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# Aminoglutethimide included in nanocapsules suspension: comparison of GC–MS and HPLC methods for control

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**Abstract**

Gas chromatography–mass spectrometry (GC–MS) and high performance liquid chromatography (HPLC) offer highly efficient and potentially sensitive separation and detection techniques. This work describes the quantification of aminoglutethimide (AG) in nanocapsules suspension with both techniques. The analysis of different lots containing known concentrations of drug (1, 2, 3 and 4 mg $ml^{-1}$) were used to investigate the quantitative capabilities of both chromatographic techniques. Both chromatographic methods were successful and on an analytical point of view the validations of aminoglutethimide dosing were suitable in both cases. In routine, the determination of the quality of nanocapsules suspension could be preferentially evaluated by difference between total AG concentration in suspension (evaluated by direct HPLC measure of the suspension diluted in acetonitrile) and free AG concentration (evaluated by direct HPLC measure of simple dilution of the supernatant).


*Keywords:* Nanocapsules; Aminoglutethimide; Adsorption; GC–MS; HPLC

## 1. Introduction

Nanocapsules made from biodegradable polymers as poly-ε-caprolactone have been tested as drug delivery systems [1]. These colloidal drug carriers can enhance the efficacy, modify the tissue distribution and reduce the toxicity of drugs [2]. They can also play a role in the pharmacokinetics, absorption, elimination and metabolism of drugs [3,4].

Because of their polymeric nature, these small biodegradable particles (diameter 50–300 nm) are able to encapsulate a wide variety of drugs in a stable and reproducible way [5], and can be used in order to make hydrophilic compounds pass through gastrointestinal barriers [6].

Aminoglutethimide (AG) is an antibreast cancer drug, and its metabolism is well known [7,8]. For the study of the influence of nanoencapsulation on the biodegradation of this hydrophilic drug, it was necessary to control the inclusion rates before studying metabolism in animals.

Determination of aminoglutethimide in biological fluids had already been performed by HPLC [9–11].

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HPLC had been also used for determination of aminoglutethimide enantiomers in tablet formulations [12] or in other drugs [13] but aminoglutethimide had never been quantified in nanoparticules. GC, but at that time not gas chromatography–mass spectrometry (GC–MS), had been used for determination of aminoglutethimide in biological fluids [9]. GC–MS could be a good alternative method, because it could be used not only for the control of nanoparticules but also for identification of metabolites and degradation products.

The aim of this work was then to choose the best chromatographic method for the quantification of AG in nanocapsules suspensions, which could be used for routine controls. Two methods were compared: gas chromatography with mass spectrometric detection and high performance liquid chromatography (HPLC).

The sample preparation is a determinant difference between the two methods and is described in this paper.

## 2. Experimental

### 2.1. Reagents and chemicals

Aminoglutethimide (Aldrich Chem. Co., Beerse Belgium), barbitone (veronal, VE) (Serva, Heidelberg, Germany), benzyl benzoate (Prolabo, Paris, France), poly-ε-caprolactone flakes (Sigma–Aldrich, Saint-Quentin Fallavier, France), methanol (Carlo Erba, Milan, Italy), methylene chloride and acetonitrile (Prolabo, Paris, France) were used. All the solvents were HPLC grade.

### 2.2. Nanocapsules suspension

Nanoprecipitation was the method chosen to prepare nanoparticles [14,15]. Aminoglutethimide was dissolved in benzyl benzoate. Poly-ε-caprolactone polymer was dissolved in acetone and added to AG solution to form solution A. Solution B was an hydroalcoholic solution containing surfactant (Synperonic®). Nanocapsules were formed by mixing solutions A and B. Acetone, alcohol and a part of water were evaporated.

Two lots of four suspensions of respectively approximate concentrations of 1, 2, 3, and 4 mg ml$^{-1}$, were prepared according to the procedure described by Al Khoury et al. [16]. The first lot was used for GC–MS dosing and the second lot for HPLC determination. In order to control extraction conditions, a standard lot of exactly known concentration of 4 mg ml$^{-1}$ was also prepared.

### 2.3. GC–MS apparatus

A Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA, USA) equipped with a splitless capillary inlet system was used. A fused-silica capillary column (25 m × 0.25 mm i.d., 0.25 μm film thickness) coated with cross-linked 5% phenylmethyl-silicone (Macherey-Nagel, Düren, Germany) was used. The carrier gas was helium at an inlet pressure of 62 kPa.

A Hewlett-Packard 7673A liquid autosampler, operated in the fast mode for splitless injection, was used in conjunction with the gas chromatograph. Before each injection, the 10 μl injection syringe was automatically rinsed out first with 10 μl of methanol and then three times with 10 μl of the sample solution. Then 1–3 μl of the sample solution were injected.

A Hewlett-Packard 5970A MSD mass spectrometer, operated in the electron-impact mode, was directly interfaced with the 5890 gas chromatograph by the capillary column and was used either in the full-scan mode or in selected-ion monitoring mode (SIM).

### 2.4. HPLC apparatus

The HPLC system consisted of an isocratic pump Beckman Model 110A (Beckman, San Ramon, CA, USA), a sample injector with 20 μl loop (Rheodyne, Cotati, CA, USA), a variable wavelength detector (Beckman Model 166) and a chromatography column Nucleosil 100, 5 μm, $C_{18}$, 250 mm × 4.6 mm (Macherey-Nagel).

### 2.5. Ultracentrifugation apparatus

Ultracentrifugation was performed with a Beckman L8-55 ultracentrifuge (Beckman Instruments, Berkeley, CA, USA), equipped with a 40TR rotor.

### 2.6. Chromatographic conditions

#### 2.6.1. GC–MS conditions

The oven temperature was maintained at 120 °C for 1 min, increased at 10 °C $min^{-1}$ to 270 °C, and then held at 270 °C for 2 min. The injector and transfer line temperatures were maintained at 240 °C. A preliminar injection in the full-scan mode, from *m/z* 50 to 400, allow to choose the *m/z* values for the SIM mode for quantitative determination. Retention times (Fig. 1) were 15.08, 10.32 and 7.25 min respectively for aminoglutethimide, benzyl benzoate and barbitone used as internal standard (IS).

The mass spectra of barbitone, benzyl benzoate and aminoglutethimide showed that the following ions should be monitored in the SIM mode: $m/z = 156$ and 141 for barbitone, corresponding to $(M\text{-}C_2H_4)^{+\bullet} = 156$ and $(M\text{-}HNCO)^{+\bullet} = 141$, *m/z* 212 and 105 for benzyl benzoate, corresponding to $(M)^{+\bullet} = 212$ and $(M\text{-}C_7H_7O)^{+} = 105$ and $m/z = 232$ and 203 for aminoglutethimide, corresponding to $(M)^{+\bullet} = 232$ and $(M\text{-}C_2H_4)^{+} = 204$.

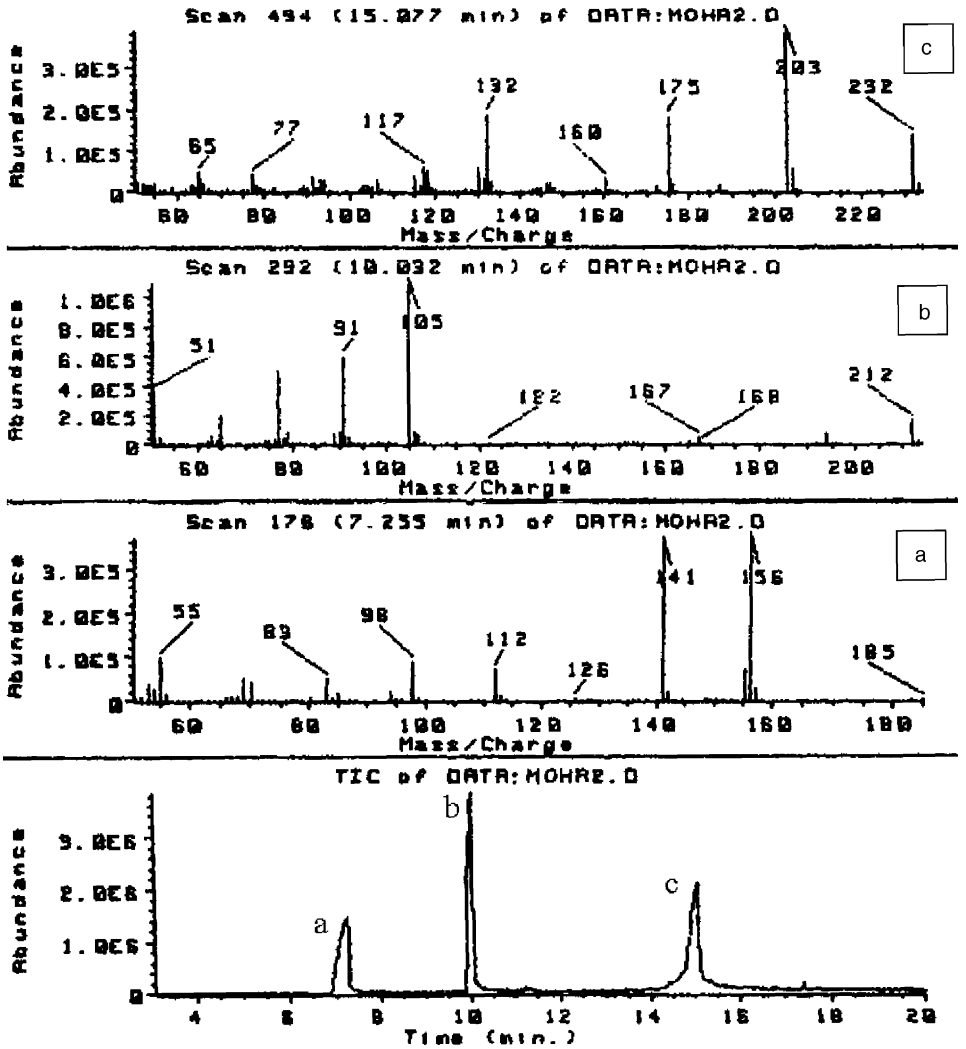


Fig. 1. GC–MS chromatogram and mass spectral data of nanocapsules suspension treated with methanol barbitone (VE), (b) benzyl benzoate (BB), (c) aminoglutethimide (AG).

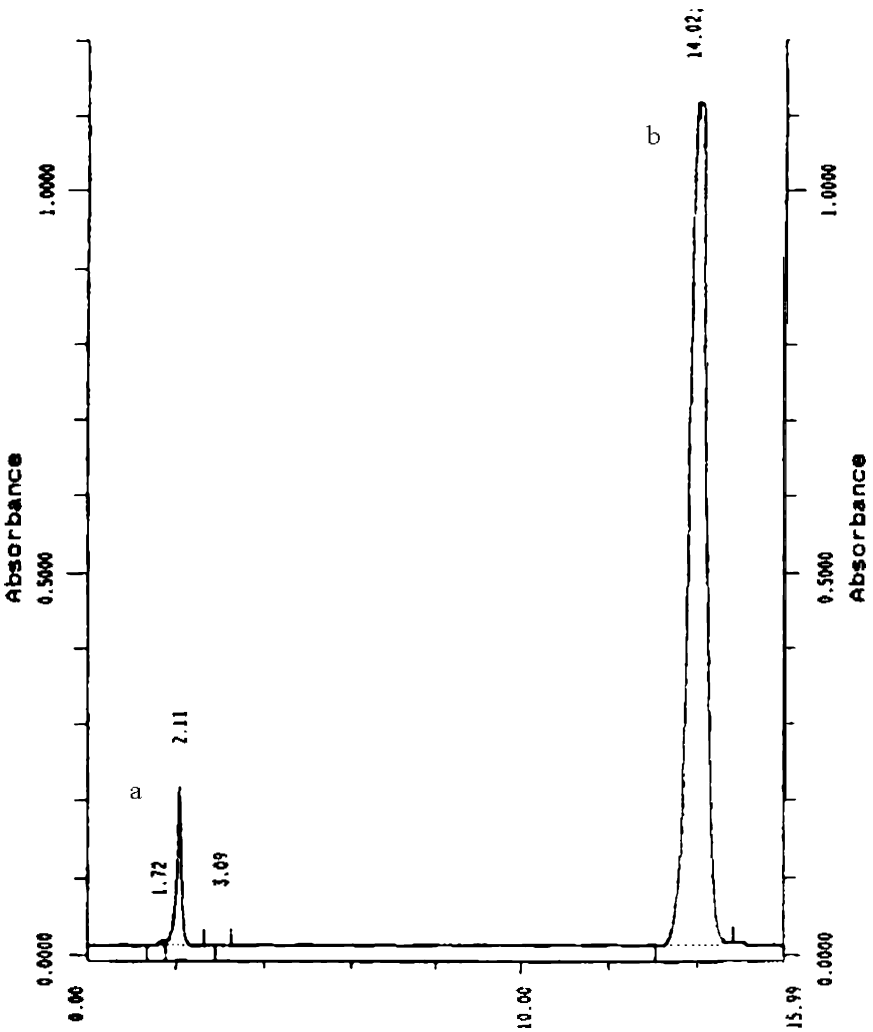


Fig. 2. HPLC chromatogram of (a) nanocapsules suspension aminogluthetimide (AG), (b) benzyl benzoate (BB).

*2.6.2. HPLC conditions*

Acetonitrile-distilled water-perchloric acid (70%) (55:45:0.02, v/v/v) was used as mobile phase at a flow of 1 ml $min^{-1}$. The UV detector was used at a wavelength of 224 nm. With these conditions, the retention times were 2.1 min for AG and 14 min for benzyl benzoate (Fig. 2).

*2.7. Standard curves*

*2.7.1. GC–MS*

The stock solution of barbitone (international standard, IS) in methanol (1 mg $ml^{-1}$) was prepared weekly and stored at 4 °C. Solutions of aminoglutethimide in methanol (2 mg $ml^{-1}$) were prepared each day before use. For standard curves, working solutions with methanol were prepared in order to obtain concentrations of 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05 mg $ml^{-1}$ for aminoglutethimide, and 0.25 mg $ml^{-1}$ for IS.

*2.7.2. HPLC*

Fifty milligrams were dissolved in 50 ml of mobile phase to obtain a 1 mg $ml^{-1}$ solution. This solution was prepared daily before use. Working solutions were prepared to obtain concentration of 0.075, 0.050,

0.025, 0.0125 and 0.00625 μg ml$^{-1}$ to study the standard curves.

### 2.8. Sample preparations

#### 2.8.1. Sample preparations for the determination of AG in nanocapsules suspension by GC–MS

1. Nanocapsules suspension (10 ml) was centrifuged at 50,000 × *g* for 45 min in order to obtain a clear supernatant liquid which was then removed by aspiration.
   - The liquid phase, was extracted twice with methylene chloride (50 ml). Organic phases were collected and dried with anhydrous sodium sulfate, then evaporated under reduced pressure. The residue was dissolved in methanol (20 ml) (solution $A_1$) and this solution was used to determinate free drug.
   - The nanocapsule sediment, was extracted twice with methylene chloride (50 ml), the organic phases were collected and dried using anhydrous sodium sulfate, then evaporated to dryness under reduced pressure. The residue was dissolved in methanol (20 ml) (solution $B_1$) and this solution was used for determination of adsorbed and incorporated drug.
2. Nanocapsules suspension (10 ml), were extracted twice with methylene chloride (50 ml), the organic phases were collected and dried using anhydrous sodium sulfate, then evaporated to dryness under reduced pressure. The residue was dissolved in methanol (20 ml) (solution $C_1$) and this solution was used for determination of total AG present in the suspension.

To 1 ml of each methanolic solution ($A_1$, $B_1$, $C_1$) were added 0.5 ml IS solution and 0.5 ml methanol. An aliquot of this solution (1–3 μl) was injected into the GC–MS system.

#### 2.8.2. Sample preparations for the determination of AG in nanocapsules suspension by HPLC

1. Nanocapsules suspension (5 ml) were centrifuged at 50,000 × *g* during 45 min.
   - The nanocapsules sediment, was dissolved in distilled water and the aqueous suspension extracted three times with methylene chloride (10 ml). The organic phase was evaporated to dryness, and the dried residue dissolved with mobile phase (20 ml) (solution $A_2$) and this solution used for determination of weakly adsorbed drug.
   - Another nanocapsules sediment was extracted twice with methanol (10 ml). The methanolic phase was evaporated onto dryness and the dried residue dissolved with mobile phase (50 ml) (solution $B_2$). This solution was used for determination of adsorbed AG.
   - The supernatant was extracted three times with methylene chloride (10 ml). The organic phase were then evaporated to dryness, and the dried residue recuperated with mobile phase (20 ml) (solution $C_2$). This solution was used for determination of free drug.
   - Another supernatant was diluted with mobile phase to obtain a suitable concentration (solution $D_2$). This solution was used for direct determination of free drug.
2. The lot of nanocapsules suspension at exactly 4 mg ml$^{-1}$ was directly dissolved in acetonitrile to obtain a clear solution for the determination of the total AG (solution $E_2$).

Each solution ($A_2$, $B_2$, $C_2$, $D_2$, $E_2$) was diluted with mobile phase to a suitable concentration and 20 μl of each diluted solution were injected in HPLC.

## 3. Results and discussion

### 3.1. Validation

HPLC and GC–MS methods have been validated [17,18].

#### 3.1.1. GC–MS

Linearity studied in the SIM mode was observed for concentrations between 0.5 and 0.05 mg ml$^{-1}$. The straight-line equation was $y = 0.147x + 0.00384$ ($y$: response ratio AG/VE; $x$: concentration ratio AG/VE). The determination coefficient, $r^2$, was 0.999. The validation results established are listed in Table 1. Intra-assay variability and inter-assay variability were established respectively for five

Table 1
Validation of the GC–MS method

| Approximate concentration (mg ml$^{-1}$) | Found concentration (mg ml$^{-1}$) | Intra-assay variability R.S.D. (%) | Accuracy (recovery %) | Inter-assay variability R.S.D. (%) |
|---|---|---|---|---|
| 0.05 | 0.0519 | 5.8 | 103 | 6.9 |
| 0.1 | 0.1058 | 4.4 | 106 | 6.5 |
| 0.2 | 0.2026 | 4.8 | 101 | 4.9 |
| 0.3 | 0.3150 | 4.6 | 105 | 5.1 |
| 0.4 | 0.4012 | 1.8 | 100 | 3.0 |
| 0.5 | 0.4852 | 2.9 | 97 | 7.3 |

Table 2
Validation of the HPLC method

| Approximate concentration (mcg ml$^{-1}$) | Found concentration (mcg ml$^{-1}$) | Intra-assay variability R.S.D. (%) | Accuracy (recovery %) | Inter-assay variability R.S.D. (%) |
|---|---|---|---|---|
| 6.25 | 5.151 | 4.1 | 82.4 | 7.9 |
| 12.5 | 12.390 | 0.6 | 99.1 | 7.0 |
| 25 | 26.308 | 1.7 | 105 | 7.4 |
| 50 | 51.021 | 1.7 | 102 | 6.5 |
| 75 | 73.922 | 1.4 | 98.6 | 6.7 |

injections and for 15 injections per concentration, and linearity was studied with six different concentrations.

#### 3.1.2. HPLC

Linearity was verified for concentration between 6.25 and 75 μg ml$^{-1}$. The straight-line equation was $y = 0.001692x - 0.00314$ ($r^2 = 0.998$). The validation results are listed in Table 2. Intra-assay variability and inter-assay variability were established respectively for five injections and for 15 injections per concentration, and linearity was studied with five different concentrations.

### 3.2. Effect of drug concentration on adsorption of AG into poly-ε-polycaprolactone

#### 3.2.1. GC–MS

Four lots of nanocapsules suspension respectively at concentration of about 1, 2, 3 and 4 mg ml$^{-1}$ were treated. The results are shown in Table 3.

The influence of the concentration of AG in the nanocapsules suspension is shown in Table 3. The amount of AG adsorbed per mg of polymer (12.5 mg of polymer by ml of suspension) increased with drug in the suspension, but this phenomenon was not very significant for concentrations between 2 and 4 mg ml$^{-1}$.

Table 3
Results of free AG and AG associated with nanocapsules by GC–MS method

| | | | Lot | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| Solution A1 | Free AG | mg ml$^{-1}$ | 0.78 | 0.82 | 1.23 | 1.64 |
| | | % | 70.3 | 42.05 | 41.2 | 39.5 |
| Solution B1 | Adsorbed and incorporated AG | mg ml$^{-1}$ | 0.33 | 1.13 | 1.76 | 2.51 |
| | | % | 29.7 | 57.9 | 58.8 | 60.5 |
| Solution C1 | Total AG | mg ml$^{-1}$ | 1.11 | 1.95 | 2.99 | 4.15 |

The best ratio between free and adsorbed AG was obtained in the case of 4 mg ml$^{-1}$. This observation and the fact it was a suitable concentration for in vivo experiments leaded us to choose a theoretical concentration of 4 mg ml$^{-1}$ of AG in nanocapsules suspension for later studies of drug administration to animals.

Another lot of nanocapsules suspension was prepared in order to obtain an exact concentration of 4.00 mg ml$^{-1}$, for the validation of the extraction method. The results were as follows: 1.23 mg ml$^{-1}$ (R.S.D. = 5.5%) in the supernatant, and 2.89 mg ml$^{-1}$ (R.S.D. = 4.8%) in the nanocapsule sediment.

A directly measured concentration, on 10 ml of this nanocapsules suspension treated by twice 50 ml of methylene chloride, was 3.74 mg ml$^{-1}$ (R.S.D. = 7.4%). This result was consistent with the theoretical values of 4 mg ml$^{-1}$.

### 3.2.2. HPLC

As in the case of GC–MS method, four other lots of nanocapsules suspension at concentrations close to 1, 2, 3 and 4 mg ml$^{-1}$ and one lot at exactly 4 mg ml$^{-1}$ were treated.

The results are shown in Table 4 and are similar to those obtained with the GC–MS method.

The best ratio between free and adsorbed AG was obtained for the concentration close to 4 mg ml$^{-1}$.

The sediment treated with methanol ($B_2$) allowed to recover greater quantities of AG than those treated with methylene chloride ($A_2$). This could be a consequence of various kinds of adsorption of AG on the polymeric film as it was previously described for phenobarbitone nanocapsules [10].

The easier method for the determination of encapsulated AG could then be:

1. To determine total AG concentration by direct measure of a solution obtained by dilution of the suspension in acetonitrile (method used to prepare solution $E_2$).
2. To determine free AG concentration by direct measure of a solution obtained by simple dilution of supernatant (method used to prepare solution $D_2$).
3. The difference between the two measures corresponding then to AG included in or adsorbed on nanocapsules, i.e. to efficient AG.

## 4. Conclusion

For the later metabolism studies and in order to permit oral administration by gavage, it was important to have a nanocapsules suspension with the highest rate of encapsulated AG and the most important quantity of AG in the lowest volume of suspension. In this work, GC–MS and HPLC lead to show that the best concentration was 4 mg ml$^{-1}$.

Both chromatographic methods were successful and on an analytical point of view the validations of AG dosing were suitable in both cases. The major argument in favor of the routine utilisation of HPLC consisted of an easier sample preparation. GC–MS cannot be performed without extraction due to the presence of water in the nanocapsules suspension, while HPLC

Table 4
Results of free AG and AG associated with nanocapsules by HPLC method

| | | | Lot | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| Solution A2 | Weakly adsorbed AG | mg ml$^{-1}$ | 0.474 | 0.997 | 1.674 | 2.252 |
| | | % | 47.4 | 49.3 | 55.8 | 56.3 |
| Solution B2 | Adsorbed and incorporated AG | mg ml$^{-1}$ | 0.546 | 1.193 | 1.919 | 2.580 |
| | | % | 54.6 | 59.6 | 64 | 64.5 |
| Solution C2 | Free AG (extraction) | mg ml$^{-1}$ | 0.248 | 0.435 | 0.531 | 0.728 |
| | | % | 24.8 | 21.7 | 17.7 | 18.2 |
| Solution D2 | Free AG (directly measured) | mg ml$^{-1}$ | 0.506 | 0.890 | 1.192 | 1.210 |
| | | % | 50.6 | 44.5 | 39.7 | 30.2 |
| | Total AG (calculated) | mg ml$^{-1}$ | 1.052 | 2.083 | 3.111 | 3.79 |

can be used for direct analysis of the suspension. Extraction being time-consuming, and being a cause of fiability lowering of the analytical procedure, when performed in routine conditions, HPLC procedure was then preferred.

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