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Short communication

Ion-pair reversed-phase liquid chromatographic determination of dihydralazine

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Abstract

Dihydralazine, a vasodilator drug chemically related to the structural class of the hydrazinophthalazines, was used as a tracer to monitor (water–oil–water) multiple emulsion stability. The hydrophilic character of the ionized form of dihydralazine hinders its chromatographic behaviour by inducing a low retention with alkyl-grafted supports. In addition, the two hydrazine functions induce some important interactions with the polar sites of the stationary phase. This yields highly tailing and poorly retained peaks, hence there is a need to develop an ion-pair technique on a stationary phase recommended for the analysis of basic drugs. After optimization, the optimum chromatogram was obtained with a C_8 RP-Select B column using a mobile phase consisting of a mixture of methanol (50%) and an aqueous solution of phosphate ($2.5 \cdot 10^{-2}$ *M*) and sodium heptanesulfonate ($2.5 \cdot 10^{-2}$ *M*) at pH 4.4. This method was found to be accurate and precise for a range of dihydralazine concentrations from 1 to 120 μg/ml.

1. Introduction

Dihydralazine is a vasodilator drug with antihypertensive properties. Several methods have been reported for its determination in biological fluids by UV or visible spectrophotometry [1–3], gas chromatography [4], potentiometry [5] and also electrochemical methods [6]. However, direct spectrophotometric and potentiometric determinations can show a lack of specificity when performing the analysis in complex matrices, and a derivatization step is essential for gas chromatographic assay. In liquid chromatography, the determination of dihydralazine is complicated by the amine functions of this drug. Serious peak tailing, due to the interaction with free silanol groups of the stationary phase, is encountered with various chromatographic columns. To overcome this problem, precolumn derivatization can be used. An HPLC procedure has been reported that is suitable for determining dihydralazine in human plasma [7].

In this paper, we describe an ion-pair RP-HPLC technique without derivatization that allows the rapid and precise determination of dihydralazine. The influence of two ion-pair reagents and buffers on solute retention using various stationary phases is discussed.

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2. Experimental

2.1. Instrumentation

Chromatographic measurements were made with a Jasco PU 980 pump (Prolabo, Paris, France) equipped with a Rheodyne model 7125 injection valve with a 20 μl loop. UV detection at 310 nm (maximum absorbance of dihydralazine in the mobile phase) was effected with a Shimadzu SPD-2A UV spectrophotometer (Touzart et Matignon, Vitry-sur-Seine, France). The flow-rate was set at 1 ml/min. The chromatograms were recorded using a Hewlett-Packard Model 3395 integrator.

Two RP columns filled with chromatographic supports from different manufacturers were tested: 5-mm C_{18} SGE 250 GL 4 P815, 250 × 4 mm I.D. (SGE, Villeneuve-St.-George, France), and 5-mm C_8 LiChrospher RP-Select B, 125 × 4 mm. I.D. (Merck, Nogent-sur-Marne, France).

The mobile phase was filtered through a 0.22-μm Millipore filter under vacuum.

2.2. Chemicals

Method validation was carried out using the Merck C_8 column and a LiChrospher 100 RP-18 end-capped C_{18} guard column (10 × 4 mm I.D.) (Merck).

Methanol, obtained from Prolabo, was of HPLC grade. Ultra-high quality water was obtained from a Milli-Q plus 185 system (Millipore, St.-Quentin, France).

Potassium dihydrogenphosphate was obtained from Merck. The pH of the mobile phase was adjusted to 4.4 using orthophosphoric acid. Sodium 1-heptane sulfonate and sodium dodecyl hydrogensulfate LiChropur quality were obtained from Merck. Dihydralazine was obtained from Ciba-Geigy.

3. Results and discussion

The optimization of the chromatographic conditions was achieved by considering two parameters: the capacity factor k' and asymmetry factor (B/A). The latter was calculated at 10% of the peak height using the ratio of the widths of the rear and front sides of the peak.

In a recent study devoted to the analysis of basic drugs using various chromatographic supports, Vervoort et al. [8] reviewed some of the factors affecting the peak symmetry and solute retention. First, the peak symmetry can be related to the interaction between the ionized functions of a drug and the free silanols of the packing. Interactions between silanols and solutes can be lowered by the use of tetrabutylammonium phosphate or phosphate buffer at pH <3.5. This pH could not be met in our case as lowering the pH could compromise the emulsion stability, and hence could lead to overestimation of the emulsion breakdown.

Second, the peak asymmetry increases with increasing k' values. In our case, the capacity factor was not retained as critical, as only one compound was to be determined. The target k' value for dihydralazine was set at $k' \approx 1$.

Third, the peak asymmetry increases with increasing pK_a of the solute and also with the flexibility of the protonated amine function. Owing to its two hydrazine functions, dihydralazine appears as a strongly interacting substance. The determination of the pK_a of dihydralazine was achieved by potentiometric titration: in aqueous solution, this compound exhibits two pK_a values, 6.4 and 10.5, corresponding to the two amine functions. The pH of the mobile phase was set at 4.4, which corresponds to the pH of the inner aqueous phase of the water–oil–water (W/O/W) multiple emulsion. At this pH, dihydralazine is in its fully ionized form.

These three factors explain the poor results encountered with two conventional (C_{18} and phenyl) bonded supports in earlier stages of the method development. Those preliminary experiments showed a poor retention of this analyte and serious peak tailing. When observing the chromatographic behaviour of dihydralazine at different operating conditions, two cases were generally noticed: either the compound eluted very close to the solvent front as a symmetrical

peak, or its retention time was longer but the peak tailing was excessive.

The experiments were continued with two chromatographic supports recommended for the analysis of basic drugs: RP-Select B (C_8) and SGE 250 (C_{18}). The SGE 250 column is based on a C_{18} chain grafted on a polymeric support. The advantage over silica-based columns is that there is no hydroxyl group present within the support [9]. The RP-Select B column is offered as a stationary phase specially deactivated for the chromatographic analysis of basic compounds [10]. Three factors were investigated. First, the influence of the phosphate buffer concentration on the capacity factor and asymmetry factor was studied; second, we inspected the effect of the type and concentration of two counter ions with increasing phosphate buffer concentration; finally, mobile phase refinement was undertaken.

3.1. Influence of the buffer concentration

The influence of the phosphate buffer concentration on the capacity and asymmetry factors was studied by plotting those two values against five concentrations of buffer from 0.01 to 0.1 *M*. As expected, the solute k' decreases with increasing ionic strength of the mobile phase [11–13]. The asymmetry factor increases with increasing k' values. However, on both the C_8 and C_{18} columns, even if the ionic strength is critical, a proper chromatogram cannot be obtained by only varying the buffer concentration. To overcome the dependence between asymmetry factor and capacity factor [14], ion pairing was investigated.

3.2. Nature and concentration of counter ion

The performances of sodium 1-heptanesulfonate (HS) and sodium dodecyl hydrogensulfate (DS) were compared. These two counter ions differ by the lipophilicity of the hydrocarbon chain and the strength of the functional group [11]. Two levels of concentration of each counter ion (0.025 and 0.05 *M* for DS and 0.05 and 0.1 *M* for HS) and four levels of buffer molarity, from 0.01 to 0.1 *M*, were investigated. The upper limit of the counter ion concentration was due to its solubility [15].

The effectiveness of complexation was demonstrated by the increase in k' with increasing concentration of the counter ion (Fig. 1a). Owing to its higher lipophilic character, the minimum concentration required to obtain ion-pair formation is lower with DS than HS and the retention times are significantly longer.

The *B/A* factors encountered with DS using the two chromatographic supports were too high (1.5–3.5). They can be lowered by increasing the

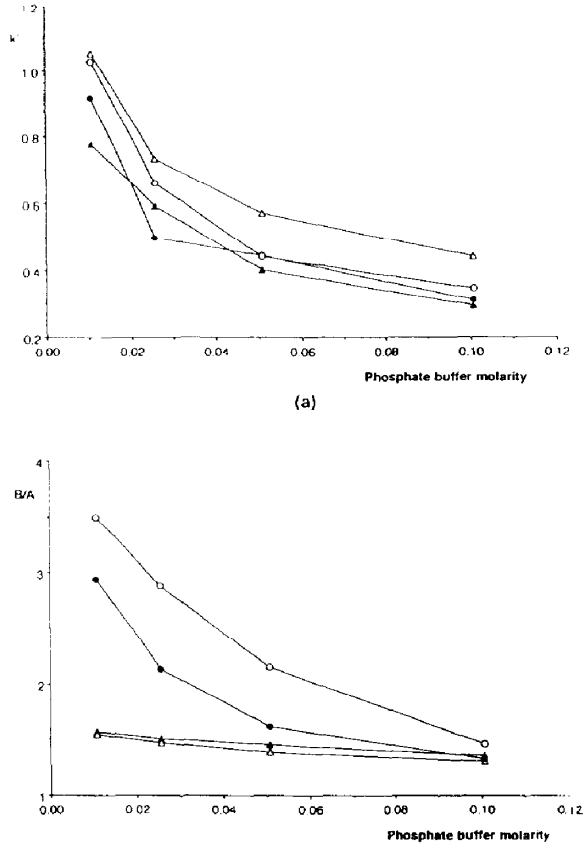

Fig. 1. Comparison of (a) k' and (b) *B/A* values vs. phosphate buffer concentrations with heptanesulfonate at two levels, from two stationary phases: RP-Select B and SGE 250 GL. Chromatographic conditions: mobile phase, methanol–buffer (50:50); flow-rate, 1 ml/mn; detection, UV at 310 nm. ▲ = RP-Select B, 0.005 *M* HS; △ = RP-Select B, HS = 0.01 *M*; ● = SGE 250 GL, HS = 0.005 *M*; ○ = SGE 250 GL, HS = 0.01 *M*.

buffer molarity but high buffer concentrations may deteriorate the chromatographic system, so DS was rejected.

An ion pair is not formed on C_{18} grafted polymeric support: when the HS concentration increased, the retention times were not altered. In contrast, ion-pair formation is observed from the large effect on solute retention when using the RP-Select B.

The asymmetry factor remains nearly unchanged (1.3–1.5) whatever the HS concentration when using RP-Select B (Fig. 1b). Therefore, the method development was carried out with the use of HS and the RP-Select B column.

3.3. Final mobile phase refinement

In the preceding experiments using HS on RP-Select B, the capacity factor remained inadequate. As the peak symmetry appears to be roughly independent of the capacity factor (Fig. 2), when the ion pair is effective, three parameters were then taken into account: the buffer concentration, the HS concentration and the organic modifier content of the mobile phase.

From Fig. 1a, a low buffer concentration was preferred as it induces a higher k'. The buffer molarity was set at 0.025 M.

From Fig. 2, the k' of dihydralazine increases from 0.2 to reach a maximum of 1.4 at HS concentrations between 0.015 and 0.020 M. For higher concentrations of HS, k' decreases with increasing ionic strength of the mobile phase. From this plot, the best compromise between retention and peak asymmetry is at $2.5 \cdot 10^{-2}$ M HS.

As proposed by Goldberg et al. [15], the organic modifier content of the mobile phase was kept constant during the preceding steps of the optimization. A final refinement of the elution strength was studied by varying the methanol content of the mobile phase from 35 to 50%, as this could affect the hydrophobic interactions and the surface concentration of the counter ion [16]. As expected, the k' of dihydralazine decreases with increasing percentage of organic modifier. As no improvement could be expected, we decided to keep the methanol content of the mobile phase at 50%.

The resulting mobile phase used for method validation with the C_8 column was phosphate buffer $2.5 \cdot 10^{-2}$ M, heptanesulfonate $2.5 \cdot 10^{-2}$ M and MeOH–H_2O (50:50).

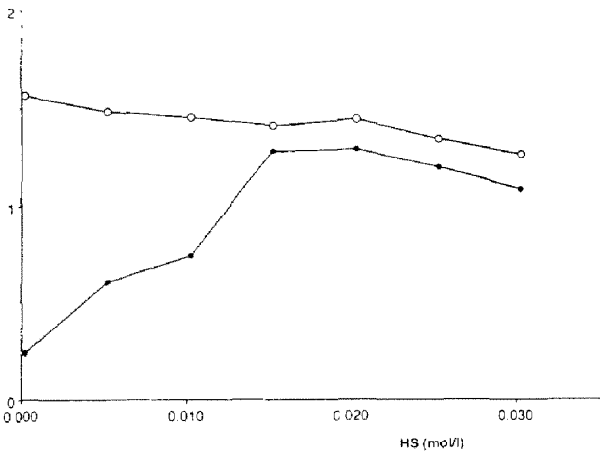

Fig. 2. Plots of (●) k' and (○) B/A vs. heptanesulfonate concentration of the mobile phase. Chromatographic conditions: column, LiChrospher RP-Select B; mobile phase, methanol–buffer (50:50), with $2.5 \cdot 10^{-2}$ M phosphate buffer at pH 4.4.

4. Application

4.1. Validation of the analytical method

The method was applied to determine the yield in the preparation of W/O/W multiple emulsions. Dihydralazine was used as a UV-tracer incorporated in the internal aqueous phase. The efficiency of entrapment of this marker molecule was determined through the concentration of residual dihydralazine in the external aqueous phase. Consequently, the calibration must take into account a broad range of concentrations in order to detect 0–100% entrapment.

The area and height of the dihydralazine peaks were recorded for nine injections at seven levels ranging from 0.001 to 0.12 g/l. The corresponding calibration graphs exhibit good lineari-

ty ($r^2 = 1.000$) in both instances. The plot of residuals (e_i) vs. C shows a uniform variance and a within-group error proportional to C. For both area and height this error is about 1% from 0.001 to 0.025 g/l and 4% at 0.12 g/l.

4.2. Method specificity

The components included in the emulsion formula (emulsifier, oil and inorganic salts) do not lead to significant UV absorbance at 310 nm. Further, the injection of the emulsion without dihydralazine shows that the small peaks generated by those compounds do not interfere with the dihydralazine peak (Fig. 3).

To verify if one of the components included in the W/O/W emulsion generates an analytical bias (matrix effect) by inhibiting or enhancing the solute response, a simplified calibration graph was constructed with five injections at three levels. In this solution, all the components of the W/O/W emulsion were added. For both area and height, the slope of the relationship does not differ significantly for the dihydralazine diluted in mobile phase and the spiked solution (with $\alpha = 0.05$ and 76 df).

The method specificity is also confirmed by the values of the intercept of the calibration graphs for the spiked solutions, which are close to zero (with $\alpha = 0.05$ and 13 df).

The limit of detection [17], defined as the lowest concentration of dihydralazine that the method can detect with a signal-to-noise ratio of 3, is about $1.6 \cdot 10^{-6}$ g/l (area) and $3 \cdot 10^{-5}$ g/l (height).

The accuracy was calculated as the difference

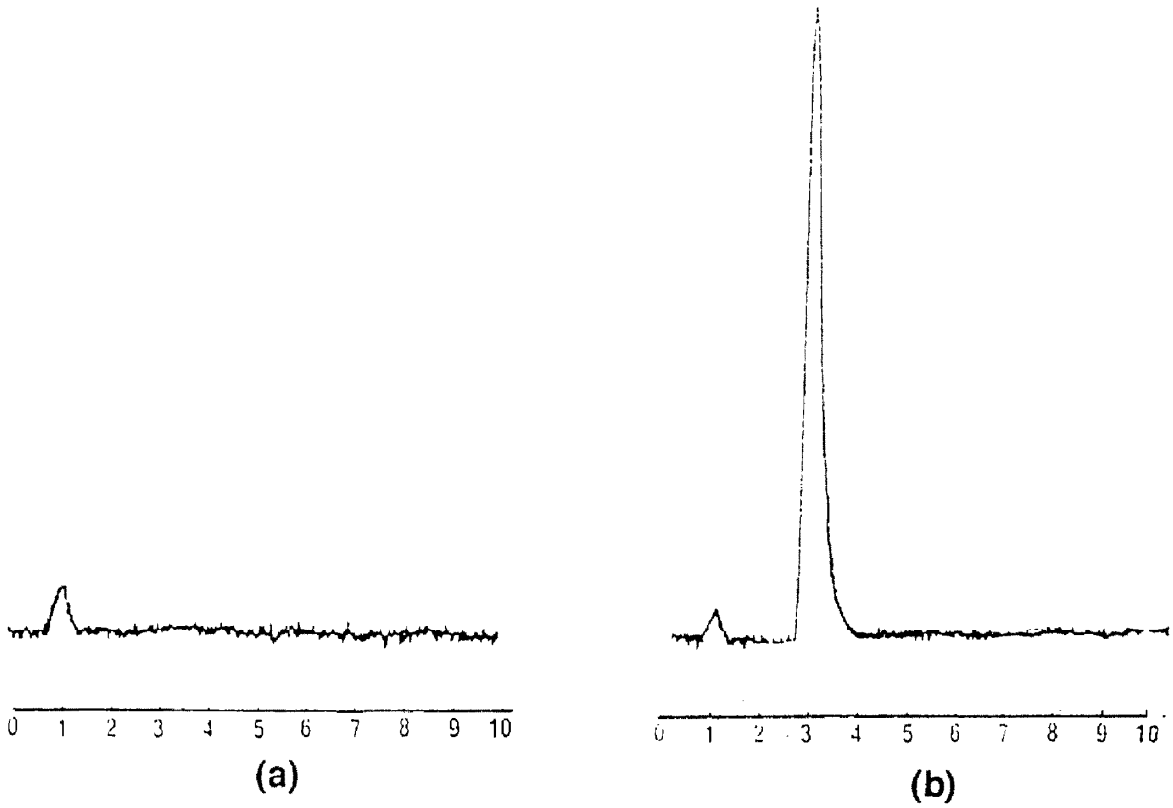

Fig. 3. Chromatogram of the components of the W/O/W multiple emulsion (a) without and (b) with dihydralazine. Chromatographic conditions: column, LiChrospher RP-Select B; mobile phase, methanol–buffer (50:50), with $2.5 \cdot 10^{-2}$ M phosphate buffer at pH 4.4 and heptanesufonate $2.5 \cdot 10^{-2}$ M.

between the mean value and the true value of known concentrations. Student's t-test for height and area showed that the method is accurate with $\alpha = 0.05$ and $df = 4$.

The repeatability was established by performing five consecutive calibrations within the same day and the reproducibility was calculated from a unique calibration repeated on each of five days. The R.S.D. was below 1% for the area-based calculation and about 2% for height, hence the method is both repeatable and reproducible.

The yield in the preparation of a W/O/W multiple emulsion was determined on three batches of 100-g size. During the preparation, 0.03 g of dihydralazine was incorporated in each batch. After dilution and centrifugation of the emulsion, the concentration of residual dihydralazine in the supernatant was measured by HPLC. The efficiency of entrapment was calculated as 91 ± 1%.

5. Conclusions

This work has demonstrated that the ion-pair technique improves the liquid chromatographic behaviour of dihydralazine. Ion-pair formation with heptanesulfonate allows the peak retention to be increased without affecting the peak symmetry when using the RP-Select B stationary phase. The described method allows the rapid, simple and reliable determination of this basic compound. The application of the technique will be extended to stability studies of multiple emulsions.

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