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The use of a response surface methodology on HPLC analysis of methyldopa, amiloride and hydrochlorothiazide in tablets

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Abstract

A multifactor optimisation technique is successfully applied to develop a new HPLC method in which methyldopa, hydrochlorothiazide and amiloride were analysed and determined on a C18 column with detection at 286 nm. The optimal conditions of HPLC separation were determined with the aid of the response surface diagram — 'window diagram'. The effect of simultaneously varying the pH, proportion aqueous acetic acidum and methanol in the mobile phase were studied to optimise the separation. The mobile phase composition that provides an acceptable resolution methyldopa, hydrochlorothiazide and amiloride in a short elution time is water–methanol (75:25) and pH 3.60. The *k'* values for methyldopa, hydrochlorothiazide and amiloride after optimisation were 1.40, 2.50 and 5.33, respectively. Relative retention (α) for ratio hydrochlorothiazide/methyldopa and amiloride/hydrochlorothiazide were 1.767 and 2.159, respectively. Correlation coefficients of the calibration curves for all analytes were greater than 0.995 and the R.S.D. values for the slope and the intercept with respect to the linearity were less than 2%. A method is applied for the quantitative analysis of Alatan® tablets (Lek-Ljubljana). The powdered tablets are extracted with methanol, containing caffeine as the internal standard and assayed by comparison of peak areas after liquid chromatography. The high recovery (for all analytes about 100%) and the low R.S.D. ($<2\%$) confirm good precision and reproducibility of the chromatographic method.

Keywords: Methyldopa; Amiloride; Hydrochlorothiazide; High-performance liquid chromatography; Internal standard; Window diagram

1. Introduction

Methyldopa, amiloride hyadrochloride and hydrochlorothiazide are applied in treatment of hypertensis in tablet form. Methods for their

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determination in pharmaceutical formulations are based on spectrophotometry [1–4], GLC [5,6] and high-performance liquid chromatography (HPLC) [7–18].

Hydrochlorothiazide and amiloride hydrochloride are analysed separately (without methyldopa) by spectrophotometry and GLC method. Numerous HPLC methods for the determination of hydrochlorothiazide [7–11], of amiloride [12–14], and both hydrochlorothiazide and amiloride [15,16] in biological fluids exist. Published HPLC methods for determination of amiloride includes fluorescence detection. Methyldopa are determined in capsules [17,18] and human plasma by HPLC method with fluorescence detection [19].

This report describes a new, sensitive and reproducible reversed-phase HPLC technique for the simultaneous separation and quantitation of hydrochlorothiazide and amiloride hydrochloride in tablets with UV detection at 286 nm. The major goal of this investigation was to obtain quality separation of methyldopa, hydrochlorothiazide and amiloride hydrochloride in a reasonable analysis time by adjusting acceptable chromatographic factors. Good chromatography requires capacity factors to be neither too low (bad resolution), nor too high (long analysis time, pure detection sensitivity). A mathematical description of such a goal is called an optimisation criterion. Usually, the methods are based on the optimisation of the mobile phase composition, i.e. on the concentration of the organic modifier and the optimisation of pH. The degree of ionisation of solutes, stationary phase and mobile phase additives may be affected by the pH and may lead to better selectivity. On varying the pH, the selectivity varies, but so does retention. In order to allow work at the pH value that yields the best possible selectivity, it is necessary to compensate for changes in retention. The best way is to vary the pH and aqueous/organic ratio simultaneously.

The 'window diagram' technique of Laub and Purnell [20–24] 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.

Single-factor systems for which the window diagram technique has been used successfully include variation of stationary phase composition in gas chromatography [20], variation of pH in liquid chromatography [25–27], and variation of lanthanide-induced-shift reagent concentration in nuclear magnetic resonance spectrometry [24].

Laub and Purnell [20] have shown that plotting the relative retention (α) as a function of a single chromatographic factor (e.g. pH) for all the possible pairs of compounds in a mixture gives a 'window diagram' that can be used to locate the globally optimal experimental condition. The 'windows' consist of the areas below the curves showing lowest relative retention. The experimental condition corresponding to the top of the tallest window gives the best possible separation of the two worst separated pairs of compounds [20].

This paper extends the single-factor window diagram technique to the multifactor case. Results are presented for the two-factor study in which values of pH and mobile phase composition are chosen to give the optimal chromatographic performance. The effects of methanol were examined in the range of 10–50% and pH at 2.85–6.00. The best set of conditions was chosen for further investigation.

2. Experimental

2.1. Equipment

Separations were made on a Waters 5 μm μ Bondapak C-18 column (300 × 3.9 mm i.d., Waters Milford, MA, USA). The injection volume was 10 μl, 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 at 286 nm. The mobile phase was water–methanol (75:25; v/v), pH 3.60 (adjusted with CH_3COOH). Hardware used for the applied response surface methodology was Pentium II PC and statistica for Windows software.

2.2. Solvents and chemicals

Standards of methyldopa, amiloride hydochloride and hydrochlorotiazide and Alatan® tablets (containing amiloride hydochloride 2.5 mg, hydrochlorotiazide 25 mg and methyldopa 250 mg) were supplied by Lek, Ljubljana, Slovenia. The chromatographic internal standard was caffeine. 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

A 80 μg ml^{-1} solution of caffeine in methanol was prepared.

2.3.2. Stock solution

About 1000 mg of methyldopa, 100 mg of hydrochlorothiazide and 10 mg of amiloride hydochloride reference material was weighed precisely, dissolved in internal standard solution and diluted to 100 ml.

Then, 1 ml of this solution was diluted to 10 ml with the same solvent to form a stock solution.

2.3.3. Standard solutions

Working standard solutions were prepared by diluting 0.5 ml volume of this solution to 10 ml with the internal standard solution. Ten solutions were prepared. The standard solutions were stable during the assay.

2.3.4. Preparations of standard curve

Thus, 90, 300, 500, 700 and 900 μm of stock solutions were accurately transferred into five 10-ml volumetric flasks and diluted to volume with the internal standard solution.

2.4. Sample preparation

A finely powdered tablet was accurately transferred to a 50 ml calibrated flask and diluted to volume with internal standard solution. The mixture was sonicated for 5 min at room temperature and then centrifuged at $2500 \times g$ for 5 min. The supernatant liquid was filtered through a 1.5-μm membrane filter. This solution (1 ml) was diluted to 10 ml with internal standard solution. A 1.0-ml volume of this solution was diluted to 10 ml with the internal standard solution. Ten solutions were prepared. The solutions of analytes were stable during the assay.

2.5. Procedure

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

3. Results and discussion

3.1. Optimum conditions for chromatographic procedure

This work presents the results of an experimental study design to determine the combine effect of pH and mobile phase composition on the reverse-phase liquid chromatographic behaviour of methyldopa, hydrochlorothiazide and amiloride hydochloride. The effects of these factors were examined in the range of conditions where they provided acceptable retention and resolution. The effect of the ratio of methanol was tested at a proportion of 10–50% and the effect of pH was tested at pH 2.85–6.

A response surface methodology was used to specify the retention time of methyldopa, hydrochlorothiazide and amiloride hydochloride to all the combination of pH values (2.85, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) and the six combinations of methanol — 0.5% water solution of acetic acid ratio in mobile phase (10:90, 15:85, 20:80, 30:70, 40:60, 50:50).

The 'window diagram' technique pioneered by Laub and Purnell for the single factor optimisa-

tion was applied to the present multifactor case to obtain optimal separation.

Fig. 1 shows the predicted retention behaviour of methyldopa, hydrochlorothiazide and amiloride hydochloride as functions of both pH and mobile phase composition. The retention time response sufaces of these three components have been superimposed. Under the experimental conditions investigated, the three surfaces do not intersect, so there is no possibility of elution order reversal and identical retention times for these three components. Relative retention, (α) is a better measure of separation than is the difference in retention times [28]. 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 combination of pH and mobile phase composition. The ratios of these capacity factor surfaces than give the relative retention surface. The domains giving acceptable separations are evident in Fig. 2 as the higher parts of the surface. Values greater than 1.4 were set equal to 14.4 [29]. The unacceptable domain occurs in the lower parts of the figure.

The mobile phase composition (of those tested) that provides acceptable resolution of methyldopa, hydrochlorotiazide and amiloride in a short elution time (10 min) is water–methanol (75:25) and pH is 3.60 (adjust at CH_3COOH). There are

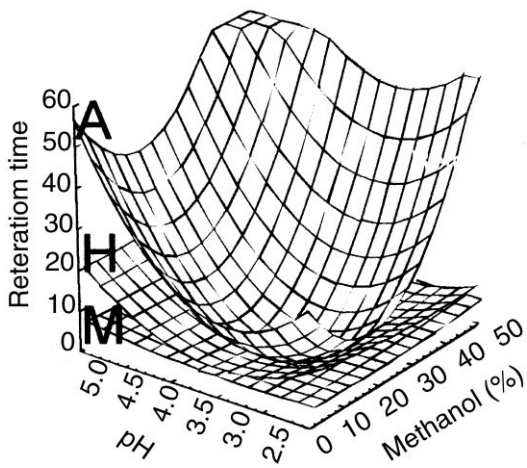

Fig. 1. Predicted retention behaviour of methyldopa (M), amiloride (A) and hydrochlorothiazide (H) as a function of pH and methanol ration in mobile phase.

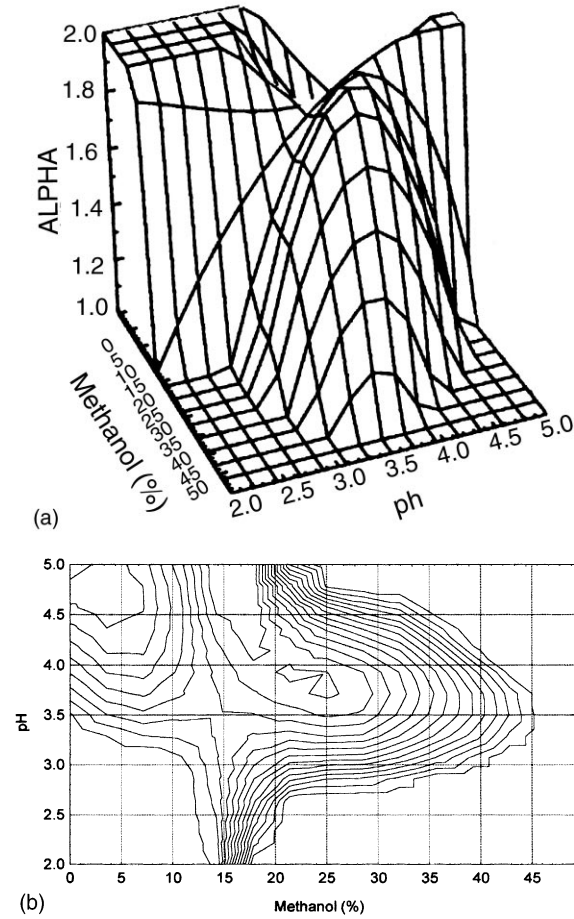

Fig. 2. Predicted relative retention (alpha) values for methyldopa, amiloride and hydrochlorothiazide as a function of pH and methanol percentage.

other domains in Fig. 2 that give the same optimum results ($\alpha \cong 1.4$, quality separation within 10 min). The domain on the left is preferable because it is more rigid, and not so sensitive to the small changes in pH and methanol percentage.

Fig. 3 (a) presents chromatogram for standard solution showing the separation under the best (optimised) conditions. Fig. 3 (b) presents chromatogram for Alatan® tablets showing the separation under the best (optimised) conditions.

3.2. Quantitative determinations

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

cificity of the method was investigated by observing any interference between methyldopa, hydrochlorothiazide and amiloride hydochloride and with tablet excipient. No interfering peaks and no peaks that indicate degradation products were present in the chromatograms. It is confirmed by the appearance of the baseline of chromatogram analytes (Fig. 3b) and recovery value of analytes (Table 2). The described method may use as stability-indicating assay. The k' values for methyldopa, hydrochlorothiazide and amiloride were 1.40, 2.50 and 5.33, respectively. HPLC allows the direct analysis of amiloride in pharmaceutical dosage forms not only in the presence of the excipient, but also in formulation containing hydrochlorothiazide and vice versa. Eluting sample and standard peaks were collected and a complete ultraviolet spectrum of each peak was obtained. In all cases, sample and standard peaks were found to be identical.

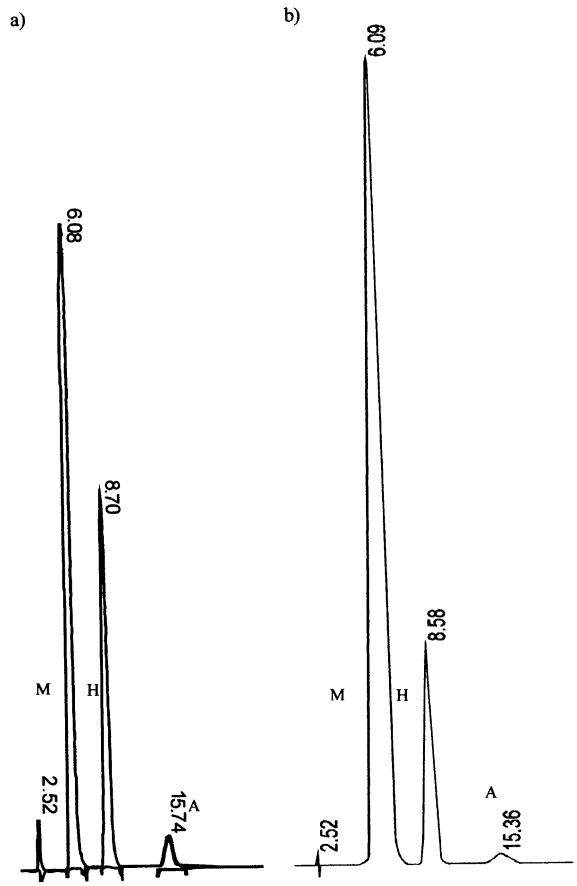

Fig. 3. (a) Separation of methyldopa (M), hydrochlorothiazide (H), amiloride (A) on optimal conditions; Eluent: methanol–water (25:75); pH 3.60; flow rate 1.0 ml min^{-1}.(b) Chromatogram of methyldopa (M), hydrochlorothiazide (H), amiloride (A) in Alatan® tablets on optimal conditions; Eluent: methanol–water (25:75); pH 3.60; flow rate 1.0 ml min^{-1}.

The linearity of the relationship between peak area and concentration was determined by analysing five standard solutions over the concentration range 9.0–90 μg ml^{-1} for methyldopa, 0.9–9.0 μg ml^{-1} for hydrochlorothiazide 0.09–0.90 μg ml^{-1} for amiloride. The parameters of the linear regression equation were calculated for each component. The regression equation was $Y = -0.0083 + 0.1545X$ for amiloride and $Y = -0.0063 + 0.1372X$ for hydrochlorothiazide and for methyldopa $Y = 0.022 + 0.034X$. For all analytes, the relationship between peak area 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 0.995 and the R.S.D. values for the slope and the intercept with respect to the linearity were 1.2, 1.5 and 1.7%, respectively, calculated at the 100% analyte level [30]. This allows only one standard solution to be used for the determination. Test to determine the limit of detection (LOD) and limit of quantitation (LOQ) for the procedure are performed on samples, containing very low concentrations of analyte.

LOD was measured as the lowest amount of analyte that may be detected above baseline noise. LOD for amiloride was 0.045 μg ml^{-1} and LOD for hydrochlorothiazide was 0.45 μg ml^{-1} and 4.5 μg ml^{-1} for methyldopa.

LOQ was measured as the lowest amount of analyte that can be reproducibly quantified above baseline noise, for which duplicate injections resulted in a R.S.D. of $\leq 3\%$. A practical LOQ giving a good precision and acceptable accuracy was 0.09 μg ml^{-1} for amiloride and 0.90 μg ml^{-1} for hydrochlorothiazide and for methyldopa 9.00 μg ml^{-1}.

The precision of the chromatographic procedure was assessed by analysing ten solutions containing known quantities of investigated compounds. (0.5 μg ml^{-1} for amiloride and 5 μg

Table 1
Precision of the assay expressed as percentage R.S.D. of ten samples

Sample number	Response [a]		
	Methyldopa (50.0 μg ml^{-1})	Hydrochlorothiazide (5.0 μg ml^{-1})	Amiloride (0.5 μg ml^{-1})
1	1.687	0.664	0.065
2	1.668	0.669	0.067
3	1.688	0.682	0.067
4	1.670	0.676	0.067
5	1.660	0.676	0.068
6	1.677	0.760	0.070
7	1.697	0.672	0.070
8	1.667	0.684	0.068
9	1.652	0.679	0.068
10	1.672	0.684	0.069
S.D.	0.3874	0.0414	0.0087
R.S.D.	0.7787	0.83	1.73
Sx	0.1222	0.0131	0.0027
R (%)	100.00	100.00	100.00

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

Table 2
Statistical analysis of results in the determination of methyldopa, hydrochlorothiazide and amiloride

($n = 10$)	Conc. (μg ml^{-1})	Found (μg ml^{-1})	S.D. (μg)	R.S.D. (%)	Mean recovery (%)
Standard solution (bulk drug)					
Methyldopa	50	50	0.387	0.77	100.00
Hydrochlorothiazide	5.0	5	0.041	0.83	100.00
Amiloride	0.5	0.5	0.009	1.73	100.00
Sample solution (Alatan® tbl.)					
Methyldopa	50	50.27	0.677	1.346	100.55
Hydrochlorothiazide	5.0	4.992	0.094	1.88	99.84
Amiloride	0.5	0.501	0.015	3.15	100.22

ml^{-1} for hydrochlorothiazide and 50 μg ml^{-1} for methyldopa). The relative standard deviation (R.S.D., %) shows the satisfactory repeatability of the system (Table 1).

Reproducibility studies were performed by analysing ten Alatan® tablets. A summary of results is presented in Table 2. Recoveries are calculated as response of sample divided by response of drug. The high recovery and the low R.S.D. confirm the suitability of the proposed method for the routine analysis of amiloride, hydrochlorothiazide and methyldopa in pharmaceutical preparations.

4. Conclusion

The RP HPLC provides a convenient and efficient method for the separation and determination of methyldopa, hydrochlorothiazide and amiloride hydochloride in its dosage forms. The method provides nanogram sensitivity and adequate linearity and repeatability. 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 analysis.

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