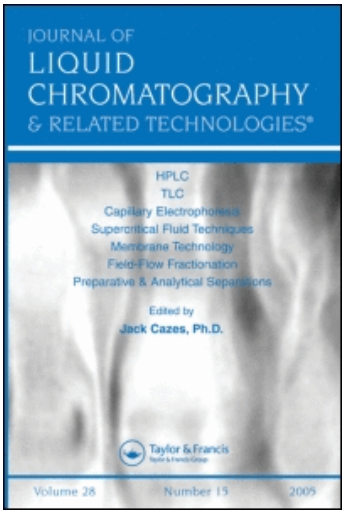

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DETERMINATION OF PROGESTERONE IN NANOCAPSULES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

In order to determine progesterone concentration in nanocapsules consisting of benzyl benzoate in a poly-ε-caprolactone shell, various methods were tested for preparation of samples. The opening of nanocapsules by a dissolution-dilution in acetonitrile was chosen. The method was validated. A reversed phase HPLC method using an acetonitrile-water mixture (75:25) as mobile phase, demonstrated that the entrapping of progesterone was almost total in the nanocapsules, and that, only traces of progesterone were present in the aqueous phase of the suspension. The lack of adsorption on the polymeric wall was also demonstrated.

INTRODUCTION

Colloidal drug carriers, nanoparticles, are used as drug delivery systems[1] and can enhance efficacy of drugs and reduce their toxicity.[2]

These new drug carriers can modify the distribution of drugs and play a role in the pharmacokinetics : adsorption, metabolism and elimination of drugs.[3,4]

Progesterone, a steroid sex hormone, can be used in many pathologies.[5,6] By oral route, its hormonal activity is low.[7] Indeed, the bioavailability of progesterone is reduced by an intense intestinal and liver metabolism. So, it would be interesting to use a carrier system for progesterone administration. In order to study potential modification induced in progesterone metabolism, after its administration to rats under nanocapsules form, it was necessary to control the quality of these nanoparticle suspensions. To our knowledge, no method had been described for the determination of progesterone in nanoparticles.

For analytical purpose, the separation of nanocapsules of various xenobiotics from the aqueous supernatant was generally performed by ultracentrifugation,[8,9] and seldom by ultrafiltration centrifugation.[10] On another hand, the determination of progesterone in biological media was performed either by gas chromatography,[11] or both normal[12] and reversed phase[13,14] high performance liquid chromatography (HPLC). In the latter case, the mobile phase was methanol/water, acetonitrile/water or methanol/acetonitrile/water.

In this paper, we describe a reversed phase HPLC method for the determination of progesterone in a nanocapsules suspension.

MATERIALS

Chemicals and Reagents

Progesterone was purchased from Upjohn, Fine Chemical Division (Michigan, USA). Acetonitrile, methylene chloride, ethyl acetate, and methanol were of liquid chromatographic grade and purchased from Prolabo (Paris, France). Distilled water, used for the mobile phase, was obtained from Fresenius (Malakoff, France).

Nanocapsules Suspension

Nanoprecipitation was the method chosen to prepare the nanoparticles.[15] Progesterone was dissolved in an oily solvent, benzyl benzoate. Poly-ε-caprolactone polymer was dissolved in acetone and added to the progesterone

solution to form solution A. Solution B was an hydroalcoholic solution containing surfactant (Synperonic®). After mixing solutions A and B, nanocapsules were formed. Evaporation of acetone, alcohol, and of a part of water, allowed us to obtain a concentration of 5 $mg.mL^{-1}$, which was in agreement with the requirements of administration to rats.

Apparatus

The HPLC system consisted of an isocratic solvent delivery pump (110A Beckman pump, San Ramon, CA, USA), a sample injector (Rheodyne Model 7125, Latek, Eppelheim, Germany) equipped with a 20 µL loop, a reversed phase column (Macherey Nagel C18 Nucleosil, 250 mm x 4 mm I.D., 5 µm particle size), and a variable-wavelength UV detector (Beckman Model 166). The data recording system consisted of an IBM personal computer PS/2 Model 8550.Z with Gold software system (version 5.1, Beckman). Ultracentrifugation was performed with a Beckman L8-55 Ultracentrifuge (Beckman Instruments), equipped with a 40TR rotor.

METHODS

Chromatography Conditions

Liquid chromatography was performed at room temperature. The mobile phase was an acetonitrile-water mixture (75 : 25 V/V) programmed to be delivered at a flow-rate of 1.0 $mL.min^{-1}$. The wavelength used for detection was 254 nm.

Standard Curves

A working solution of progesterone (0.055 $mg{\cdot}mL^{-1}$) was prepared in acetonitrile. This solution was diluted with acetonitrile to give the following final concentrations : 0.0055, 0.011, 0.022, 0.033, 0.044, 0.055 $mg{\cdot}mL^{-1}$ for the construction of standard curves. Stability of progesterone solutions in acetonitrile at various concentrations (0.0055 to 0.055 $mg.mL^{-1}$) was studied. These solutions were stored at 4°C and analysed periodically. Progesterone was eluted as a single peak, with a constant peak area, over a period of two weeks under these conditions, and no other peak of degradation was seen at λ = 210, 230 or 254 nm.

Preparations of Samples

Progesterone in the aqueous phase

Five mL of the nanocapsules suspension were centrifuged at 50,000 g for 45 min; it gave a clear supernatant liquid which was directly injected into the HPLC system. Concentrations were very low, and no dilution was necessary.

Progesterone in the nanocapsules suspension

The suspension of nanocapsules was treated according to either method A, B or C. For checking adsorption on the polymer surface, method D was used.

Method A

It consisted of an opening of nanocapsules by a dissolution-dilution in acetonitrile. 0.5 mL of nanocapsules suspension, previously kept under magnetic agitation, were diluted 1:200 with acetonitrile. A solution was obtained with formation of a residue of poly-ε-caprolactone insoluble in acetonitrile. This solution was filtered through a microfilter before being injected into the HPLC system.

Method B

Methylene chloride was used as solvent for nanocapsules opening. 5 mL of nanocapsules suspension were evaporated, and diluted with 150 mL of methylene chloride. The solution was dried using anhydrous sodium sulfate and then evaporated under reduced pressure. The residue was dissolved in 50 mL of methanol. This solution was diluted 1:20 with methanol and was filtered through a microfilter before being injected into the HPLC system.

Method C

Nanocapsules suspension was treated according to method B, using ethyl acetate instead of methylene chloride as solvent.

Method D

A progesterone solution in acetonitrile at a concentration of 5 $mg.mL^{-1}$ was diluted 1:200 with acetonitrile before being injected into the HPLC system. A progesterone and poly-ε-caprolactone solution in acetonitrile at concentrations of 5 and 12.5 $mg.mL^{-1}$ respectively, was diluted 1:200 with acetonitrile, filtered and injected into the HPLC system.

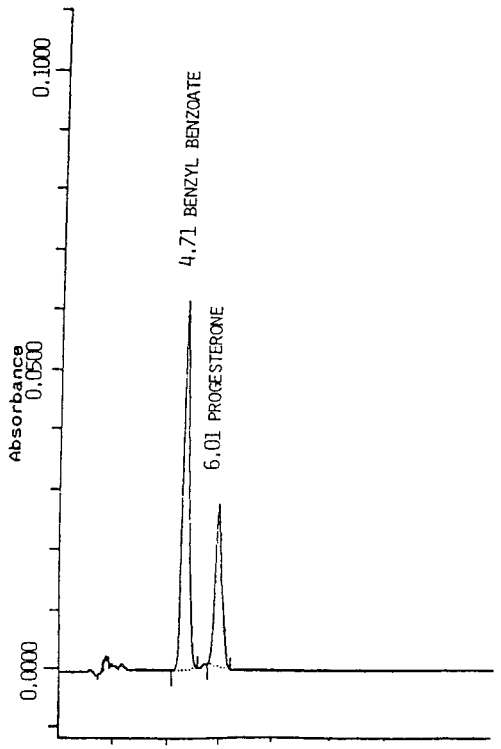

Figure 1. Chromatogram of nanocapsules suspension.

RESULTS AND DISCUSSION

Chromatograms

Typical chromatogram of progesterone and benzyl benzoate is reproduced in Figure 1.

Retention times for these compounds were 6 min and 4.7 min respectively; symmetrical peaks were obtained. Under these conditions, the synperonic® (component of nanocapsules) does not adsorb at 254 nm.

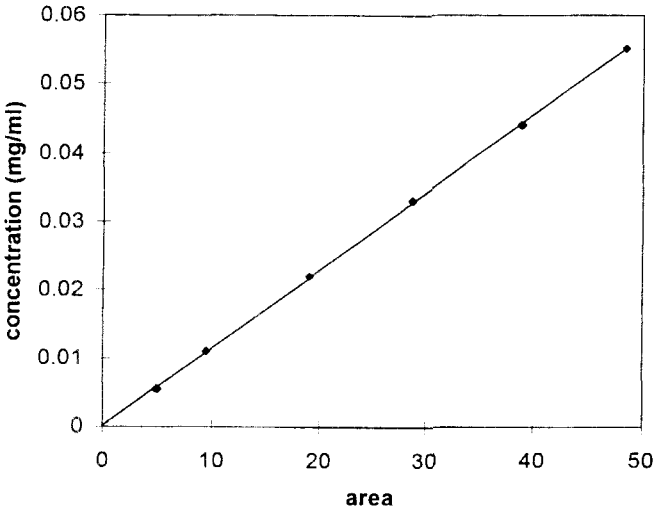

Figure 2. Linearity curve.

Validation

Linearity

Linearity was observed for concentrations of 0.0055 to 0.055 mg.mL^{-1}. The straight-line equation was Y = 0.001132x + 0.000245 (Figure 2). The correlation coefficient, r, was 0.9998. The validation results were established for two injections per concentration and for six concentrations.

Reproductibility

This study was performed on lots of nanocapsules suspension at a concentration of 5mg.mL^{-1}, and using method A for determination of progesterone in nanocapsules suspension. For this purpose, the intra-assay and inter-assay (RSD) were established for six injections per determination on six lots (I,II,III,IV,V,VI), and the values are listed in Table 1.

The limit of detection was defined as the minimum drug concentration corresponding to twice the signal-to-noise ratio. It was evaluated as 0.5 µg·mL^{-1} in 0.5mL of nanocapsules suspension.

Table 1

Validation of the Method

Lots	Found Conc. ($mg \cdot mL^{-1}$)	Accuracy (%)	Intra-assay Variability RSD(%)	Inter-assay Variability RSD(%)
I	4.87	97.4	1.00	0.99
II	4.82	96.3	0.76	0.83
III	4.86	97.1	0.75	1.10
IV	4.92	98.4	0.26	1.00
V	4.87	97.3	1.03	0.72
VI	4.88	97.5	0.34	0.98

Table 2

Results of the Three Extraction Methods for Nanocapsules Suspension ($5mg \cdot mL^{-1}$)

	Concentration ($mg \cdot mL^{-1}$)	Precision (%)	Concentration (%)
Nanocapsules suspensions Method A (CH_3CN)	4.89	0.79	98
Nanocapsules suspensions Method B (CH_2Cl_2)	5.05	3.70	101
Nanocapsules suspensions Method C (AcOEt)	5.05	3.60	101

Comparison of Extraction Methods

In order to check the consistency of the values of total progesterone, measured, three parts of the same lot at the concentration of 5 $mg.mL^{-1}$ were treated separately according to method A, B and C. The values are compared in Table 2.

Table 3

Results of Encapsulation Checking

	Concentration ($mg \cdot mL^{-1}$)	Precision (%)	Concentration (%)
Aquesous phase free progesterone	0.0026	1.10	0.05
Nanocapsules suspension Method A(CH_3CN)	4.89	0.79	98

These results showed that the behaviour of these different solvents towards progesterone nanocapsules was identical. Acetonitrile, methylene chloride, ethyl acetate, identically extract the progesterone present in the nanocapsules suspension.

The precision obtained for the three methods showed that method A was more accurate. Moreover, this method was a simple dissolution-dilution in acetonitrile, so it was more reliable than the two other methods which were more complicated. Measures performed in the presence of an internal standard, diazepam, and without internal standard, gave identical results.

Checking the Quality of the Encapsulation

Progesterone determinations were performed simultaneously in the aqueous phase and in the whole nanocapsules suspension, on aliquots of the same lot of nanocapsules suspension at the concentration of 5 $mg \cdot mL^{-1}$. The experimental results listed in Table 3 are given both in $mg \cdot mL^{-1}$, and in percentage of the total amount of progesterone.

These results showed that progesterone was almost totally encapsulated, since the concentration of free progesterone (i.e. progesterone dissolved in the aqueous phase) was very low: 0.0026 $mg \cdot mL^{-1}$ (0.05 % of progesterone theoreticaly present). This result was expected because progesterone is a lipophilic drug which is not soluble in water (s = 12 $\mu g.mL^{-1}$ at 37°C).[16]

A question remained. Was progesterone entirely dissolved in the oily solution entrapped in the nanocapsules, or was it partly adsorbed on the polymer surface? In order to solve this question, experiments were performed to clear up the ways of trapping progesterone in nanocapsules.

Lack of Adsorption of Progesterone on Poly-ε-caprolactone

The results obtained on solutions of progesterone in acetonitrile and of progesterone and poly-ε-caprolactone in acetonitrile, prepared according to method D, showed that the same values of progesterone concentration were found for the two solutions. This observation confirms that the same results were obtained when the extraction from nanocapsules suspension was performed, either by methylene chloride (method B) or by ethyl acetate (method C). So this lipophilic drug does not adsorb on the polymer. This leads us to conclude that progesterone is entrapped by simple inclusion inside the nanocapsules.

This lipophilic drug has a different behaviour than a hydrophilic compound studied in our laboratory, such as phenobarbitone,[17] which aside from being present in the aqueous phase of the suspension, was both entrapped in the oily solution and adsorbed on the nanocapsules wall. In that case, the results were different using method B or method C.

CONCLUSION

The HPLC method presented here, allows the evaluation of the total progesterone included in the nanocapsules suspension. This lipophilic drug is almost entirely entrapped in the nanocapsules without adsorption on the polymer constituting the nanocapsule wall. The method was validated and it can be used for the evaluation of the quality of the preparation of nanoparticule suspension.

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