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Statistical optimization of a reversed-phase liquid chromatographic method for the determination of amiloride and methyclothiazide in tablets[1]

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Abstract

A quantitative high-performance liquid chromatographic method in which amiloride is separated from methyclothiazide on a C_{18} column with detection at 286 nm was developed with the aid of the 'window diagram' technique of Laub and Purnell. The effect of simultaneously varying the pH and methanol to water ratio in the mobile phase were studied to optimize the separation. The method was applied to the quantitative analysis of Lometazid tablets. The powdered tablets were extracted with methanol, containing phenacetin as the internal standard, and assayed by comparison of peak heights after liquid chromatography.

Keywords: Amiloride; High-performance liquid chromatography; Internal standard; Methyclothiazide; Window diagram technique

1. Introduction

Amiloride hydrochloride and methyclothiazide are diuretics that are mainly used in tablet form. Various methods have been proposed for their determination in pharmaceutical formulations, including spectrophotometry [1–4], GLC [5,6] and HPLC [7–10].

The major goal of this investigation was to obtain an adequate separation of amiloride and methyclothiazide in a reasonable time by adjusting appropriate chromatographic factors. The 'window diagram' technique of Laub and Purness [11] has been shown to be an effective means of locating the global optimum if a mathematical functional relationship between chromatographic retention and a single variable factor is known or can be assumed. This paper extends the single-factor window diagram technique to the multi-factor case. Results are presented for a two-factor study in which values of pH and mobile phase composition were chosen to give the optimum chromatographic performance. The best set of conditions was chosen for further investigation.

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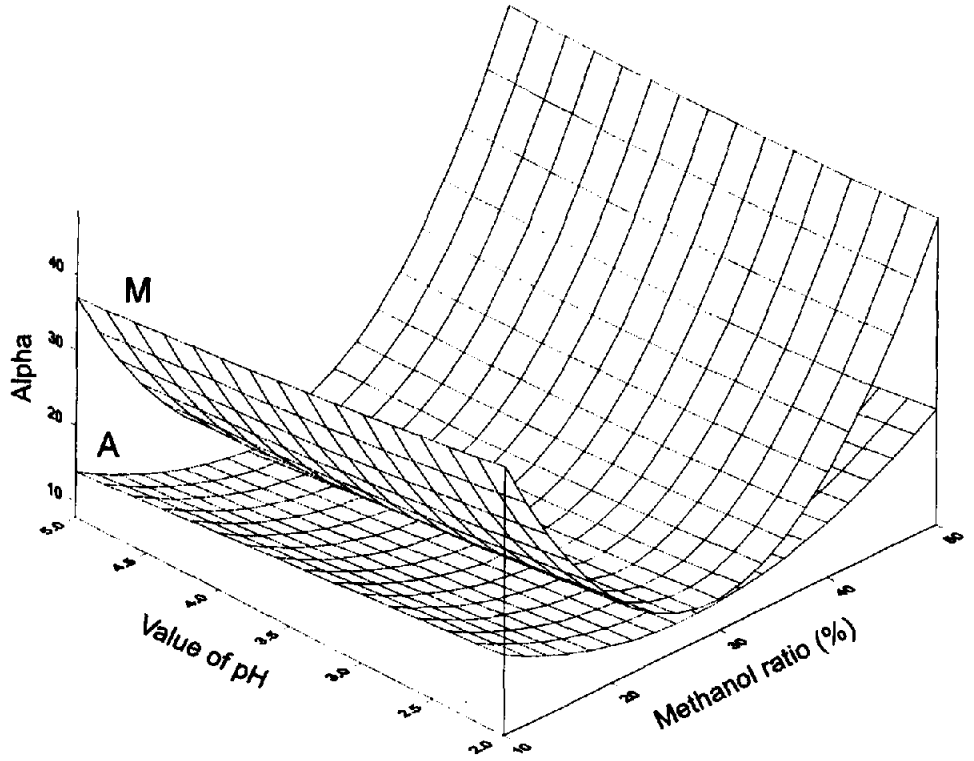

Fig. 1. Predicted retention behaviour of amiloride (A) and methyclothiazide (M) as functions of pH and methanol ratio in mobile phase.

2. Experimental

2.1. Equipment

Separations were made on a 5 μm μBondapak C_{18} column (300 × 3.9 mm i.d.) (Waters, Milford, MA). The injection volume was 10 μl, elution was performed at a flow rate of 1.5 ml min^{-1} and the column was maintained at ambient temperature. The absorbance was monitored at 286 nm. The mobile phase was 0.05 M KH_2PO_4–methanol (pH adjusted 3.0 with phosphoric acid (30:70, v/v).

2.2. Solvents and chemicals

Standards of amiloride and methylchlothiazide and Lometazid tablets were supplied by ICN Galenika (Belgrade, Serbia). The internal standard was phenacetin. All the solvents used for the preparations of the mobile phase were of HPLC grade and the mixtures were filtered and degassed before use.

2.3. Solutions

2.3.1. Internal standard solution

An 800 μg ml^{-1} solution of phenacetin in methanol was prepared.

2.3.2. Stock standard solution

About 10 mg of amiloride reference material and 5 mg of methyclothiazide reference material were precisely weighed, dissolved in internal standard solution and diluted to 100 ml with the same solvent to form a stock standard solution.

2.3.3. Working standard solutions

Working standard solutions were prepared by dilution of 4 ml of the stock standard solution to 10 ml with the internal standard solution. Ten solutions were prepared.

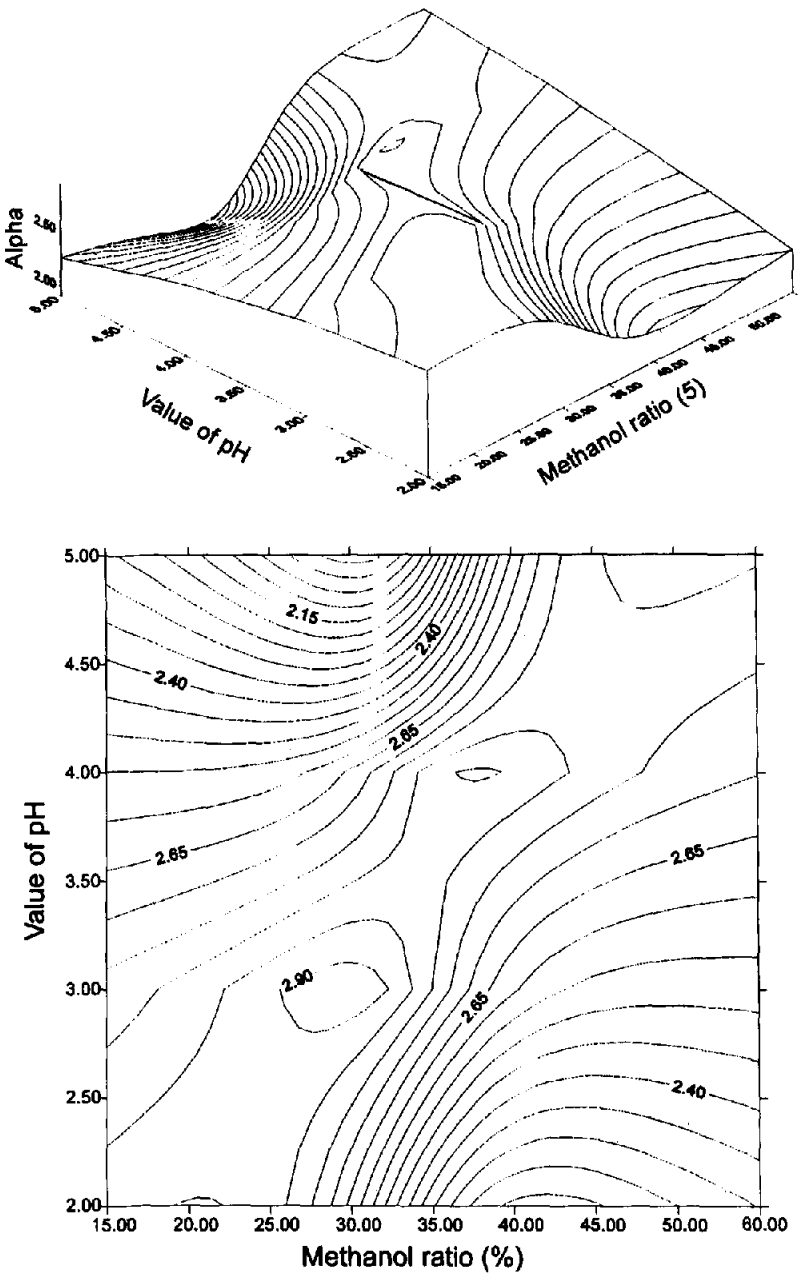

Fig. 2. Predicted relative retention surface (α) of amiloride and methyclothiazide and contours of constant response for the retention surface as a function of pH and mobile phase composition.

2.3.4. Preparations of calibration curve

Volumes of 1, 2, 4, 6, 8 and 10 ml of the stock standard solution were accurately transferred into six 10-ml volumetric flasks and diluted to volume with the internal standard solution.

2.3.5. Sample solutions

A finely powdered tablet was accurately transferred into a 100 ml calibrated flask and diluted to volume with internal standard solution. The mixture was sonicated for 5 min at room temperature

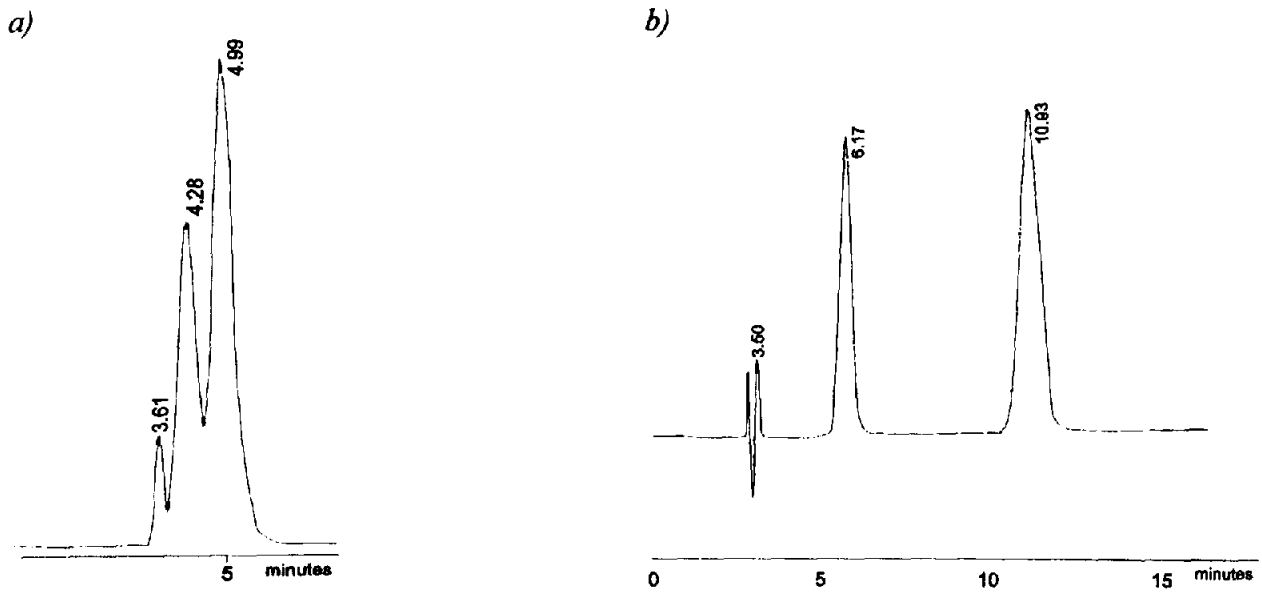

Fig. 3. (a) Separation of amiloride and methyclothiazide before optimization. Eluent, methanol–water (50:50, v/v) (pH 5.2); flow rate, 1.5 ml min^{-1}. (b) Separation of amiloride and methyclothiazide after optimization. Eluent, methanol–water (30:70, v/v) (pH 3.0); flow rate, 1.5 ml min^{-1}.

and then centrifuged at 2500*g* for 5 min. The supernatant liquid was filtered through a 1.5 μm membrane filter. A 4 ml volume of this solution was diluted to 10 ml with the eternal standard solution. Ten solutions were prepared.

2.4. Procedure

Three injections (10 μl) of each of these solutions and of undiluted phenacetin standard solution were made into the chromatographic system. The areas of the peaks were measured and the ratios of the peak area of amiloride and methyclothiazide to that of the internal standard were calculated for each injection. For the calibration curve the average peak-area ratio for each dilution was plotted against the quantity of amiloride and methyclothiazide in the solution.

3. Results and discussion

3.1. Optimum conditions for chromatographic procedure

The combined effects of pH and mobile phase composition on the reversed-phase liquid chromatographic behaviour of amiloride and methyclothiazide were studied. The effects of these factors were examined in the range of conditions where they provided acceptable retention and resolution. The effect of methanol concentration was tested at proportions from 10 to 50% and the effect of pH was tested in the range 3–6.

A response surface method was used to specify the retention times of amiloride and methyclothiazide for all combinations of pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) and five combinations of

methanol–water ratios in the mobile phase (10:90, 20:80, 30:70, 40:60 and 50:50, v/v).

Fig. 1 shows the predicted retention behaviour of amiloride and methyclothiazide as functions of both pH and mobile phase composition. The retention time response sufaces of these two components have been superimposed. Under the experimental conditions investigated, the two surfaces do not intersect, so there is no possibility of elution order reversal and identical retention times for these two components. We have a broad range of experimental conditions that will give an acceptable separation of amiloride and methyclothiazide.

The 'window diagram' technique pioneered by Laub and Purnell [11] for single-factor optimization was applied to the present multi-factor case to obtain an optimum separation. Because the relative retention, α, is a better measure of separation than is the difference in retention times, the two-dimensional 'alpha diagram' shown in Fig. 2 was produced by dividing the higher capacity factor surface by the lower capacity factor surface at all combinations of pH and mobile phase composition. The ratios of these capacity factor sufaces then give the relative retention surface. The two domains giving acceptable separations are evident in Fig. 2 as the higher parts of the surface.

Table 1
Precision of the assay expressed as RSD for 10 samples

Sample No.	Response[a]	
	Amiloride	Methyclothiazide
1	0.312	0.029
2	0.313	0.026
3	0.314	0.029
4	0.323	0.028
5	0.309	0.029
6	0.321	0.028
7	0.310	0.029
8	0.314	0.029
9	0.307	0.028
10	0.312	0.028
RSD (%)	1.61	2.55

[a] Response = peak-area response of drug divided by peak area of internal standard.

Table 2
Recoveries from ten Lometrazid tablet sample solutions

Sample No.	Recovery (%)	
	Amiloride	Methyclothiazide
1	100.60	96.90
2	97.64	95.14
3	98.42	101.58
4	98.80	99.79
5	97.92	95.36
6	98.28	98.47
7	99.32	102.18
8	95.55	100.65
9	104.95	99.91
10	105.72	100.15
Mean	99.72	99.01

The unacceptable domain occurs in the 'basin' of the figure.

The mobile phase composition (of those tested) that provides an acceptable resolution of amiloride and methyclothiazide in a short elution time is KH_2PO_4–methanol (30:70 v/v) and the optimum pH is 3.0. Fig. 3 presents chromatograms showing separations under the worst (non-optimized) and best (optimized) conditions.

3.2. Quantitative determinations

The method was tested for specificity, linearity, precision and reproducibility.

The specificity of the method was investigated by observing potential interferences between amiloride and methyclothiazide and with tablet excipients. No interfering peaks were present in the chromatograms. The k' values for amiloride and methyclothiazide were 0.98 and 2.1, respectively. HPLC allows the direct determination of amiloride in pharmaceutical dosage forms not only in the presence of the excipients, but also in formulations containing methyclothiazide and vice versa. Eluted sample and standard peaks were collected and a complete UV spectrum of each peak was obtained. In all cases the sample and standard peaks were found to be identical.

The linearity of the relationship between peak area and concentration was determined by analysing six standard solutions over the concen-

Table 3
Statistical analysis of results for the determination of amiloride and methyclothiazide

Sample[a]	Concentration (μg/ml)	Found (μg/ml)	SD (μg)	RSD (%)	Recovery (%)
Standard solution (bulk drug):					
Amiloride	40	40.00	0.06	1.61	97.94–103.04
Methyclothiazide	20	20.00	0.51	2.55	94.85–102.14
Sample solution (Lometazid tablets):					
Amiloride	40	39.89	1.28	3.23	95.55–104.95
Methyclothiazide	20	19.80	0.47	2.39	95.14–102.18

[a] $n = 10$

tration range 10–100 μg ml^{-1} for amiloride and 5–50 μg ml^{-1} for methyclothiazide. The parameters of the linear regression equation were calculated for each component. The regression equation was $y = -0.0101 + 0.0087x$ for amiloride and $y = -0.0012 + 0.0015x$ for methyclothiazide. For all analytes the relationship between peak-height ratio of drug to internal standard and concentration was highly linear over the entire concentration range (correlation coefficients of the calibration curves were greater than 0.999 and the intercept was not significantly different from zero ($P = 0.05$)). This allows only one standard solution to be used for the determination.

The limit of quantification (LOQ) was determined as the smallest amount of analyte which can be reproducibly qualified above baseline noise, for which duplicate injections resulted in a RSD of $\leqslant 3\%$. A practical LOQ giving a good precision and acceptable accuracy was 5 μg ml^{-1} for amiloride and 3 μg ml^{-1} for methyclothiazide.

The precision of the chromatographic procedure was assessed by analysing ten solutions containing known quantities of investigated compounds (40 μg ml^{-1} for amiloride and 20 μg ml^{-1} for methyclothiazide). The RSD shows the satisfactory repeatability of the system (Table 1).

Reproducibility studies were performed by analysing ten Lometazid tablets (Table 2). A summary of results is presented in Table 3. The high recovery and the low RSD confirm the suitability of the proposed method for the routine determination of amiloride and methclothiazide in pharmaceutical preparations.

4. Conclusion

HPLC provides a convenient and efficient method for the determination of amiloride and methyclothiazide in its dosage forms. There was no interference in the product examined, so no addition extraction or separation procedures are required. The method is rapid and sensitive enough to be used for single tablet analyses.

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