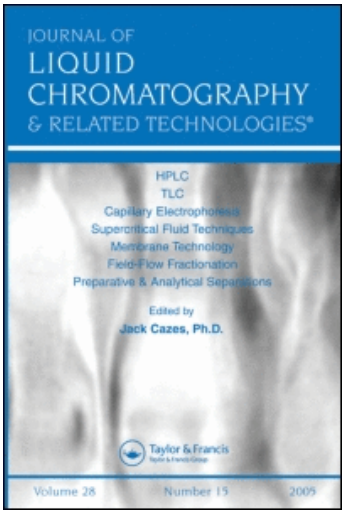

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HPLC METHOD DEVELOPMENT AND VALIDATION FOR FORMALDEHYDE IN ENTERIC COATING OF HARD GELATIN CAPSULES

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ABSTRACT

A reversed-phase high-performance liquid chromatography (HPLC) method for determination of formaldehyde in enteric coating of hard gelatin capsules is described and fully validated. This method entails the separation of formaldehyde as its 2,4-dinitrophenylhydrazone derivative using isocratic solvent eluition and its quantification with appropriate internal standard and ultraviolet detection. The results for selectivity, linearity, precision, accuracy and recovery were in agreement with validation parameters.

INTRODUCTION

The method to prepare hard gelatin capsules, which are resistant to gastric juice by crosslinking with formaldehyde and their stabilisation, succintly includes, for determined time, different stages such as: immersion of capsules in hidroalcoholic solutions of formaldehyde, first drying followed by "washing" and second drying. So the quantification of this aldehyde is very important besides other requirements (1).

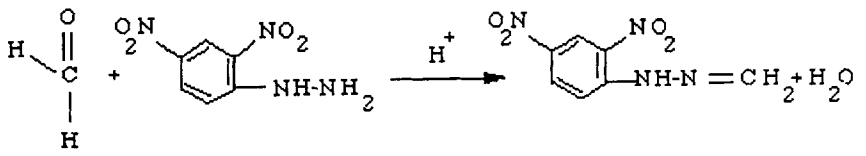

FIGURE 1. Chemical reaction of formaldehyde with 2,4-dinitrophenylhydrazine.

For the analysis of formaldehyde, colorimetric determination has generally been used (2, 3, 4, 5). However the colorimetric assay is not necessarilly reliable because the samples are sometimes coloured or the excipients (e.g. lactose) can give different reactions with formation of colour (6, 7).

So, we attempted to develop a precise high-pressure liquid chromatography (HPLC) method based on the formation of the derivative formaldehyde 2,4-dinitrophenylhydrazone by reaction of the formaldehyde its carbonyl group with 2,4-dinitrophenylhydrazine under acidic conditions (Figure 1).

After optimization and validation, the method was used to determine the amount of spent and extracted formaldehyde by analysing respectively "coating" and "washing" solutions applied to prepare the gastro-intestinal tract resistant gelatin capsules. It is also possible to assay the residual formalin content immediately after preparation and during storage time of gelatin capsules. As formaldehyde is the crosslinking agent of gelatin, this methodology is very important for a rigorous and reproducible control of the process.

MATERIALS AND METHODS

Chemicals

Water (HPLC degree, Milli-Q). Methanol (Lichrosorb, Merck). Formaldehyde (36,6 % w/w), hydrochloric acid, n-hexane, methylene chloride and 2,4-dinitrophenylhydrazine, all proanalyse (Merck).

2,4-Dinitrophenylhydrazine reagent

Dissolve powdered 2,4-dinitrophenylhydrazine (0,025g) in 6NHCl and bring the volume to 10 ml. This solution should be freshly prepared on the day of use.

Instrumentation

The HPLC apparatus consisted of a Hewlett Packard model 1050, a injector with a 20μl loop, a variable wavelenght UV/Vis detector set a 350 nm with a sensitivity range of 0,016 AUFS and an Hewlett Packard model 3396A. Recorder/integrator using chart speed at 0,5 cm min^{-1}.

The melting points were determined on a Buchi model 512 apparatus.

The proton nuclear magnetic resonance (^{1}HNMR) spectra were recorded on a Varian XL-Spectrometer.

Chromatographic conditions

A reversed-phase column Rp-18 Lichrosorb (200x4,6 mm) 5 μm was used at ambient temperature. The mobile phase was methanol: water (70:30) filtered using 0,20 μm membrane filters (Schleicher & Schuell) and degassed prior to use.

Injection volume was 10 μl and flow rate 1,4 ml/min.

2,4-Dinitrophenylhydrazone standards

Formaldehyde 2,4-dinitrophenylhydrazone and acetone 2,4-dinitrophenylhydrazone (internal standard) were prepared by standard procedure described by Shriner et al.(8) and used by Papa and Turner (9). They were purified by different recristallizations from ethanol to a constant melting point. The purities were checked by this method (HPLC) and methanolic solutions showed 100 by area

per cent. The standards were also characterized by proton nuclear resonance magnetic (^{1}HMNR) (10-12).

Formaldehydde 2,4-dinitrophenylhydrazone:

MP = 165 - 166 °C (ethanol/water) (13)
^{1}H-NMR - (p p m, δ) - 6,739 (d; J 10,8 Hz, 1H, -N = C<u>H</u>) 7,149 (d, J 10,8 Hz, 1H, -N = C<u>H</u>); 7,981 (d, J 10,5 Hz, 1H; 6 -$H_{arom.}$); 8,362 (q; J_{ortho} 10,5 Hz; J_{meta} 2,4 Hz, 1H; 5 -$H_{arom.}$); 9,151 (d, J 2,4 Hz, 1H, 3 -H_{arom}); 11,025 (s, 1H, -N<u>H</u> -)

Acetone 2,4-dinitrophenylhydrazone:

MP = 128 - 129 °C (ethanol/water) (13)
^{1}H-NMR - (p p m, δ) - 2,089 (s, 3H, C<u>H</u>$_3$); 2,183 (s, 3H, C<u>H</u>$_3$); 7,960 (d, J 6,5 Hz, 1H, 6 $H_{arom.}$); 8,295 (q; J_{ortho} 6,5 Hz; J_{meta} 2,6 Hz, 1H; 5-$H_{arom.}$); 9,190 (d, J 2,6 Hz, 1H; 3- $H_{arom.}$); 11,026 (s, 1H, -N<u>H</u> -)

Stock solutions of these standards (100 μg ml^{-1}) were prepared by their dissolution in a mixture n-hexane: methylene chloride (70:30 v/v) and preserved from light.

Calibration curve

Six standard solutions of formaldehyde 2,4-dinitrophenylhydrazone (2,5-10 μg ml^{-1}) corresponding to formaldehyde (0,357-1,428 μg ml^{-1}) containing 20 μg ml^{-1} of acetone 2,4-dinitrophenylhydrazone (internal standard) were prepared according to Table 1. A 10 μl volume was then injected in the chromatograph and the calibration curve was calculated by linear regression of the peak-area ratios of formaldehyde 2,4-dinitrophenylhydrazone to internal standard versus concentrations. Unknown formaldehyde concentrations were determined from the regression equation.

TABLE 1
Standard and Sample Preparation

Standard	Sample
- 2 ml of distilled water	-1 ml of distilled water
-different volumes of formaldehyde 2,4-dinitrophenylhydrazone solution	-0,1 ml of 2,4-dinitrophenylhydrazine reagent
-1 ml of internal standard (100 μg ml $^{-1}$)	-1 ml of assay solution
-5 ml of organic mixture	-wait 5 min
	-1ml of internal standard (100 μg ml-1)
	-5 ml of organic mixture
. Stirring for 20 min. . Removing of aqueous phase . Washing of organic phase . Separation of organic extract . Drying of organic phase . Recovery of residue . Filtration . Injection	

Sample preparations

Samples were prepared in parallel to the standards according to Table 1. The solutions to analyse were derived from solutions used in enteric coating of hard gelatin capsules and obtained by three manners: [1] Before and after immersion (with stirring) of hard gelatin capsules in a clean vial well stoppered, 1 ml of coating solution was withdrawn and used for determine spent formaldehyde. [2] Twelve formalin treated capsules were placed in a clean vial containing 100 g of

hidroalcoholic solution. After closing with rubber stoppers the vial was stirred on a mechanical agitation apparatus, 1 ml was withdrawn and used for determination of extracted formaldehyde. [3] Two formalin treated capsules were placed in a clean vial containing 20 ml of distilled water. After closing the vial was stirred on a mechanical agitation apparatus for two hours. Subsquently 1 ml was used to analyse residual formalin content.

RESULTS AND DISCUSSION

Optimization of the derivatization reaction

To each of six Erlenmeyer flasks, 1 ml of water, 0,1 ml of the 2,4-dinitrophenylhydrazine reagent and 1 ml of formaldehyde solution (5 μg) was added. After five minutes, 5 ml of the mixture n-hexane: methylene chloride (70:30 v/v) and 1 ml of acetone 2,4-dinitrophenylhydrazone (internal standard) were added. The mixtures were stirred by mechanical agitation for 5, 10, 15, 20, 25 and 30 minutes. After these intervals, aqueous phase was recoverd and organic extract washed with deionized water to remove the acid and most of the unreacted 2,4-dinitrophenylhydrazine reagent.

The results from Figure 2 indicate that formaldehyde was quantitatively converted to its 2,4-dinitrophenylhydrazone in 20 minutes, which is in accordance with Selim (14) in a analogous study with propionaldehyde.

Selectivity

In Figure 3A and B typical chromatograms can be seen from blank solution containing 20 μg ml^{-1} of acetone 2,4-dinitrophenylhydrazone (internal standard) and from standard solution containing 5 μg ml^{-1} of formaldehyde 2,4-dinitrophenylhydrazone and 20 μg ml^{-1} of internal standard respectively. In Figure 3C a typical chromatogram from sample can be seen.

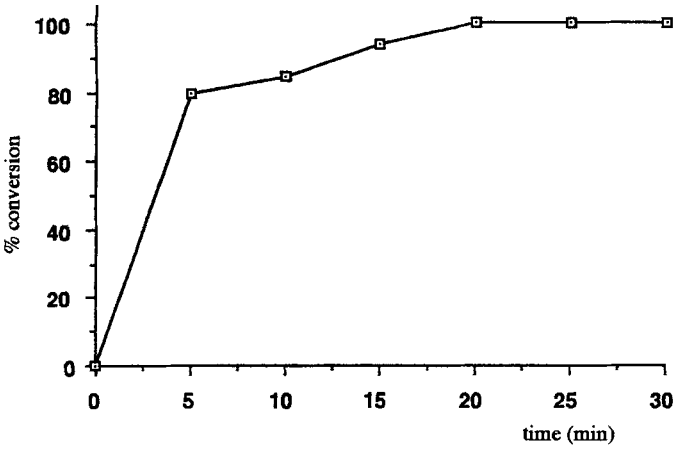

FIGURE 2. Rate of conversion of formaldehyde to its 2,4-dinitrophenylhydrazone.

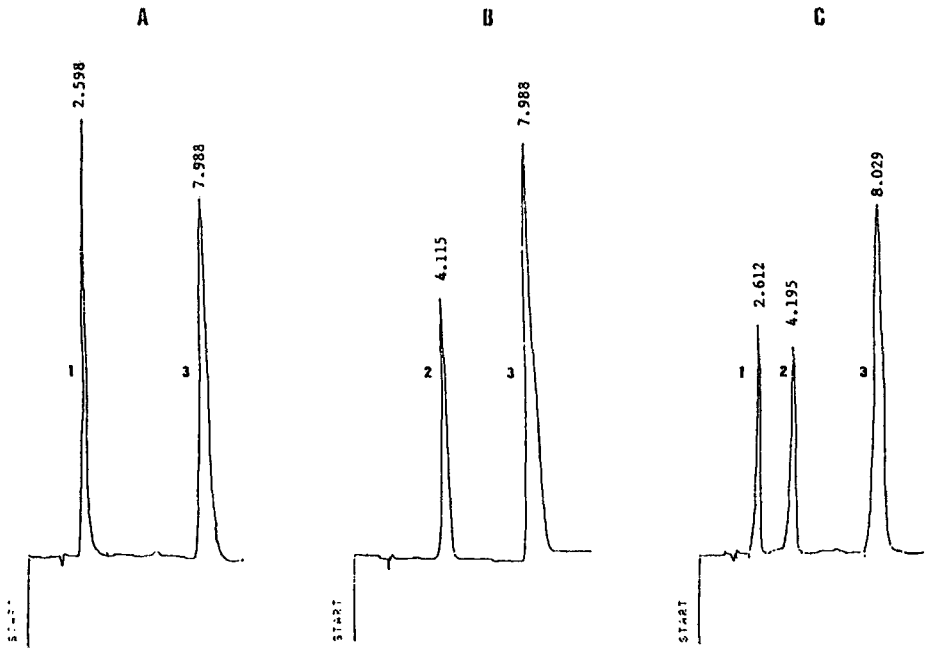

FIGURE 3. Chromatograms: (A) blank solution containing 20 μg ml^{-1} of internal standard; (B) standard solution containing 5 μg ml^{-1} of formaldehyde 2,4-dinitrophenylhydrazone and 20 μg ml^{-1} of internal standard; (C) sample solution.

(1) 2,4-dinitrophenylhydrazine reagent
(2) formaldehyde 2,4-dinitrophenylhydrazone
(3) internal standard

No interference of formaldehyde 2,4-dinitrophenylhydrazone with internal standard or with 2,4-dinitrotrophenylhydrazine reagent were observed.

The retention times of 2,4-dinitrophenylhydrazine reagent, formaldehyde 2,4-dinitrophenylhydrazone and internal standard were 2,6, 4,1 and 8,0 minutes, respectively. It can be concluded that the proposed method is selective for formaldehyde.

Linearity

The regression equation obtained was : A = 0,532 C - 0, 0094325 where A-peak-area ratios and C-formaldehyde concentrations $\mu g\ ml^{-1}$. The coefficient of variation of calibration curve of 2,45 % and the correlation coefficient of 0.999511 (N = 6), proved excellent linearity between peak-area ratios and concentration.

Precision

Run variation within day (repeatability) and run by run (reproducibility) on three different days were calculated for known formaldehyde contents. Coefficient of variation for the repetability test was 1,79 (N=5). For the reproducibility the coefficient of variation was 1,88% (N=9). The low results proved that this analytical method had acceptable precision for formaldehyde quantification.

Accuracy

For determination of the accurancy we calculated the closeness of agreement between the value accepted as the conventional true value and the value found applying the Student' s test (N=8) for a 0,05 probability. The results were t theoretical = 2,37 and t experimental = 2,39. So we can affirm the exactness of the analytical method.

Recovery

The recovery of known formaldehyde concentration added to the blank subjected to sample treatment was analysed. The results varied between 96% and 100,6% which indicates good effectiveness.

Detection Limits

Detection limits are of the order of a few nanograms (15).

CONCLUSIONS

It was demonstrated that the procedure developed is simple, sensitive precise and accurate, making it important for assay of formaldehyde during coating of hard gelatin capsules (16-23).

The selectivity of this procedure make it likely that this assay can also be utilised to analyse formaldehyde in a wide variety of other pharmaceutical samples .

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