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HPLC method for determination of the purity of a mebrofenin-ligand for ^{99m}Tc-complexes

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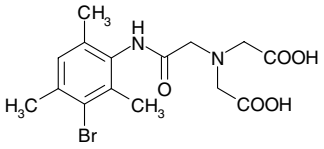
A reversed-phase HPLC method has been developed for the estimation of purity and quantitative determination of mebrofenin UV detection at 205 nm was used. The stability of mebrofenin alone and in the presence of stannous chloride at 25 °C was studied. No decomposition product was found and there was no influence of $SnCl_2$ on the stability of mebrofenin.

1. Introduction

Mebrofenin, *N*-[2-[(3-bromo-2,4,6-trimethylphenyl)amino]-2-oxoethyl]-*N*-(carboxymethyl)glycine represents the third generation of iminodiacetic acid (IDA) derivatives designed especially for ^{99m}Tc labeled complexes for diagnostic imaging of the hepatobiliary system. ^{99m}Tc-IDA complexes exhibit high specificity and rapid transit through the hepatobiliary system, high resistance to competition from compounds such as bilirubin and rapid radiolabeling with high radiochemical purity and stability (Loberg et al. 1976; Nunn et al. 1983; Doo et al. 1991; Kapuściński et al. 1986). ^{99m}Tc-Mebrofenin (Choletec®), with its high hepatic uptake and fast biliary excretion, provides superior image resolution and enables diagnosis of various hepatobiliary diseases, especially in the early stages (Burns et al. 1978; DeJuliis et al. 1980; Callery et al. 1976; Peters et al. 1998).



The non-radioactive product contains all the necessary ingredients, including stannous chloride as a reducing agent for the sodium pertechnetate that is used for preparation of the technetium ^{99m}Tc-Mebrofenin complex. There are no literature data on quantitative determination of mebrofenin in a kit for the preparation of ^{99m}Tc complexes. The only exception is a paper by Zodda et al. (1994), where an HPLC method for the simultaneous quantitative determination of methyl paraben and propyl paraben in Choletec® in the presence of the major ingredient, mebrofenin, is described.

The main goal of this study was to establish whether the earlier preparation of the solutions for complexation has any influence on the stability of the ligand. This paper reports results for the stability of mebrofenin conditioned at 25 °C and in the presence of stannous chloride as well as the results for a two – factor study in which the pH value and composition of the mobile phase were chosen to give an optimal chromatographic performance for the quantitative determination of mebrofenin.

2. Investigations, results and discussion

An HPLC method for the quantitative analysis of mebrofenin substance is included in Official Monographs USP XXIV (USP 2000) in which a reverse-phase HPLC system consisting of a 4.6×250 mm, 10 μm RP C-18 column and a mobile phase of methanol/0.05 M phosphate buffer (pH 5.0) (60 : 40, v/v) with detection at $\lambda = 220$ nm is proposed. We used this method at first for our study of the stability of mebrofenin. We found that using a RP C-18 chromatography column of 250×4.6 mm i.d., 5 μm with a mobile phase of methanol: 0.05 M phosphate buffer in a ratio of 60 : 40 results in unstable chromatographic conditions that make it impossible to obtain repeatable chromatographic performance. Consistent stability was realized using the same mobile phase in ratio 68 : 32. Since the degree of ionization of solutes, the stationary phase and the mobile phase additives may be affected by the pH and may lead to better selectivity we studied the influence of a variation of pH with simultaneous variation of mobile phase composition.

We established the pK_1 and pK_2 of mebrofenin by potentiometric titration using a 0.1 M solution of sodium hydroxide as 3.07 and 5.65 respectively. Therefore, mebrofenin is probably present in the solution at pH = 4.0 as a mixture of monoanionic and acidic forms that may be confirmed by the broad peak of mebrofenin. The effect of pH was tested at pH 3–7. We used for that purpose phosphate buffers of the appropriate pH and a mobile phase of methanol : phosphate buffer (68 : 32 v/v) at a flow rate of 1.0 ml min^{-1} with detection at $\lambda = 220$ nm. The relationship between log k and pH indicates a clear dependence between the pH of the mobile phase and resolution ($y = -0.2843x + 0.7947$, $R^2 = 0.9957$). We observed k values increasing with diminishing pH values. Moreover, there was a single narrow peak for mebrofenin at pH = 3.0

Table 1: Suitability results for USP and proposed methods

Methods and columns	t_R (min)	k′	N	USP Tailing
USP Supelcosil™ 250 × 4.6 mm i.d.	5.46 RSD% 0.10	1.81 RSD% 0.16	294	1.24 RSD% 5.14
USP Waters Symetry® 150 × 3.9 mm i.d.	2.77 RSD% 0.33	0.79 RSD% 0.76	1817	1.19 RSD% 4.58
Proposed Waters Symetry® 150 × 3.9 mm i.d.	3.43 RSD% 0.33	1.27 RSD% 0.24	3330	1.29 RSD% 0.30

t_R – retention time, k′ – capacity factor, N – numbers of theoretical plates (USP method), T – tailing factor (USP method)
Mobile phase: a) USP method-methanol : 0.05 M phosphate buffer (68 : 32 v/v), pH = 5.0; b) Proposed method-acetonitrile: methanol : 0.25% of H_3PO_4 (38 : 6 : 56 v/v/v), pH = 3.0

while at pH = 4.0 and at pH = 5.0 two narrow signals and one broad signal, respectively, were present. This clearly shows the existence of two detectable, monoanionic and acidic, forms for mebrofenin in these chromatographic systems.

Moreover we have found that using acetonitrile as a stronger eluent for mebrofenin and 0.25% aqueous phosphoric acid solution instead of methanol and phosphorane buffers, respectively, gives better chromatographic parameters. The effect of acetonitrile was tested using three combinations of acetonitrile (42%; 38%; 34%) and a 0.25% aqueous solution of phosphoric acid. The regression equation for the relationship between log k and ratio of acetonitrile was $y = -0.045x + 1.8057$ and the correlation coefficient was 0.9996 indicating an unchanged elution mechanism. The final composition of the mobile phase for mebrofenin chromatographic analysis was established as being acetonitrile : methanol : 0.25% phosphoric acid (38 : 6 : 56 v/v/v). Significant UV absorbance for mebrofenin was observed at λ = 205 nm. Table 1 contains the results of the comparison for both the USP and proposed methods.

The specificity of the method was checked by adding possible impurities (nitrilotriacetic acid-NTA and 3-bromo-2,4,6-trimethylaniline-BrTMA) to a pure mebrofenin sample and analysing it (Fig. 1). The resultant assay results were compared with the results for the pure sample. There was a good match between the results (Table 2). The detection limit from the signal to noise method for NTA and BrTMA was 0.4 μg ml^{-1} and 0.01 μg ml^{-1} respectively.

Six samples of different concentration levels ranging from 0.005 to 0.500 mg ml^{-1} were prepared and checked for linearity. A calibration curve between peak area and concentration of mebrofenin was drawn. The regression equation for the calibration curve was $y = 5.542 \cdot 10^4 x - 5.249 \cdot 10^4$. Standard error for slope and intercept were $3.560 \cdot 10^2$ and $7.338 \cdot 10^4$ respectively. The correlation coefficient of the plot was found to be 0.997 indicating good linearity. Limit of detection (LOD) and limit of quantification (LOQ) were calculated by the signal to noise method (i.e. peak height equal six times baseline noise). LOD and LOQ for mebrofenin were 0.026 μg ml^{-1} and 0.088 μg ml^{-1} respectively.

Table 2: Test for specificity of the method

Sample with impurities		Pure sample	
Amount (μg/ml)	Recovery (%)	Amount (μg/ml)	Recovery (%)
11.47	98.58	12.41	98.52
11.49	98.71	12.63	100.28
11.52	98.93	12.67	100.56
Mean: 98.743		Mean: 99.783	
SD: 0.178		SD: 1.106	
RSD%: 0.180		RSD%: 1.109	

(n = 3); Mobile phase: acetonitrile : methanol : 0.25% of H_3PO_4 (38 : 6 : 56 v/v/v), pH = 3.0; Column : Waters Symetry® (150 × 3.9 mm i.d.) with guard

Accuracy of the method was checked at three concentration levels, at 0.025, 0.108, 0.176 mg ml^{-1}. The RSD% was below 1.5% and shows the satisfactory repeatability of the system. The relevant details are presented in Table 3.

The stability of mebrofenin were checked in two solutions: the first containing mobile phase and second containing stannous chloride. Stannous chloride as a reducing

Table 3: Precision methods. Statistical analysis of results for the determination of mebrofenin solution

Concentration (μg/ml)	Measured conc. (μg/ml)	S.D.	RSD (%)	Recovery (%)
25.2	24.7	0.12	0.51	99.43–100.36
107.6	107.71	0.45	0.42	99.63–100.42
176.0	177.74	1.35	0.76	99.18–100.69

(n = 3) Conditions: see Table 2

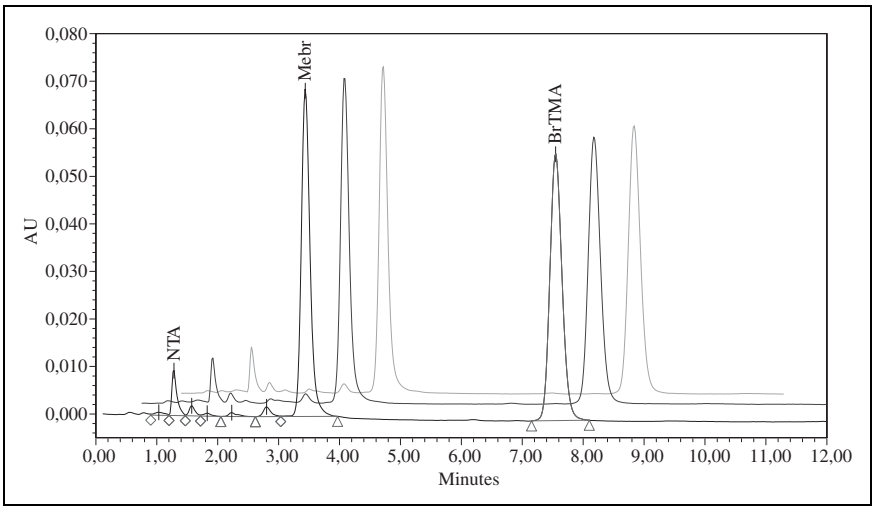


Fig. 1:
Chromatogram of mebrofenin (Mebr) spiked with the related substances: nitrilotriacetic acid (NTA) and bromotrimethylaniline (BrTMA) Chromatographic conditons: Mobile phase: ACN : methanol : 0.25% phosphoric acid, 38/6/56 (v/v/v), flow rate 1 ml/min, detection 205 nm UV Stationary phase: Waters Symetry® C18, 5 μm, 150 × 3,9 mm, i.d.

agent for sodium pertechnetate is used for the formation of technetium ^{99m}Tc-mebrofenin complexes. That is why we decided to check the stability of mebrofenin in the presence of stannous chloride. The stability in mobile phase was determined by keeping a sample at ambient temperature under normal lighting conditions and checking its assay on three successive days (Table 4). The stability of mebrofenin in the solution containing the mobile phase with stannous chloride was determined by incubating samples at 25 °C in a water bath for 3 h and comparing with freshly prepared samples and samples incubated under the same conditions (Table 5). No interfering peaks and no peaks that indicate degradation products were present in the chromatograms (Fig. 2). It is confirmed by the appearance of baseline chromatogram analytes and the recovery value of analytes. Recovery values of assay were found to be 98.8–101.6%. The described method may be used as a stability-indicating assay.

In conclusion, the determination of mebrofenin can be performed by a sensitive, rapid, direct reversed – phase HPLC method. The criteria for a satisfactory system were established with RSD of below 1,5% for five replicate injections of the working standards. The method developed can also be used as a stability – indicating assay and well as a quantitative assay for mebrofenin.

Table 4: Stability of the mebrofenin in mobile phase

Concentration (μg/ml)	Measured conc. (μg/ml)	S.D.	RSD (%)	Recovery (%)
24.16	1 day 23.85	0.28	1.16	97.85–100.00
	2 day 23.33	0.08	0.36	96.19– 96.85
	3 day 23.33	0.08	0.36	96.19– 96.85
40.16	1 day 40.05	0.11	0.26	99.48–100.00
	2 day 40.34	0.90	2.23	97.91–102.09
	3 day 39.31	0.02	0.04	97.83– 97.91
112.8	1 day 112.2	0.54	0.48	99.08–100.00
	2 day 111.8	0.88	0.79	98.22– 99.65
	3 day 112.72	0.59	0.52	99.53–100.52

Conditions: see Table 2

Table 5: Stability of mebrofenin solution containing $SnCl_2$

Concentration (μg/ml)	Amount (μg/ml)	S.D.	RSD (%)	Recovery (%)
89.92	91.25 0 h, 25 °C	0.14	0.15	101.37–101.58
	89.49 1 h, 25 °C	0.15	0.17	99.40– 99.64
	88.90 3 h, 25 °C	0.03	0.04	98.89– 98.84

Conditions: see Table 2

3. Experimental

3.1. Chemicals

Mebrofenin was synthesized using a patented method (Nunn and Loberg 1983). All the solvents used for the preparation of the samples and mobile phases were HPLC grade. HPLC isocratic grade methanol and acetonitrile were supplied by J. T. Baker, Deventer Holland. Potassium dihydrogen phosphate, sodium hydrogen phosphate and ortho-phosphoric acid were purchased from Merck, Darmstadt, Germany. Nitrilotriacetic acid was obtained from Sigma, St Louis, USA. Phosphoric acid (0.25%) was prepared by appropriate dilution of conc. 65% phosphoric acid with water. All the mixtures were filtered before use and mobile phases were sparged with helium. Sodium hydroxide (0.1 mol/l) was prepared from standard carbonate-free solution sodium hydroxide 0.1 mol/l DILUT-IT® from J. T. Baker, Deventer Holland.

3.2. Equipment

The Waters LC system consisted of a Waters 600 pump and Waters 996 Photodiode detector with a Waters 717 plus autosampler, and Millennium 4.0 chromatography manager and System Suitability software were used. Separations were made using a Symetry® C18 column 150 × 3.9 mm, i.d., 5 μm with 2 cm guard column (Waters, Milford USA) and a Supelcosil™ LC18 column 250 × 4.6 mm, i.d., 5 μm (Supelco Park, Bellefonte USA). Elution was performed at a flow rate of 1.0 ml min^{-1} and the column was maintained at ambient temperature. The absorbance was monitored in the range 200 nm–400 nm. The mobile phases were: methanol : buffer (68 : 32, v/v) and acetonitrile : methanol : 0.25% aqueous phosphoric acid solution (38 : 6 : 56; v/v/v). Water bath was a Techne, TE-8D (Cambridge Ltd.). An ESKP – 309W combined glass electrode (Eurosensor Gliwice, Poland) and an HI 9318 pH meter (Hanna Instruments, Germany) with a Solarus Digital Titrator (Hirschmann Laborgerate, Germany) were used for potentiometric titration.

3.3. Preparation of phosphate buffers

Aqueous solution of Na_2HPO_4 (0.025 mol l^{-1}) and 0.025 mol l^{-1} aqueous solution of KH_2PO_4 were mixed in the ratio 1 : 1 and adjusted to the appropriate pH by adding 10% aqueous H_3PO_4 solution.

3.4. Preparation of the standard solutions

About 5 mg of mebrofenin was precisely weighted, dissolved in methanol and diluted to 5 ml with methanol to form a stock solution. Working standard solutions were prepared in a 5 ml volumetric flask by dilution of suitable volumes of the stock solution with the mobile phase. Six solutions with the following concentrations of mebrofenin: 0.005, 0.01, 0.050, 0.100, 0.250, 0.500 mg ml^{-1} were then prepared. Three injections (10 μl) of each of these solutions were made into the chromatographic system.

3.5. Preparation of the samples

The solution with stannous chloride was prepared by dissolving mebrofenin in 0.5 M sodium hydroxide (using 2 mol sodium hydroxide for 1 mol of the ligand) to give a solution with final pH of about 7 to 7.5. Then, a suitable amount of a 50 mg ml^{-1} solution of stannous chloride in 0.05 M

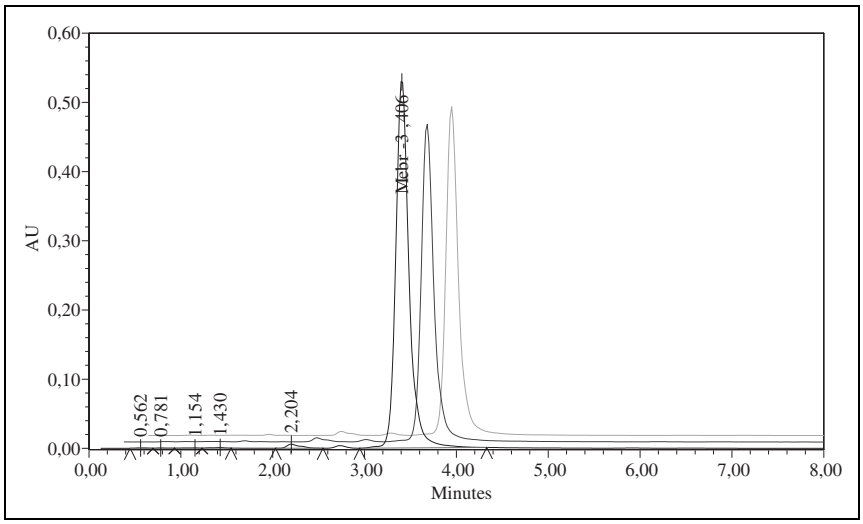


Fig. 2:
Chromatogram of mebrofenin incubated with stannous chloride Chromatographic conditions: see Fig. 1

hydrochloric acid was added to this. The final solution was diluted to 5 ml with water. The sample was incubated in a water bath at 25 °C for 3 h. After incubation was completed, 100 µl of the solution was placed in a 5 ml calibrated flask and diluted to volume with the mobile phase to give a concentration of about 0.1 mg ml^{-1}.

3.6. Determination of pK

All potentiometric measurements were performed in a titration cell thermostated at 25.0 ± 0.1 °C. Ionic strength was kept constant at 0.10 M with $NaNO_3$ under a nitrogen atmosphere. 50 ml of about 1.3×10^{-3} mol l^{-1} aqueous mebrofenin solution was titrated using 0.1 M sodium hydroxide solution. The two proton association constants were found and K values were calculated from the potentiometric data by a direct algebraic method. The average value of pK_1 was determined as 3.07 (±0.04) and pK_2 5.65 (±0.10) respectively. Titrations were performed at least three times.

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