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Packed-column supercritical fluid chromatography of a new dihydropyridine drug based on direct injection of emulsion samples

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Abstract

In this paper, a method based on direct injection in packed-column supercritical fluid chromatography (SFC) for the determination of a dihydropyridine drug in emulsion samples is described. To optimize the chromatographic system, an experimental design approach with four factors was applied. The parameters studied were modifier–carbon dioxide ratio, flow-rate, column temperature and back-pressure. A face-centred central composite design (CCF) yielded a model with good fit and high predictive power. Using plates per second as the response parameter, a fast method for quantitative determination of the analyte was developed. Within the experimental space investigated, the concentration of the polar component in the mobile phase and the flow-rate were found to be the important parameters. Peak compression effects, resulting in high apparent peak numbers (>80 000 versus a nominal value of 7000) were encountered when emulsion samples were injected.

Keywords: Direct injection; Injection methods; Emulsion samples; Pharmaceutical analysis; Experimental design; Central composite design; Dihydropyridines

1. Introduction

Quantitative analysis of target compounds present in emulsion samples normally requires breaking of the emulsion to enable analyte availability. This may be achieved through, e.g., dilution in a suitable solvent [1–4], sometimes followed by ultrasonication [3]. Alternatively, the sample handling step can be omitted if a chromatographic system, suitable for direct injection of the emulsion samples is employed. Such a system should preferably be designed in such a manner that the oil fraction separates from the compounds of interest. Furthermore, the emulsion components should not have a detrimental effect on the separation process.

Reversed-phase liquid chromatographic analysis, which often is the method of choice in pharmaceutical quality control, is not the appropriate choice for emulsion applications since the lipid fraction would adsorb strongly to the non-polar stationary phase. Normal-phase liquid chromatography is a better alternative, allowing the lipid fraction to elute with the front. The technique nevertheless suffers from some disadvantages. System equilibration can be a tedious process and the mobile phase is often a mixture of hazardous organic solvents. In packed silica column SFC, on the other hand, the bulk of the mobile phase consists of environmentally acceptable carbon dioxide. As in normal-phase liquid chromatography, the lipid fraction is bound to elute near the void without interfering with the target analytes. In contrast to normal-phase liquid chromatography

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(LC), phase equilibration times are usually very fast, in the order of a few minutes. Packed-column SFC applications [5–7] have shown that the technique offers fast and efficient separations. In pharmaceutical applications, packed-column SFC has been used in the analysis of antipsychotics [8], antidepressants [9], calcium channel blocking agents [10,11], proton pump inhibitors [12] and chiral compounds [13].

In this work, a method employing packed-column SFC for the determination of a dihydropyridine drug in an emulsion is described. The results from the newly developed SFC method is also compared with data obtained from a routine method employing LC. To our knowledge, this is the first approach which uses SFC in the analysis of pharmaceutical emulsion samples. The influence of various experimental parameters (type and amount of modifier, flow-rate, column temperature) on the chromatographic system was studied. The chromatographic performance was optimized using experimental design, an approach that has proven helpful in optimizations of various chromatographic systems. For instance, results from designed experiments were used to create multivariate models for predictions of optimal separations in reversed-phase LC [14–16]. Multivariate analysis also provides interesting opportunities to interpret the correlations between the experimental variables and the responses, e.g., retention and separation factors [14–16].

2. Experimental

2.1. Instrumentation

A Hewlett-Packard G1205A SFC instrument, equipped with dual reciprocating pumps, a variable-wavelength detector and an autosampler, was used throughout this work. Flow-rate, fluid composition and column outlet pressure are independently controlled by the system. The emulsion samples were injected using a 5-μl injection loop on a Hypersil (Hewlett-Packard, Wilmington, DE, USA) column (200×4.6 mm I.D.). In a few experiments this loop was exchanged for larger ones of 10 and 20 μl, respectively. Detection was performed at λ=240 nm.

For the LC runs, a system consisting of an SP 8780 autosampler (Spectra-Physics, Analytical San Jose, CA, USA), equipped with a 40-μl injection loop, a LKB 2150 LC pump (Bromma, Sweden) and a Spectra 100 UV detector (Spectra-Physics) with the wavelength set to 240 nm, was employed.

Vacuum centrifugation was done on a SVC 100H Speed Vac Concentrator/Evaporator (Savant Instruments, New York, NY, USA).

2.2. Conditions

The chromatographic conditions for the samples obtained from centrifugation were: 2.0 ml/min of carbon dioxide containing 8% of methanol, then increased by 1%/min to 14%. The back-pressure was set to 150 bar.

In the screening experiments, based on direct injection of emulsion samples, the temperature of the oven housing the column was varied from 31°C to 45°C. The column outlet pressure was in the range 100–200 bar, whereas the flow-rate was varied in the range 0.5 to 2.0 ml/min.

2.3. Chemicals

Carbon dioxide (3.5 grade, AGA, Lidingö, Sweden), delivered in a tank with a diptube, was used as the main component of the mobile phase.

In the screening and optimization processes, the polar component of the eluent was either methanol or 2-propanol, present in amounts ranging from 5 to 20% (v/v). The two analytical-reagent grade solvents were obtained from Merck (Darmstadt, Germany). Standard solutions were prepared with 2-propanol. Citric acid (analytical-reagent grade, Merck) was used as the third component in a few cases.

The compound chromatographed, shown in Fig. 1,

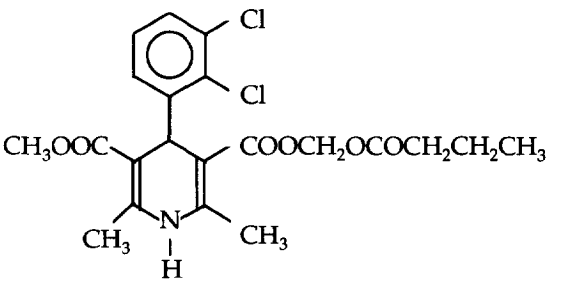

Fig. 1. The investigated compound.

was from the Department of Medicinal Chemistry, Astra Hässle. The water-based emulsion contained 20% (w/w) of vegetable oil, whereas the dihydropyridine drug concentration was either 1.0 mg/ml or 0.05 mg/ml.

2.4. Methods

The first step in a preliminary SFC method, developed prior to the direct injection approach, was to mix a 100-μl aliquot of the emulsion sample with 1.0 ml 2-propanol containing 100 μg/ml of felodipine as a marker. The vial with the now clear solution was then submitted to vacuum centrifugation for two hours. The oily residue was dissolved in 0.1–1.0 ml of 2-propanol.

In the direct injection approach the emulsions were injected as received.

The method based on reversed-phase LC demanded some sample pretreatment, which was performed according to an in-house method developed at Astra Hässle. In this scheme, 5 ml of the emulsion is mixed with 20 ml of acetonitrile–methanol (35:65, v/v) in a conical flask. The sample is shaken overnight prior to centrifugation at 4000 rpm for 10 min. The supernatant is then analyzed on the LC system described above.

Experimental designs [17] were created using Modde 2.1 software (Umetri, Umeå, Sweden). Multivariate analysis was performed by partial least squares [18]. Multivariate models were validated by cross validation [19].

3. Results and discussion

3.1. Initial experiments

Liquid–liquid extraction of the emulsion with a fourfold excess of dichloromethane resulted in a poor recovery (only 80%) of the drug. Dilution with at least a 10-fold volume of 2-propanol gave a clear sample solution that could be analyzed in an SFC system developed earlier [11]. The yield of the analyte was now similar to the values obtained in the method based on reversed-phase LC, described in Section 2. To improve the detection limit and enhance the potential of quantifying degradation products, a method based on vacuum centrifugation was now employed. This procedure required the use of an internal standard (felodipine). No losses of analyte were encountered during sample preparation.

In an effort to make the sample preparation step redundant, an approach based on direct injection was evaluated. Initially, direct injection of emulsion samples were tested using either methanol or 2-propanol as the polar component of the mobile phase. Chromatograms resulting from these experiments are shown in Fig. 2.

The lipid fraction, only slightly retained on the

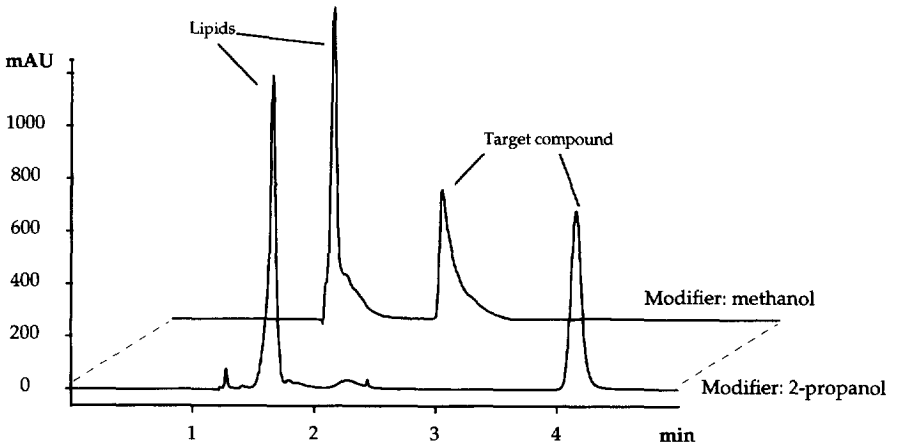

Fig. 2. Chromatogram of emulsion samples. Back-pressure, 150 bar; temperature, 40°C; modifier concentration, 15%; injection volume, 5 μl; analyte concentration, 1 mg/ml.

Table 1
Experimental design

Parameter	Abbreviation	Type	Unit	Settings
Temperature	Te	Factor	°C	31–45
Back-pressure	Pr	Factor	bar	100–200
Modifier concentration	Mo	Factor	%	10–20
Flow-rate	Fl	Factor	ml/min	0.5–2.0
Plates per second	PPS	Response	1/s	–

silica phase, elutes near the front and is well separated from the peak of interest. Considerable tailing was however obtained using methanol as the polar component. Varying the methanol content in the range 5–20% did not improve analyte peak shape. Noting that injection of standards produced gaussian peaks with both mobile phases, this indicates that the observed differences in peak shapes may be attributed to the injection process of the emulsion samples. Probably, the emulsion breaks as it enters the mobile phase, and the process occurs faster and more efficiently in the 2-propanol–carbon dioxide phase. In the following experiments, 2-propanol was used as the polar component of the mobile phase. The last results from this initial set of experiments certainly shows the importance of the injection procedure, especially in relation to the compatibility of the injection solvent and the polar component of the mobile phase, in packed-column SFC.

3.2. System optimization

To optimize the chromatographic system, an experimental design approach with four factors (Table 1) was applied. The response parameter was plates per second, i.e., the number of theoretical plates for the peak of interest divided by its retention time, expressed in seconds. In the first set of experiments, a fractionated factorial design was carried out, containing 11 experiments in all, including a centrepoint experiment in triplicate. The data obtained from these runs were loaded and the modelling phase was initiated in the software. The parameters R^2 (the fraction of variation of the response explained by the model) and Q^2 (the fraction of variation of the response that can be predicted by the model) are given by

$$R^2 = \frac{SS_{REG}}{SS} \quad (1)$$

$$Q^2 = 1 - \frac{PRESS}{SS} \quad (2)$$

where SS_{REG} is the sum of squares of Y corrected for the mean, explained by the model, SS is the total sum of squares of Y corrected for the mean and PRESS is the prediction residuals sum of squares. R^2 and Q^2 are used as indicative criteria of the model fit. R^2 is an overestimated and Q^2 an underestimated measure of the model performance. A model with values exceeding 0.9 for both these parameters has to be considered as satisfactory (Table 2). The three centrepoints generated values of 30.5, 30.0 and 31.3, respectively. Since the response for the other eight points varied from 6.0 to 50.9, it was concluded that the system was quite stable. However, in this first

Table 2
Optimization procedure

Design	Number of experiments (centrepoints)	Interaction and quadratic terms	R^2	Q^2
Fractionated Factorial	11(3)	None	0.7543	−0.1122
Full Factorial	20(4)	All interaction terms	0.8958	0.5167
Full Factorial	20(4)	Mo·Fl	0.8670	0.7541
CCF	25(5)	All terms	0.9815	0.7368
CCF	25(5)	Mo·Fl, Fl·Fl	0.9666	0.9364

round of experiments, poor values of R^2 and Q^2 were obtained. This first model, containing no interaction or quadratic terms, might profit from such expansion for more accurate predictions.

Next, a fold-over was performed, rendering a full factorial design. An extra centrepoint was measured, providing a rough measurement of the system ruggedness. The initial model included all the interaction terms. Still, the values of R^2 and Q^2 were quite poor. It appeared that the interaction term Mo·Fl had the greatest influence on the response. If all other interaction terms were removed from the model, the values of R^2 and Q^2 were improved to 0.8670 and 0.7541, respectively. Nonetheless, the model was not considered good enough yet to appropriately predict the chromatographic output. Apparently, at least one quadratic term was needed for good modelling of this particular application. A second expansion of the design was made to complete a central composite facial design (CCF), thus providing data also for the quadratic terms. Again, an extra centrepoint experiment was performed to monitor system stability. A slightly lower plates per second value (26.5) was obtained this time. Initial modelling by PLS again rendered somewhat poor cross validation. Thus, the model was refined again by removing all interaction and quadratic terms that did not contribute to improved cross validation except for the largest of each category, i.e., the linear model was expanded by Mo·Fl and Fl·Fl, providing an acceptable model with good prediction capacity (R^2 and $Q^2>0.9$).

A plot of the scaled and centred coefficients of the refined model based on the CCF designed data is shown in Fig. 3.

Apparently, temperature and back-pressure is of no or little importance within the experimental parameter intervals employed here. A high modifier content generated higher values of plates per second, whereas the dependence on flow-rate was somewhat more complex. A response surface, with the temperature and back-pressure set at 38°C and 150 bar, respectively, is shown in Fig. 4.

At higher modifier concentrations there is a continuous increase in plates per second with increasing flow, whereas a maximum in the plates per second versus flow curve is found in the lower end of the modifier interval.

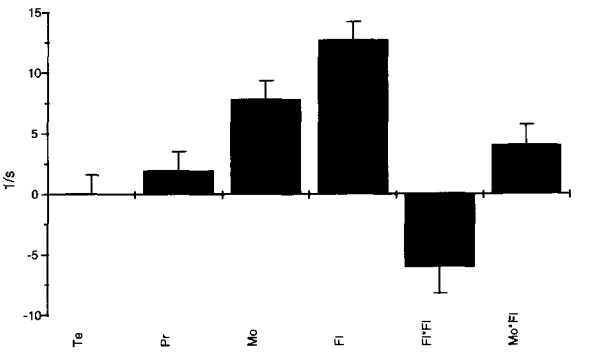

Fig. 3. Scaled and centred coefficients for the response, number of theoretical plates/s, in the CCF design, edited as described in the text. Abbreviations as in Table 1.

In accordance with the model the flow-rate and modifier values were set high in further experiments, at 2.0 ml/min and 20%, respectively. The temperature was kept low (31°C), whereas the back-pressure was set high (200 bar).

3.3. Peak compression effects

The positive influence on the response of increasing the content of 2-propanol in the mobile phase prompted an investigation where even higher values were applied. While doing so, by using, for instance, the parameters obtained from the optimization procedure but increasing the concentration of 2-propanol to 30%, this rendered very narrow peaks with extremely large plate numbers, allegedly only when

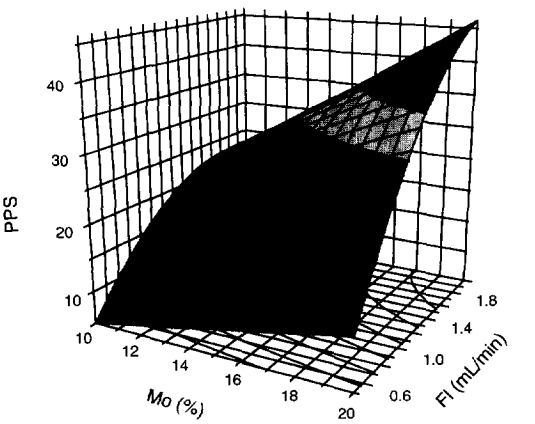

Fig. 4. Responce surface. Back-pressure, 150 bar; temperature, 38°C.

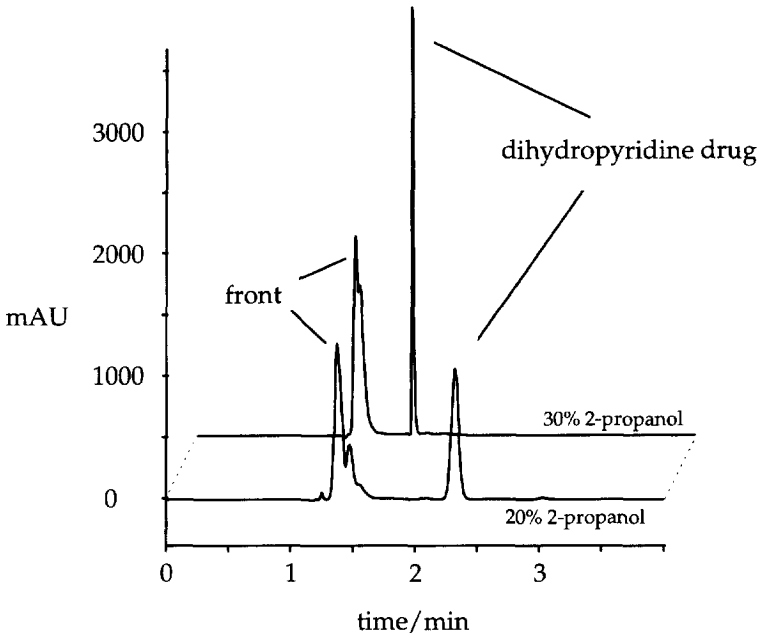

Fig. 5. Chromatograms of emulsion samples. Back-pressure, 200 bar; temperature, 31°C; injection volume, 5 μl; analyte concentration, 1 mg/ml.

emulsion samples were injected. The effect is depicted in Fig. 5.

In this particular case the analyte plate number increased from 7060 to an amazing 84 400. Increasing the 2-propanol content further beyond 30% resulted in decreasing plate numbers, indicating that the system needs to be properly tuned in order to achieve the effect. The peak compression effect may also be obtained by increasing the injection volume. Using the conditions found from the optimization scheme (31°C, 200 bar, 2.0 ml/min, 20% 2-propanol) but using injection volumes of 5, 10 and 20 μl, the resulting plate numbers were 6530, 20 175 and 5412, respectively, i.e., there was initially an unconventional increase in plate numbers with increasing volumes. Further experiments showed that the compression effect could be obtained when adding water to standard solutions made up with 2-propanol. Consequently, it is the water in the emulsion samples that causes the effect. Currently, our working hypothesis to explain the underlying fundamentals of the compression effect is that water displaces 2-propanol from the stationary phase. This secondary 2-propanol competes efficiently with the analyte for active sites on the column. This, in turn, causes the analyte to move quickly within the plug of displaced 2-propanol. Hence, the analyte plug is compressed and high apparent peak numbers are obtained. The displacement effect mentioned above is also supported by chromatograms obtained at 210 nm, showing a positive system peak originating from excess 2-propanol, followed by a negative peak caused by the water plug. The compression effect is only obtained if the analyte peak co-elutes with the front of the positive system peak.

Peak compression effects have earlier been thoroughly discussed for more traditional LC systems [20,21]. The present chromatographic system is currently being carefully studied in our laboratory, aiming to control the peak compression effect properly and use it to our advantage.

3.4. Performance

Using placebo emulsion to dilute a 1 mg/ml emulsion, samples with drug concentrations ranging from 0.002 mg/ml to 0.010 mg/ml were prepared. These samples were later analyzed using optimal

conditions, as found from the chemometric optimization schemes, i.e., without peak compression effects applied. The resulting regression plot revealed a fairly straight line, with the correlation coefficient (r) being 0.9996.

The method was also validated by comparing results obtained from the newly developed direct injection SFC method with data obtained from an in-house solvent extraction–LC method as described in Section 2. For an emulsion batch with the nominal value of 0.050 mg/ml, the SFC method gave the value 0.043 mg/ml ($n=5$), whereas the LC method provided the answer 0.045 mg/ml ($n=2$). The precision in the SFC determination was reasonable, 3.4% R.S.D. When comparing the two methods it must be noted that the SFC method is based on direct injection of the emulsion samples, with a total analysis time of less than 5 min whereas the LC method with the sample preparation step included takes some 20 h to perform.

The SFC method is clearly developed here to perform a rapid determination of the amount of drug present in the emulsion matrix. For qualitative and quantitative analysis of the degradation products, the system needs to be reoptimized to allow for full separation of these.

4. Conclusions

The method developed for quantitative analysis of a drug in emulsion samples based on direct injection packed-column SFC shows considerable promise. When compared to a routine LC method, the SFC method gave similar quantitative results but was superior in terms of speed and simplicity.

Learning to control the peak compression effect will help to improve detectability for degradation products in the formulation.

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