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A modified HPLC method for monensin analysis in liposomes and nanocapsules and its comparison with spectrophotometric and radioactive methods

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Abstract

Monensin is a carboxylic ionophore which can potentiate the immunotoxin activity against human tumors in vitro and in vivo. Currently monensin is being encapsulated in liposomes and nanocapsules in our laboratory. The reported methods for monensin analysis by spectrophotometric and HPLC lack the required sensitivity. We have developed a sensitive HPLC method for analysis of monensin. Separation was achieved on a Beckman C18 reverse phase column with methanol–acetonitrile–methylene chloride–water–acetic acid (45:20:25:9.5:0.5) as the mobile phase. The eluent was reacted with vanillin reagent in the post column reactor at 70°C. The reagent reacted with monensin and formed a pink color, which was detected at 520 nm. The retention time of monensin was found to be 6 min. By using this method it was possible to quantify monensin down to 100 ng ml^{-1}, with a signal to noise ratio of $>17:1$. Linearity was observed within the range of 10 to 100 ng ($r^2 > 0.99$). Inter-day standard deviations for monensin samples of 20, 50 and 80 ng were 0.675, 0.543 and 0.736 respectively. Alternative methods of analysis include using radioactive [^{3}H]monensin in liposomes which can be quantified by scintillation counter. The results from the HPLC, spectrophotometric and radioactive method were compared and were found to be within acceptable limits. The HPLC method is being utilized in our laboratory for quantitative analysis of monensin in liposomes and nanocapsules. © 1997 Elsevier Science B.V.

Keywords: Monensin; HPLC; Reverse phase; Liposomes; Nanocapsules; Spectrophotometric; Radioactive

1. Introduction

Monensin is a carboxylic ionophore which can potentiate the immunotoxin activity by several logs in vitro [1,2]. Monensin has been found to enhance the cytotoxicity of various immunotoxins including ricin A. At low concentrations, monensin sensitizes cells to the cytotoxic action of cell specific immunotoxins causing increased cytotoxicity and more rapid cell killing. Monensin is highly lipophilic and has a short half-life on parenteral administration [3]. Therefore, attempts were made to entrap monensin in liposomes and nanocapsules in our laboratory, which were very successful [1,4]. In order to study the efficacy of

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various monensin formulations, it is necessary to estimate the amount of monensin entrapped in liposomes and nanocapsules. Various methods have been reported for analysis of monensin, which are, a turbidimetric method [5], a spectrophotometric method [6] and bioautography [7]. High pressure liquid chromatography with post column reaction has been used for determination of monensin, narasin and salinomycin in cattle feeds [8,9]. HPLC coupled with a refractive index detector has also been utilized for analysis of monensin in feed premixes [10]. Another alternative method for analysis of the compound include using radioactive [^{3}H]monensin in formulations which can be quantified by scintillation counter [1]. The reported spectrophotometric and HPLC methods were not sensitive enough for quantitative analysis of monensin at low concentration, especially in various liposome and nanocapsule formulations. Furthermore, in our laboratory we routinely carry out pharmacokinetic studies on liposomes and nanocapsules, for which estimation of monensin in nanogram quantities is essential. The radioactive method is sensitive, but requires the use of [^{3}H]monensin, which is not commercially available.

In order to overcome the various problems associated with monensin analysis, the present work was initiated, which describes the development of a modified HPLC method which can be used for quantitative analysis of monensin at low concentration in various liposome and nanocapsule formulations. The efficiency of the modified HPLC method was compared with the spectrophotometric and radioactive methods.

2. Materials and methods

2.1. Chemicals and reagents

Monensin (sodium salt) and vanillin were obtained from Sigma (St. Louis, MO). Radioactive [^{3}H]monensin was a generous gift from Dupont NEN, North Billerica, MA. Methanol, acetonitrile, methylene chloride, water and acetic acid were HPLC grade (Fisher Scientific, PA). Other solvents and reagents were of analytical grade.

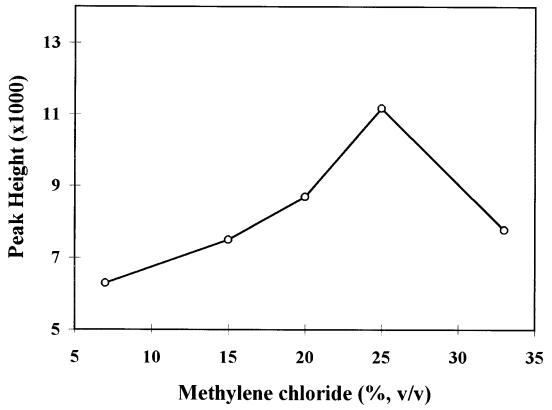

Fig. 1. Effect of methylene chloride in the mobile phase on the peak height of monensin. The other components of the solvent system were methanol–acetonitrile–water–acetic acid (45:20:9.5:0.5) and the flow rate was 0.33 ml min^{-1}.

2.2. HPLC equipment

A Beckman System Gold HPLC was used in all experiments. It consisted of an autosampler 507e, double pumps model 125, and a visible detector 166. The autosampler could deliver samples from 1–100 μl in each injection. Pump A was used for mobile phase and pump B was utilized for pumping vanillin reagent for the post column reaction. The eluent coming out of the column was passed through a Beckman 231 post column reactor, and

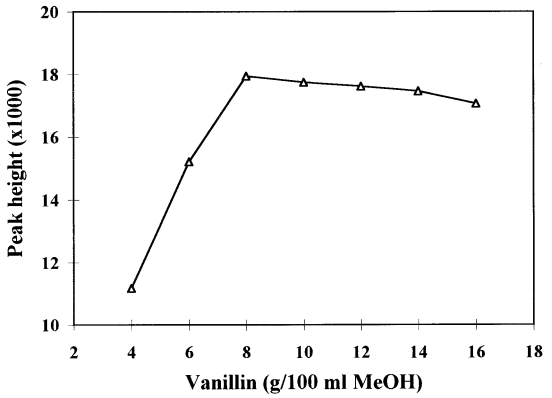

Fig. 2. Effect of varying the concentration of vanillin on the peak height of monensin. Temperature of the post column reactor was 70°C, and the flow rate of vanillin reagent was 0.67 ml min^{-1}.

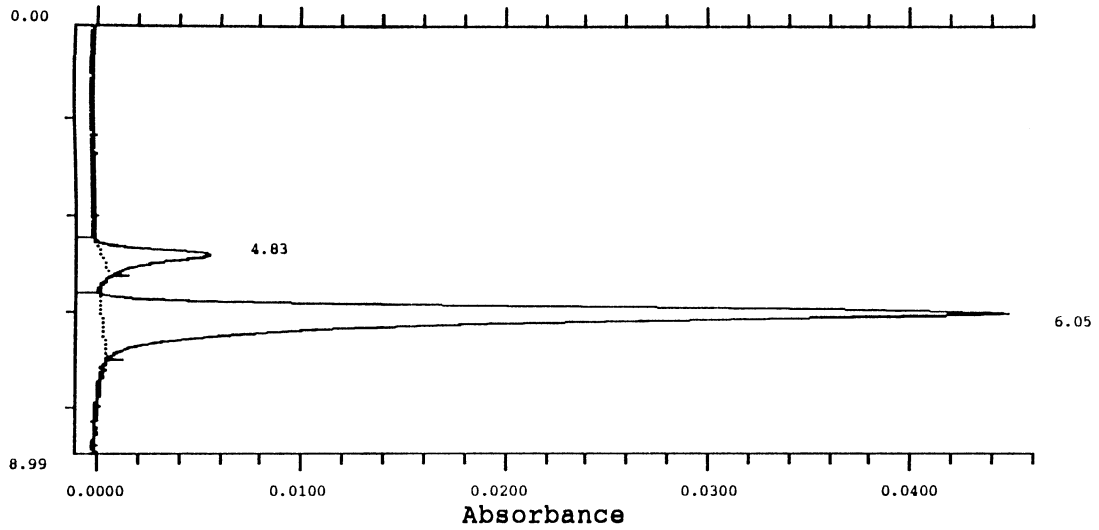

Fig. 3. The chromatogram of a standard monensin sample (60 ng) using methanol–acetonitrile–methylene chloride–water–acetic acid (45:20:25:9.5:0.5) as the mobile phase. The retention time of monensin was 6.05 min and that of impurities was 4.83 min respectively.

the detection was performed at 520 nm. The detector sensitivity range was set at 0.5 aufs. The whole system was interfaced with an IBM 433DX/Si computer and was run by the Beckman GOLDV810 software. After sample analysis, the digitized chromatogram, concentration, peak height, peak area and other data were recorded and analyzed by using the software.

2.3. Chromatographic conditions

Monensin was analyzed by using a reverse phase Beckman Ultrasphere XL-ODS 3 μm, 7 cm × 4.6 mm i.d. C18 column. An isocratic mobile phase was used which consisted of methanol–acetonitrile–methylene chloride–water–acetic acid (45:20:25:9.5:0.5), and the flow rate was 0.33 ml min^{-1}. Prior to use, the mobile phase was filtered through a 0.22 μm μStar LB filter (Costar Corporation, MA), and degassed in an ultrasonic bath for half an hour. The mobile phase was passed through the column for 15 min before sample analysis. Monensin eluting from the column was reacted with vanillin reagent in the post column reactor.

2.4. Preparation of vanillin reagent

The reagent was prepared by dissolving varying concentrations of vanillin in 100 ml of cooled methanol. To this solution concentrated sulfuric acid was added which was half the amount of vanillin. Fresh reagent was prepared every day and filtered by passing through a 0.22 μm μStar LB filter. The vanillin reagent was kept in an ice bath and pumped through pump B of HPLC. The flow rate of the reagent was maintained at 0.67 ml min^{-1}, which was twice the rate of the mobile phase. Prior to the first injection, the system was allowed to equilibrate for 30 min. Vanillin reagent reacted with monensin in the post column reactor and the reaction was carried out at 70°C. The reaction produced a pink color, which was detected by the detector at 520 nm.

2.5. Preparation of test solution and standard curve

Monensin stock solution was prepared by dissolving 10 mg of monensin in 10 ml of grade methanol. The solution was diluted 10 fold three times with methanol, so the final test solu-

Table 1
Estimation of monensin by modified HPLC method

Monensin concentration (ng)		Day 1	Day 2	Day 3	Average accuracy
20	Mean	19.93	21.12	19.97	
	S.D.	0.900	0.365	0.431	101.7
50	Mean	50.58	49.94	51.02	
	S.D.	1.708	1.640	0.841	101.0
80	Mean	80.54	79.55	80.99	
	S.D.	0.871	0.633	0.406	100.4

Triplicate samples were run for each concentration. The mean concentration along with interday S.D. are given.

tion contained monensin 1 ng μl^{-1} of methanol. A standard curve was prepared by plotting a known concentration of monensin against the corresponding peak height. It was observed that peak height data was more accurate and reproducible than the peak area for the same concentration of monensin.

2.6. Assay of monensin in liposomes and nanocapsules

To 100 μl liposome (multi lamellar vesicles, MLV), 50 μl Tween 20 was added and the sample was vortexed for 5 min to completely dissolve the contents of the liposomes. To the mixture, 600 μl methanol was added and centrifuged in a Micro-Centrifuge (Costar Corporation, MA) at 10 000 rpm for 30 min. The supernatant was diluted 10 times and analyzed for monensin concentration. A similar procedure was followed for extraction and quantification of monensin in nanocapsules. All analyses were performed in triplicate and the average results are reported.

2.7. Assay of monensin by radioactive and spectrophotometric methods

The method reported by Singh et al. [1] was used for analysis of radioactive monensin. In brief, the method consists of using a known concentration of radioactive [^{3}H]monensin in preparing liposomes which was quantified by using a LKB Wallac 1219 Rackbeta liquid scintillation counter (Wallac, MD). The spectrophotometric method consists of measuring monensin by reacting with vanillin and heating the mixture at 70°C. The absorbance of the pink color produced was measured by using a Beckman DU 640 spectrophotometer (Beckman Instruments, IL) at 555 nm [6].

3. Results and discussion

Initially monensin was analyzed by injecting 50 ng 50 μl^{-1} of methanol. The mobile phase was methanol–acetic acid (90:0.5), along with water, the concentration of which was varied from 5 to 15% (v/v). No significant change in peak height was observed in those experiments so it was decided to continue with 9.5% (v/v) water in the mobile phase. Similarly, experiments were carried out with a mobile phase of methanol–water (90:9.5) with acetic acid which was varied from 0.5 to 2% (v/v). No significant improvement of the monensin peak was observed by changing the acetic acid. Therefore, the percentage of acetic acid was kept at 0.5% (v/v) in all later experiments. Buffer salts were not tried as these might react with monensin and cause problems in the column.

Addition of acetonitrile to the mobile phase caused a wide separation of the impurities from the monensin. Therefore 20% (v/v) of acetonitrile was added to the mobile phase. Since monensin is highly soluble in non-polar solvent, methylene chloride was chosen as a component of the mobile phase. The effect of adding varying concentration of methylene chloride to the mobile phase is shown in Fig. 1. Increasing methylene chloride up to 25%/v) significantly increased the peak height of monensin. Above that concentration,

Table 2
Comparison of HPLC (previous and modified), radioactive and spectrophotometric methods of monensin analysis

Method	Minimum detectable amount	Signal to noise ratio	Amount of monensin in a sample of MLV (M)
Modified HPLC (ng ml^{-1})	100	17:1	4.88×10^{-6}
HPLC[a] (ng ml^{-1})	500	13:1	n.d.
Radioisotope[b] (ng ml^{-1})	50	n.d.	5.30×10^{-6}
Spectrophotometric[c] (μg ml^{-1})	300	n.d.	4.65×10^{-6}

[a] The HPLC method reported in [9].
[b] The method mentioned in [1].
[c] The method reported in [6].
n.d., Not done.

peak height decreased. Finally the solvent system was chosen to be methanol–acetonitrile–methylene chloride–water–acetic acid (45:20:25:9.5:0.5). By using this mobile phase the retention time of monensin was found to be 6 min.

High temperature is essential for monensin to react with vanillin reagent in the post column reactor. Several experiments were carried out by increasing the temperature from 70 to 100°C. At higher temperatures peak broadening and reduction in peak height was observed. Therefore all subsequent experiments were carried out at 70°C. The important advantage of using the post column reactor is that it neither interferes nor ruins the column at high temperature.

Vanillin reacts with monensin at high temperature and forms a pink color. The intensity of the color is dependent on vanillin concentration which is shown in Fig. 2. With up to 8% of vanillin the peak height increased but beyond which no substantial improvement was observed. Blanchflower et al. [8] reported that 10% vanillin gave the highest absorbance. The difference in our findings may be attributed to the different composition of the mobile phase.

By using the modified HPLC method a small amount of impurities could be separated and detected in the preparation which is shown in Fig. 3. It was possible to quantify monensin down to 100 ng ml^{-1}, with a signal to noise ratio of $>17:1$. Slight band broadening was observed in the chromatogram which can be attributed to the post column reaction of monensin with vanillin reagent at high temperature. The theoretical plates for the monensin peak was found to be 2930. A standard curve was prepared for monensin within the range 10–100 ng. Linearity was observed within the range ($r^2 > 0.99$). Standard samples consisting of 20, 50 and 80 ng monensin were analyzed by HPLC on three different days and the results are summarized in Table 1. After HPLC analysis of pure monensin samples, interday, intraday and R.S.D. were measured. It was observed that intraday S.D. and R.S.D. were statistically insignificant. Only the interday S.D. is reported in Table 1. Interday S.D. for those samples were found to be 0.675, 0.543 and 0.736 respectively.

The modified HPLC method of monensin analysis was compared to the reported HPLC, radioactive and spectrophotometric methods, and the results are shown in Table 2. The modified HPLC method was found to be five times more sensitive and had a better signal to noise ratio compared to the HPLC method reported earlier [9]. The radioactive method was found to be slightly more sensitive than the modified HPLC method, but it also has some limitations. Radioactive [^{3}H]monensin is not commercially available and it has to be specially prepared which is very expensive. The spectrophotometric method was found to be far less sensitive to the other two methods, and cannot be used for analysis of trace amounts of monensin.

The modified HPLC method was utilized for the analysis of monensin in liposomes and nanocapsule formulations. Comparative efficiency of the three methods was tested by quantitative analyses of monensin in liposomes (MLV) samples (Table 2). The S.D. of the three methods was 3.2×10^{-7} and was within the acceptable limit. Currently, pharmacokinetic experiments on animals using 'stealth liposomes' are underway, where the modified HPLC method could be utilized for the analysis of monensin in blood and tissue samples.

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