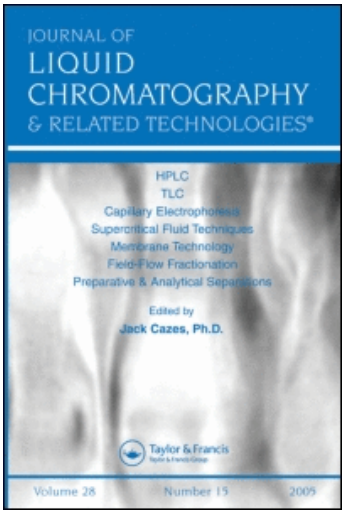

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Chromatography of Rhodamine 123 and Rhodamine 110 on Reverse-Phase Liquid Chromatographic Column

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CHROMATOGRAPHY OF RHODAMINE 123 AND RHODAMINE 110 ON REVERSE-PHASE LIQUID CHROMATOGRAPHIC COLUMN

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ABSTRACT

Rhodamine 123 (Rh 123), a cationic fluorescent dye that accumulates in mitochondria of living cells, is widely used in study of mitochondria and has potential use as anticarcinoma agent. HPLC methods for analysis of Rh 123 have been reported but detailed study of the chromatographic behavior of Rh 123 has not been previously described. Described in this paper is a simple isocratic HPLC method for optimal separation of Rh 123, and Rh 110 which is an impurity present in commerical supply of Rh 123 and also a potential metabolite of Rh 123, resulting from detailed investigation of the effect of various mobile phase parameters on retention behavior of Rh 123.

INTRODUCTION

Rhodamine 123 (Rh 123) is a cationic fluorescent dye that accumulates in the mitochondria of living cells, and is widely used to

study mitochondrial and general cellular functions [1-6]. The mitochondria of a variety of carcinoma cells retain Rh 123 for prolonged periods (2-5 days), whereas, normal cells release it within 1-16 hours [7-10]. Furthermore, it has been observed that whereas, Rh 123 is relatively non-toxic to normal cells, it selectively kills carcinoma cells. This selective accumulation of Rh 123 in tumor cells and non-toxicity to the normal cells has generated interest in Rh 123 as a therapeutic and imaging agent [11-14].

Commercial Rhodamine 123 has been shown to be contaminated by structurally related compound, Rhodamine 110 (Rh 110), which is in turn a potential metabolite of Rh 123 resulting from de-esterification in vivo. Therefore, chromatographic separation of Rh 123 from Rh 110 would be useful in analysis of Rh 123.

Recently, high performance liquid chromatographic methods were reported using a C_8 column and a phenyl column, gradient elution, and fluorescence detection [15-16]. Both methods involve complexity of gradient elution and longer analysis time (20-30 min per sample).

In this paper, we describe a simple and rapid isocratic HPLC method for optimal separation of Rh 123 from Rh 110 using RP-18 column and UV (254 nm) detection, derived from investigation of the effect of mobile phase parameters on retention behavior of Rh 123 and Rh 110.

MATERIALS AND METHODS

Rhodamine 123 and Rhodamine 110 were purchased from Kodak Laboratory and Specialty chemicals, Rochester, N.Y., U.S.A. Rose Bengal was purchased from Fisher Scientific Co., Fair Lawn, N.J., U.S.A. HPLC analyses were performed using Varian, Model 5000 liquid chromatograph equipped with Rheodyne fixed loop (20 µl) injector and Varian UV-50 variable wavelength detector. The

column used for chromatographic analysis was an Alltech C-18 column (10μm, 25 cm x 4.6 mm i.d.). The column was operated at ambient temperature and all injections were 20μl (full loop). Guard column (2 cm x 2 mm i.d.) was filled with 40μm C_{18} Analytichem material.

Acetonitrile used in analysis was HPLC grade from Burdick and Jackson, U.S.A. Buffer was made from HPLC grade ammonium dihydrogen phosphate. pH of the buffer was adjusted to the desired value with 2M ammonium hydroxide or 2M phosphoric acid before mixing with the organic phase. The mobile phase was filtered through a 0.5 μm filter and degassed ultrasonically under reduced pressure.

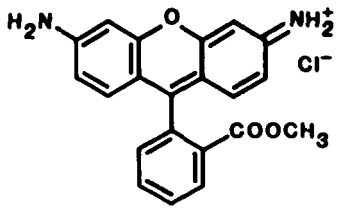
Standard solutions of Rhodamine 123 and Rhodamine 110, and Rose Bengal were made by dissolving 0.1 mg/ml of each of the compounds in HPLC grade methanol. Each of the solution was diluted 10x with mobile phase. Further dilutions with mobile phase were made as needed for construction of the standard curve.

The time required for first unretained peak was used in calculations of the capacity factor (k').

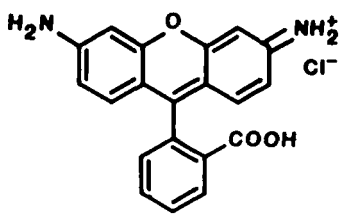
RESULTS AND DISCUSSION

Rh 123 and Rh 110 are structurally similar compounds (Fig. 1). Rh 123 displays a net positive charge at neutral pH due to protonation of imine/amine moiety, whereas, Rh 110 has net zero charge at neutral pH due to simultaneous ionization of carboxylic as well as the imine/amine moiety. Rh 110 is found as an impurity in commercial supply of Rh 123. Rh 110 is also a suspected metabolite of Rh 123 resulting from *in vivo* from de-esterification. Therefore, a method was developed which efficiently separates these two compounds.

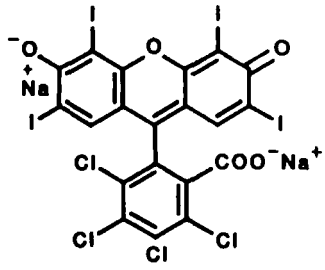
In this investigation, chromatographic behavior of Rh 123 and Rh 110, along with an internal standard (Rose Bengal) of similar



Rhodamine 123 (RH 123)



Rhodamine 110 (RH 110)



Rose Bengal

Fig. 1 Structural formulae of Rh 123, Rh 110 and Rose Bengal

chemical structure (Fig. 1) was studied in detail in order to derive the optimum conditions for separation. Mobile phase consisted of 0.05M ammonium dihydrogen phosphate buffer and acetonitrile. pH of the buffer was adjusted with 2M ammonium hydroxide or with 2M phosphoric acid before mixing with the organic phase. Three mobile phase variables: pH of the mobile phase, molarity of the buffer, and percentage of the organic modifier (acetonitrile) were addressed for their effect on retention and optimum separation of the components of interest. As it is evident from Fig. 2, pH of the buffer had great influence on retention of all three compounds. In the pH range (3.0 - 6.0) investigated, retention of Rh 123 on the chromatographic column increased with increase in pH, while the retention of Rh 110 and Rose Bengal decreased with increase in pH of the buffer. At pH above 5.0, Rh 110 and Rose Bengal were not retained on the

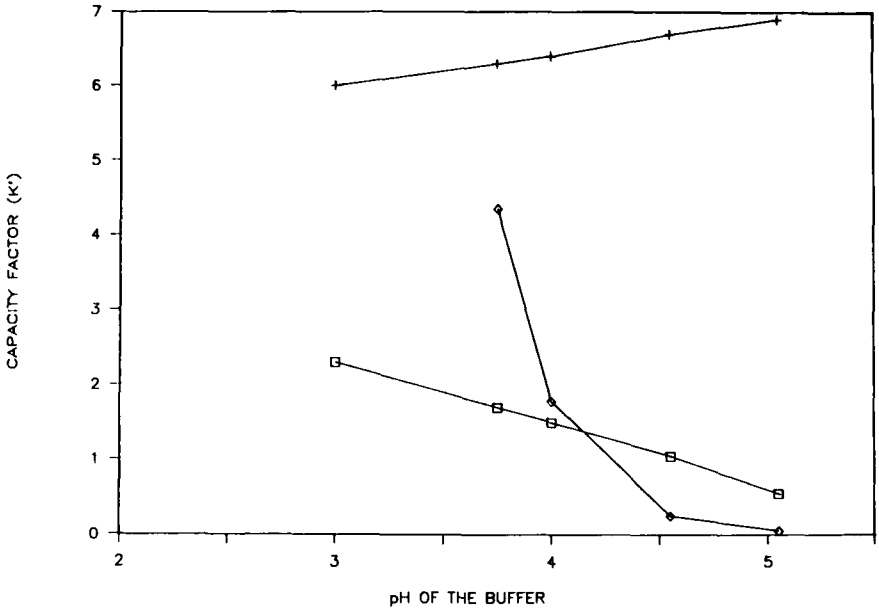


Fig. 2 Effect of pH of buffer on retention of Rh 123 (+), Rh 110 (□), and Rose Bengal (◇). Mobile phase: 0.05M ammonium dihydrogen phosphate, variable pH/acetonitrile (50/50) flow rate: 1.5 ml/min.

column, and as a result eluted almost with the first unretained peak. Also, change in retention of Rh 123 and Rh 110 with change in pH of the buffer was found to be linear in the pH range investigated. On the other hand, retention of Rose Bengal was severely affected in pH range of 3 to 4 and then a plateau was reached.

An examination of the chemical structures of Rh 123 and Rh 110 reveals that increasing pH would increase the fraction of Rh 123 in neutral form (less protonation of the imine/amine moiety) and, thus, increased interaction with the lipophilic column bed. On the other hand, Rh 110 due to the ionization of the carboxylic (-COOH) moiety would become more ionic with increase in pH, and hence, less retained on the lipophilic column bed. Similarly,

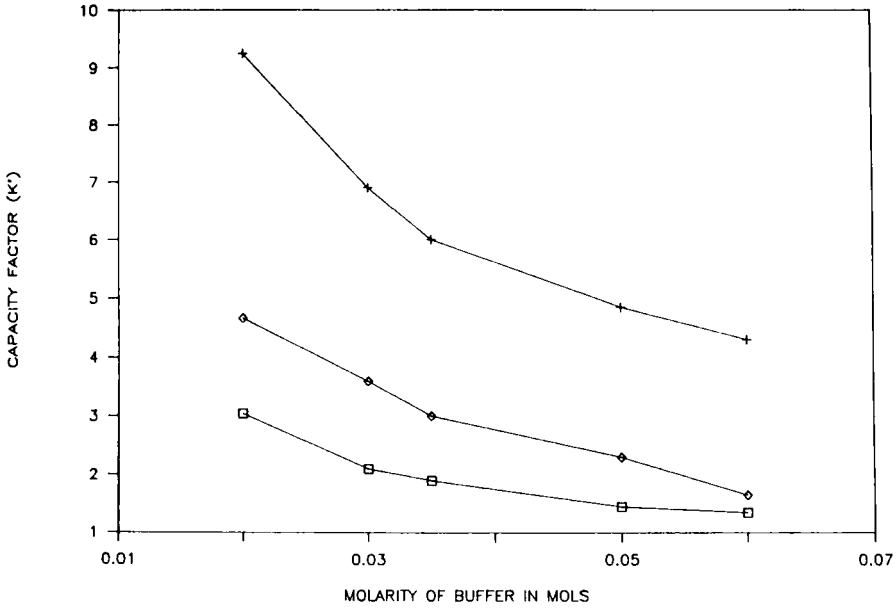


Fig. 3 Effect of molarity of buffer on retention of Rh 123 (+), Rh 110 (□), and Rose Bengal (◇). Mobile phase: variable molarity ammonium dihydrogen phosphate, pH 3.75/acetonitrile (45/55), flow rate: 1.5 ml/min.

Rose Bengal, due to its two acidic moieties, phenolic (-OH) and carboxylic (-COOH), becomes even more ionic as pH of the medium increases, and therefore, is even less retained.

The effect on retention time of Rh 123, Rh 110 and Rose Bengal, with change in molarity of buffer and percent of organic phase follows the expected pattern (Figs. 3,4). All three compounds are less retained as percentage of the organic phase is increased. Also, retention decreases when molarity of the buffer is increased. However, a close examination revealed that retention times of the compounds being analyzed is not equally affected by change in these mobile phase parameters. Optimum separation can now be easily derived from the graphic data presented here.

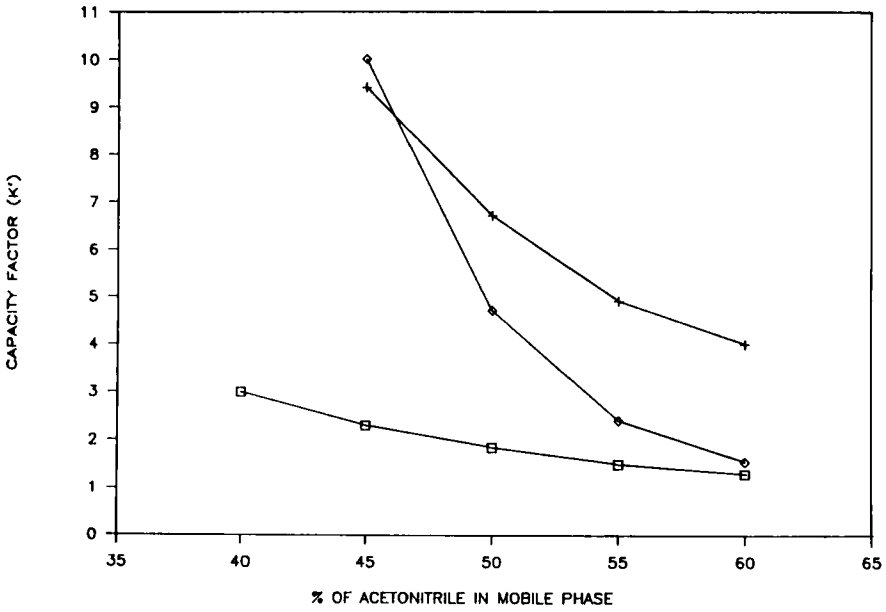


Fig. 4 Effect of percentage of acetonitrile on retention of Rh 123 (+), Rh 110 (□), and Rose Bengal (◇). Mobile phase: 0.05M ammonium dihydrogen phosphate, pH 3.75/variable percentage of acetonitrile, flow rate: 1.5 ml/min.

A mobile phase consisting of 0.05M ammonium dihydrogen phosphate at pH 3.75/CH_3CN (45/55) at a flow rate of 1.5 ml/min results in optimum retention times and resolution, such that repeat analyses can be performed within less than 10 min. (Fig. 5). This allows more sample throughput as opposed to the gradient method where 20-30 min are required for each sample [15-16].

Finally, data in Fig. 6 indicates that the detection limit for Rh 123 is 1.0 ng (∿2 picomols) or less with S/N ratio of 4. Rhodamine 110 also shows similar detection limit (data not shown). Detection of Rh 123 is linear in the range investigated (1 ng to 40 ng). Correlation coefficient = 0.998.

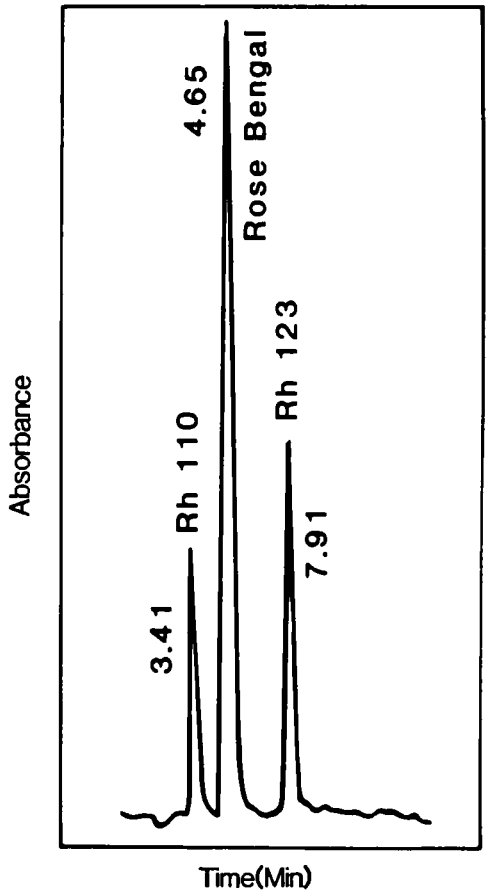


Fig. 5 Simultaneous analysis of Rh 123, Rh 110, and Rose Bengal. Column: Alltech C_{18} (10μm, 25 cm x 4.6 mm i.d.); mobile phase: 0.05 M ammonium dihydrogen phosphate, pH 3.75/ acetonitrile (45/55) flow rate: 1.5 ml/min. R_t: Rh 110 3.41 min; Rose Bengal, 4.65 min; Rh 123, 7.91 min.

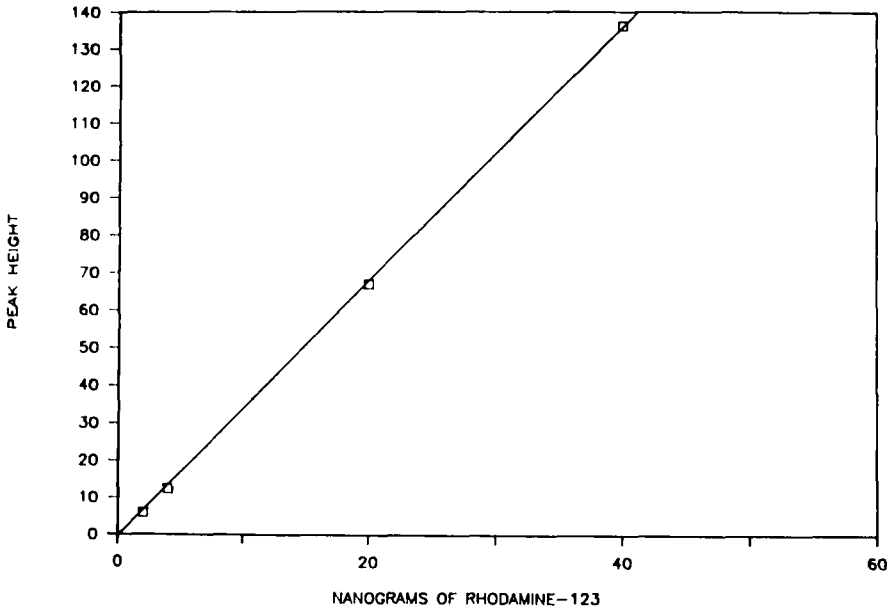


Fig. 6 Quantitation of Rhodamine 123

CONCLUSION

Chromatographic behaviour of Rhodamine 123 and Rhodamine 110 was investigated. Although, not as sensitive as the fluorescence detection, the UV detection at 254 nm provides a limit of detection in the picomole range. More importantly, we have documented a simple and rapid isocratic analysis method for determination of Rhodamine 123 and Rhodamine 110 using reversed-phase HPLC.

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