compared while the Student's t-test was used to compare the average values obtained for each series [25,26].

2.11. The effect of storage time of drug-loaded gels on the characteristics of doxorubicin-loaded gels and stability of doxorubicin

The investigation of the effect of storage time of drug-loaded gels on the characteristics of doxorubicin-loaded gels and the stability of doxorubicin revealed that there was no dependence of the characteristics of doxorubicin-loaded gels on the storage time. Consequently, these results will not be presented in this paper.

Table 2
Statistical evaluation of reproducibility of the sol–gel method of doxorubicin encapsulation carried out at independent time intervals, based on the results of investigations of the dependence of the amount of doxorubicin encapsulated in gel on the mass of doxorubicin-loaded gel

Time of repetition of synthesis of doxorubicin-loaded gels (months)	Average value and confidence interval for the amount of doxorubicin encapsulated per unit mass of gel, calculated for $n = 27$ and $P = 95\%$, ($\mu g \times g^{-1}$), S.D. ($\mu g \times g^{-1}$); R.S.D. (%)
1	201 ± 4.5; S.D. = 9.64; R.S.D. = 4.8
6	200 ± 3.7; S.D. = 7.80; R.S.D. = 3.9
12	199 ± 2.3; S.D. = 8.16; R.S.D. = 4.1
18	201 ± 2.5; S.D. = 6.43; R.S.D. = 3.2
24	202 ± 3.5; S.D. = 8.48; R.S.D. = 4.2

S.D.—standard deviation; R.S.D.—relative standard deviation.

2.12. Determination of UV–vis light influence on the transparency of the pure and doxorubicin sol–gel samples over time

The investigation of the effect of time of UV–vis irradiation of samples of pure gel and doxorubicin-loaded gels on the changes in light transmittance of pure gels and spectral changes of doxorubicin in gels aimed at determining the maximum time of their use as reference materials. The measurements were carried out on 10 samples of pure gel and 10 samples of doxorubicin-loaded gel having masses of 1 g (±0.0001 g) prepared in 10 independent series of preparations. Samples of pure gel and of doxorubicin-loaded gel were exposed to UV–vis radiation in the region from 300 nm to 800 nm using a spectrophotometer lamp with a power 30 W cm^{-2}, for a combined period of time of 360 h, in an enclosed light-box. The distance from the lamps to the samples was maintained at 10 cm. The spectral results expressed as the change in absorbance of doxorubicin measured at $\lambda = 333.4$ nm as a function of time of UV–vis exposure of the gels with encapsulated doxorubicin $A_{333.4} = f(t)$ are shown in Fig. 3.

2.13. Determination of the signal dependence of a UV–vis spectrophotometer on the mass of pellets of doxorubicin-loaded gels

To examine the spectral differences in the UV–vis range depending on the mass of doxorubicin-loaded gel taken for analysis, 10 groups of samples were used to record the UV–vis spectrum in the 300 nm to 800 nm range. Each group

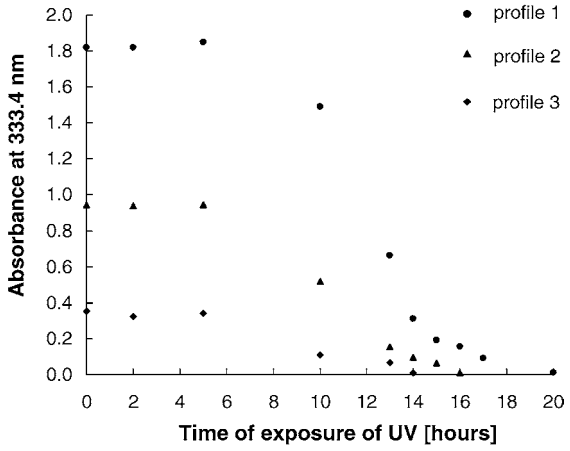

Fig. 3. Photodegradation profile of doxorubicin in gels expressed as a function of change in absorbance value at λ = 333.4 nm (average value determined for $n = 10$) for three selected masses: ($m = 0.5$ g, profile 1); ($m = 0.3$ g, profile 2); ($m = 0.1$ g, profile 3).

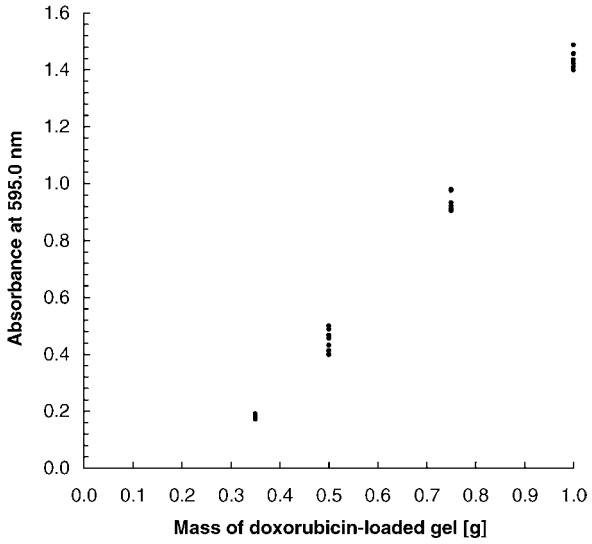

Fig. 4. Profile of linearity of readout of a UV–vis spectrophotometer expressed as the dependence of absorbance at λ = 333.4 nm (a), λ = 352.0 nm (b) and λ = 595.0 nm (c), on the mass of a sample of doxorubicin-loaded gel taken for analysis: $A_\lambda = f(x)$: x—mass of a sample of doxorubicin-loaded gel.

contained 10 different mass of the synthesized doxorubicin-loaded gels (from 0.01 g to 1 g (±0.0001 g)).

The UV–vis absorption spectra of doxorubicin in gel reveal six maxima: two strong: λ = 333.4 nm and 352.0 nm, and four weak: 388.0 nm, 575.0 nm, 595.0 nm and 630.0 nm. For the quantitative determination of doxorubicin in solid samples, three wavelengths were selected: the wavelength λ = 333.4 nm corresponding to the absorbance maximum, 352.0 nm and 595.0 nm. The 3 calibration curves for the 10 groups at these wavelengths are shown in Fig. 4a (λ = 333.4 nm), Fig. 4b (λ = 352.0 nm) and Fig. 4c (λ = 595.0 nm). Statistical reports on the linearity of spectrophotometer readout computed using a least-square method

Table 3
Linearity of spectrophotometer readout: $A_{333.4} = f(x)$: $y = a + bx$; x {mass (g)}:{0.0075; 0.01; 0.025; 0.04; 0.05; 0.1; 0.175; 0.25; 0.375; 0.5}

n	m	b	a	S_b	S_a	r	S_r	n	m	b	a	S_b	S_a	r	S_r
1	10	3.67	−0.003	0.071	0.016	0.99	0.019	6	10	3.78	−0.004	0.029	0.0066	0.99	0.008
2	10	3.91	−0.015	0.126	0.028	0.99	0.035	7	10	3.72	0.004	0.098	0.022	0.99	0.027
3	10	3.79	−0.007	0.078	0.017	0.99	0.021	8	10	3.74	−0.015	0.192	0.043	0.99	0.053
4	10	3.77	0.003	0.053	0.012	0.99	0.015	9	10	3.79	−0.007	0.093	0.021	0.99	0.026
5	10	3.79	−0.007	0.078	0.017	0.99	0.022	10	10	3.89	−0.015	0.126	0.028	0.99	0.035

n—number of series; m—number of samples in a series.

Table 4
Linearity of spectrophotometer readout: $A_{352.0} = f(x)$: $y = a + bx$; x {mass (g)}:{0.01; 0.02; 0.05; 0.08; 0.09; 0.1; 0.2; 0.35; 0.55; 0.8}

n	m	b	a	S_b	S_a	r	S_r	N	m	b	a	S_b	S_a	r	S_r
1	10	2.21	−0.108	0.131	0.044	0.99	0.056	6	10	2.15	−0.100	0.138	0.047	0.99	0.059
2	10	2.21	−0.118	0.051	0.151	0.99	0.064	7	10	2.21	−0.105	0.133	0.045	0.99	0.057
3	10	2.16	−0.105	0.133	0.045	0.99	0.057	8	10	2.22	−0.112	0.134	0.045	0.99	0.057
4	10	2.24	−0.111	0.136	0.046	0.99	0.058	9	10	2.15	−0.100	0.136	0.046	0.99	0.058
5	10	2.19	−0.105	0.133	0.045	0.99	0.057	10	10	2.22	−0.112	0.134	0.045	0.99	0.057

n—number of series; m—number of samples in a series.

Table 5
Linearity of spectrophotometer readout: $A_{595.0} = f(x)$: $y = a + bx$; x {mass (g)}:{0.01; 0.02; 0.05; 0.08; 0.1; 0.2; 0.35; 0.5; 0.75; 1}

n	m	b	a	S_b	S_a	r	S_r	N	m	b	a	S_b	S_a	r	S_r
1	10	1.46	−0.139	0.233	0.104	0.97	0.129	6	10	1.41	−0.134	0.224	0.099	0.97	0.124
2	10	1.45	−0.134	0.213	0.095	0.98	0.117	7	10	1.41	−0.135	0.226	0.101	0.97	0.125
3	10	1.38	−0.134	0.230	0.103	0.97	0.127	8	10	1.47	−0.136	0.218	0.097	0.98	0.120
4	10	1.43	−0.137	0.232	0.103	0.97	0.128	9	10	1.39	−0.134	0.228	0.102	0.97	0.126
5	10	1.41	−0.137	0.236	0.105	0.97	0.131	10	10	1.41	−0.132	0.215	0.096	0.97	0.119

n—number of series; m—number of samples in a series.

for the function: $A_\lambda = f$ (mass of gel sample) for the measurement series at a given wavelength are listed in Tables 3–5.

3. Discussion

High purity of analyte-free synthesized gel matrices is a primary criterion which has to be met if these matrices are next to be used as a source of the analyte for spectrophotometric analysis. Silica monoliths synthesized in our laboratory and dried under low-temperature supercritical with CO_2 conditions have a good transparency above 320 nm, but they are only partially transparent in the range from 300 nm to 320 nm (Fig. 1). Therefore, spectral analysis of gel matrices with encapsulated doxorubicin is possible only in the UV–vis region above 320 nm.

Consequently, the next stage in the investigation was the determination of the characteristics of doxorubicin-loaded silica gels prepared by the sol–gel method by statistical evaluation of accuracy and repeatability of the sol–gel method of analyte encapsulation as well as its reproducibility. On the basis of statistical analysis of the results, the efficiency of encapsulation of doxorubicin in gels and the degree of homogeneity of doxorubicin encapsulation during sol gelation were estimated.

The loading efficiency of doxorubicin encapsulation per 1 g of gel prepared by the sol–gel method with an acid catalyst was $100 \pm 1.2\%$ (w/w). It can thus be concluded that the acid-catalyzed sol–gel pre-doping method enables a complete encapsulation of doxorubicin in the gel without any measurable losses, and the measurement uncertainty expressed as the relative standard deviation R.S.D. = 11.2% (Table 1) is related to typical random and systematic errors. The uncertainty of the final result is a sum of many composites, including the error in mass of doxorubicin (confidence interval 99% reported by the CRM), random errors associated with the analytical procedure used (preparation of standard solutions of doxorubicin, dilution), purity of the reagents used, and the errors in weighing samples on an analytical balance as well as the instrumental errors (± 0.0001 g, 95%) and limited resolution of the device being calibrated ($A \pm 0.005$ AU).

The experimentally determined average amount of doxorubicin encapsulated per unit mass of the gel was $201 \pm 5.0\ \mu g\ g^{-1}$ (Table 1). The value of relative standard deviation (11.2%) indicating repeatability of the results obtained demonstrates indirectly a degree of homogeneity of encapsulation of the analyte in the gel matrix. The experimental results indicated that the amount of doxorubicin encapsulated per unit mass of the gel (1 g) did not depend on the total mass used. Statistical analysis of the relationship between the amount of doxorubicin encapsulated per 1 g of the gel and the total mass of the gel used in the studies based on the Student's *t*-test revealed that the slope of the straight line describing the above relationship (Fig. 2) was not statistically significantly different from zero ($t_{cri} > t_{calc}$) for the actual probabilities (95%). Also, the intercept (Fig. 2) was found to be not statistically different from the experimentally determined average amount of doxorubicin encapsulated per 1 g of the gel matrix ($t_{crit} > t_{calc}$; 95%). In addition, the calculated value of the correlation coefficient (Fig. 2) was compared with the critical value r_{crit}. Since ($r_{crit} > r_{cal}$), the hypothesis that the two variables (the amount of doxorubicin per 1 g of the gel and the mass of gel) are interdependent has to be rejected. Consequently, due to the uniform distribution of doxorubicin throughout the gel matrix, a UV–vis spectrophotometer can be quantitative calibrated over a wide measurement range by using various masses of the doxorubicin-loaded gels.

Statistical analysis of the method's reproducibility based on the results shown in Table 2 revealed that the values of relative standard deviation of the mean R.S.D.% for each of the 5 series of measurements for doxorubicin-loaded gels prepared after: 1, 6, 12, 18, and 24 months varied from 3.2% to 4.8% (at $P = 95\%$). The application of Hartley's test demonstrated that there was no reason for rejection of the null hypothesis of homogeneity of variance in all the series being investigated ($F_{max(calc)} < F_{max(crit)}$ at the confidence level 95%). In practice, this means that the series of gels prepared at different time intervals do not differ with respect to precision.

A comparison of each measurement series with respect to accuracy using Student's *t*-test for one expected value revealed no grounds for rejection of the null hypothesis that the average values obtained were the same as the expected value ($t_{calc} < t_{crit}$). Such a conclusion was reached for each series of measurements. On the basis of statistical analysis, it can thus be concluded that the sol–gel method of doxorubicin encapsulation allows obtaining reliable calibration results over extended periods of time.

The next criterion of the application of silica gels as matrices for the analyte in spectrophotometric analysis was examination of the effect of storage time of pure and doxorubicin-loaded gels and long-term irradiation with UV–vis radiation on the characteristics of doxorubicin-loaded gels and the stability of doxorubicin and pure gel matrices. A 12-month storage of gels in a desiccator at room temperature was found to have no effect on the characteristics of the gels. It can thus be concluded that the storage conditions of gels (desiccator, room temperature) are sufficient and require no further attention, which is another advantage of the sol–gel method. No changes in light transparency of pure gels were observed after 360-h irradiation of pure gels with UV–vis light, which demonstrates that UV–vis radiation does not destroy bonds of the gel polymer; hence, the same gel can be used many times as a blank (reference).

In contrast, in case of doxorubicin-loaded gels, photodegradation of the analyte begins after 6–8 h of continuous irradiation with the UV–vis lamp (Fig. 3), which limits their multiple uses. However, our previous investigations on degradation of free doxorubicin in solution [17] indicate that the analyte undergoes irreversible destabilization at room temperature and in the presence of light already after 4 h. Furthermore, it was found that a gradual irreversible photodegradation of doxorubicin in solution began to take place after as

little as 1 h of exposure to UV–vis light. Thus, encapsulation of doxorubicin in a solid gel matrix extends the time of its use and allows multiple use of a batch of samples for spectrophotometric analysis.

The final step included the determination of readout of a UV–vis spectrophotometer as a function of mass of pellets of doxorubicin-loaded gels (Fig. 4). It was established that all spectra of doxorubicin in solid materials with masses from 0.01 g to 1 g revealed the same qualitative information.

The calibration curves $A_\lambda = f$ (mass of gel sample) (Fig. 4; Tables 3–5) obtained using a least-square procedure demonstrate the linear dynamic range for masses from 0.01 g to 0.5 g (correlation coefficient $r = 0.99$) at $\lambda = 333.4$ nm, from 0.1 g to 0.8 g, $r = 0.99$, at $\lambda = 352$ nm and from 0.2 g to 1 g, $r = 0.97$, at $\lambda = 595$ nm. This means that at the above wavelengths the Lambert–Beer law is followed for the range of gel masses indicated and a single-point calibration is feasible. A visual inspection of the calibration curves obtained at the wavelengths $\lambda = 388$ nm, 575 nm and 630 nm revealed small deviations from linearity in the mass range from 0.01 g to 1 g. Similar deviations from the Lambert–Beer law at these wavelengths were also observed for solutions of doxorubicin.

On the basis of results obtained for doxorubicin-loaded gels, it can thus be concluded that the gels with masses from 0.01 g to 1 g can be used for the calibration of spectrophotometers based solely on the mass of the gel taken for analysis.

4. Conclusion

The paper presents a new approach to the generation of a standard doxorubicin in the form of a monolithic, crack-free material prepared by the acid-catalyzed sol–gel method and the possibility of its use in the future for the calibration of a UV–vis spectrophotometer.

Considering the fact that this new approach to preparation of analytical standards offers a number of advantages, including good repeatability and reproducibility, high accuracy, long-term sample stability, and wide range of analyte concentrations available as compared to the applicability range of an analytical method, we claim that the sol–gel method described here can be a reliable way of generation of solid standards, which can be applied to both a single-point calibration and the calibration of an analytical instrument over a wide measuring range.

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