Use of methyl and ethyl acetate as organic modifiers in reversed-phase high-performance liquid chromatography

Application to impurity control in bulk drug steroids

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ABSTRACT

We assayed methyl and ethyl acetate as organic modifiers for mobile phases used in reversed-phase high-performance liquid chromatography for the removal of impurities from steroids of pharmaceutical interest. By using ternary mobile phases consisting of one of the esters, water and a third component that was miscible with both and applying a systematic experimental methodology, we developed an optimal procedure for the isolation of the compound of interest (9α-fluoroprednisolone acetate) from the impurities resulting from its synthesis in a minimum time. Inclusion of an acetic ester in the reversed-phase high-performance liquid chromatographic mobile phase was found to improve the resolution of chemically similar substances.

INTRODUCTION

Methyl and particularly ethyl acetate have been frequently used in mobile phases for normal-phase high-performance liquid chromatography (HPLC) in order to modify the eluting power and selectivity of the technique, and hence facilitate separations [1–3]. However, they have only rarely been used as modifiers in reversed-phase (RP) HPLC.

One of the potential reasons for the infrequent use of acetic esters in RP-HPLC may be their marked absorption in a broad region of the ultraviolet spectrum. Methyl and ethyl acetate are virtually opaque to wavelengths below 256 nm, unlike other solvents commonly used in RP-HPLC [*e.g.* methanol (205 nm), acetonitrile (190 nm) and tetrahydrofuran (212 nm)] [4]. This implies that both solvents have to be used in amounts small enough to avoid interfering with the detection of the compounds of interest. As shown below, the high eluting power of these esters and the risk of forming immiscible phases compels the use of quite low concentrations in the mobile phase, which poses no technical problem on the analyses in our case.

The main reason why esters have systematically been avoided in RP-HPLC probably lies in their immiscibility with water, which is without doubt the most frequently employed eluent in this chromatographic mode. However, the miscibility of an ester–water system can be increased by using a third solvent provided that it is miscible with both the ester and water.

The third solvent in question is normally chosen in such a way as to ensure the maximum possible difference in selectivity between the three in order to obtain maximal differences in the behaviour of the mixtures to be dealt with and hence find the optimal conditions for their resolution. On Snyder's [5]

selectivity triangle, aliphatic esters, like acetonitrile, belong to group VIa, while water belongs to group VII. Choosing methanol as the third solvent therefore seems appropriate inasmuch as it is fully miscible with the other two and belongs to selectivity group II, which is distant enough from the other two for the selectivity of the resulting mobile phase to vary markedly with its composition.

The effect of the acetic esters as organic modifiers was studied on the resolution of a mixture of nine steroids by RP-HPLC. Because of the synthetic procedure used for its preparation, 9α-fluoroprednisolone acetate (FPA) can be accompanied by the impurities shown in Fig. 1 [6]. In a previous work [7] we developed a procedure to determine the optimal composition of the ternary mobile phase and applied
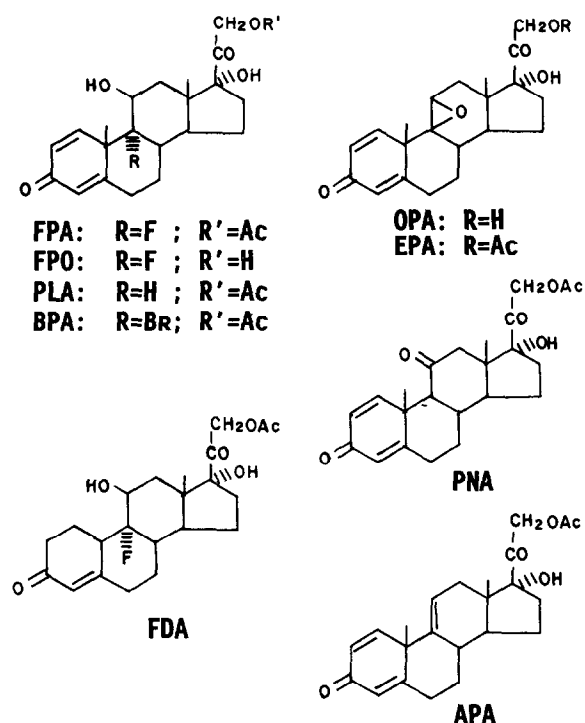
it to the acetonitrile–tetrahydrofuran–water system for the complete isolation of FPA from its related impurities. The reason for using the same type of sample here was the possibility of determining whether the acetic esters offer any advantages in terms of selectivity or rapidity. The procedure applied here was similar, but involved mobile phases including one of the acetic esters. Also, the impurities contest of the synthesized steroid must be reduced to less than 1 in 1000 in order to comply with the recommendations from most pharmacopeias [8,9].

EXPERIMENTAL

The chromatographic set-up used consisted of a CM4000 multiple solvent partitioning pump and an SM5000 photodiode-array detector, both from LDC Analytical (Riviera Beach, FL, USA). Data were processed and integrated with the aid of ThermoChrom PDA software, also from LDC Analytical. Samples were injected by means of a Rheodyne 7125 injector (Cotati, CA, USA) with a fixed-volume loop of 20 μl. The mobile phase was thoroughly homogenized prior to reaching the column by using a dynamic mixer (Dinamixer from LDC Analytical) immediately before the injector. Calculations were done and plots constructed with the aid of commercially available computer software.

The solvents used included methanol (Scharlau, Barcelona, Spain), ethyl acetate (SDS, Peypin, France) and methyl acetate (Fluka, Buchs, Switzerland), all of which were of HPLC grade. The water used was purified by passage through a Nanopure II system from Barnstead (Newton, MA, USA). All eluents were filtered through 0.5 μm mesh and degassed by bubbling with helium. The column used was a Spherisorb octadecylsilane (ODS) column, 5 μm particle size, 150 m × 0.46 mm I.D., supplied by Phenomenex (Torrance, CA, USA).

Chromatograms were recorded at a flow-rate of 1 ml/min and room temperature (*ca.* 20°C). The dead time (1.4 min) was the same for all the mobile phases assayed and was assigned to the first baseline distortion observed upon injection of pure acetonitrile. Steroids standards were supplied by Cesquisa (Segovia, Spain) and were dissolved in HPLC-grade acetonitrile (SDS) at a concentration of 1000 mg/l in

Fig. 1. Chemical structures of 9α-fluoroprednisolone and its related impurities resulting from its synthesis process. FPA = 9α-fluoroprednisolone acetate; FPO = 9α-fluoroprednisolone; PLA = prednisolone acetate; BPA = 9α-bromoprednisolone acetate; FDA = fludrocortisone acetate; PNA = prednisone acetate; OPA = 9β,11β-epoxy-17α,21-dihydroxypregne-1,4-dien-3,20-dione; EPA = 9β,11β-epoxy-17α-hydroxypregne-1,4-dien-3,20-dione 21-acetate; APA = 17α-hydroxypregne-1,4,9(11)-trien-3,20-dione 21-acetate. Ac = Acetyl.

FPA and 1 mg/l in all of the other components except for 9β,11β-epoxy-17α,21-dihydroxypregne-1,4-dien-3,20-dione (OPA), the concentration of which was 0.1 mg/l.

RESULTS AND DISCUSSION

Construction of the phase diagrams

First, we determined which compositions of the mobile phase can be used. The range of useful concentrations was constrained by the partial immiscibility of the phase components. This involved examining the phase equilibrium diagrams of the systems as compiled by Sörensen and Artl [10]. We strived to maintain the retention of all the com-

pounds to be resolved within a preset range given by $1 < k' < 100$, where k' is the so-called "capacity factor". The range in question was determined experimentally because the solvent strength parameter of the acetic acid esters in reversed phase was unknown.

The phase diagram of the ethyl acetate–methanol–water system was obtained by converting the experimental data reported by Beech and Glasstone [12] into percentage volumes by using the molecular masses and densities of the components at 20°C (Fig. 2).

We found no phase diagram in the literature for the methyl acetate–methanol–water system with the exception of an approximate description by Craw-
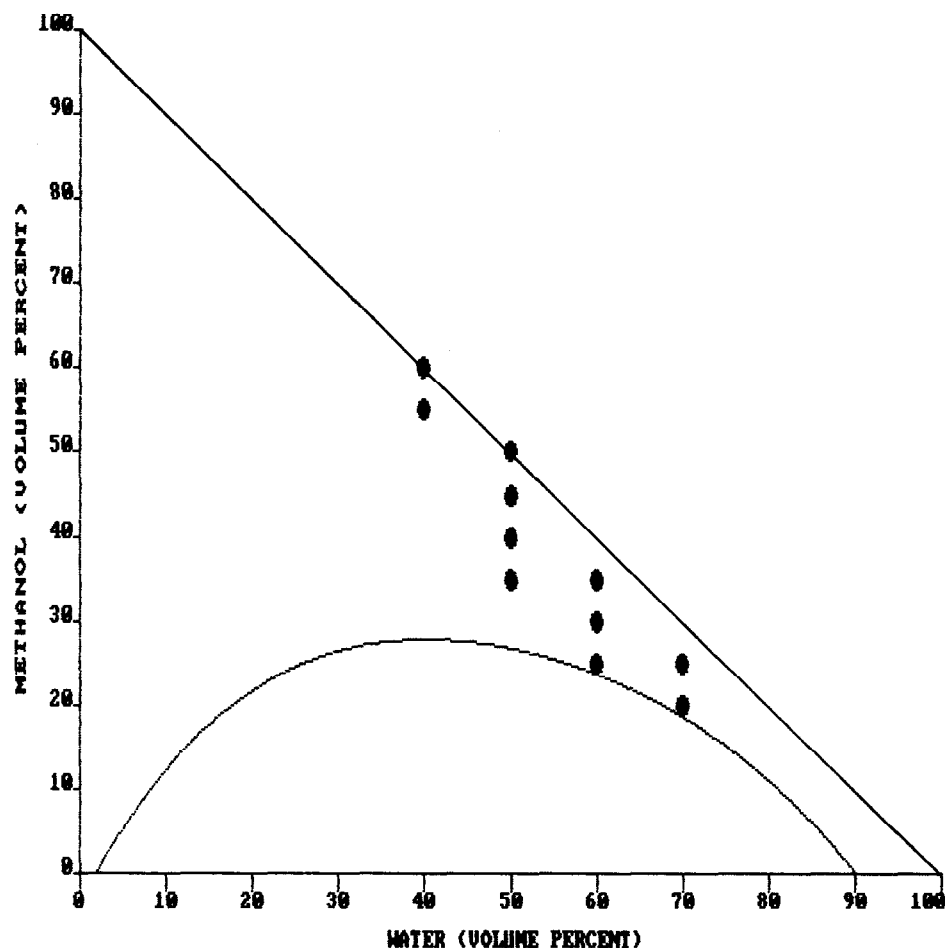
Fig. 2. Phase diagram and experimental design points for the ethyl acetate–methanol–water mobile phase.

ford *et al.* [13], which was used as a reference for precise construction of the diagram.

For convenience, we chose to use isoceles rectangular triangles, which can accommodate the Cartesian coordinates for two of the components, the composition of the third being obtained by difference.

Study of the ethyl acetate methanol–water mobile phase

The optimization procedure used involved the construction of three-dimensional window diagrams. The response surface (*viz.* the minimal resolution *vs.* the composition of the mobile phase) was obtained from the equation of Schoenmaker *et al.* [11], which correlates retention and composition:

$$\log k' = A_1\varphi_1 + A_2\varphi_2 + B_1\varphi_1^2 + B_2\varphi_2^2 + C_{\varphi_1\varphi_2} + D \tag{1}$$

where φ_1 and φ_2 denote the volume fractions of methanol and ester, respectively, k' is the capacity factor for each solute in the sample and A–D are constants. The coefficients of eqn. 1 can be calculated by multiple regression from the data points obtained in the experimental design. From this point, one can develop a mathematical relation between the resolution (R_s) of the chromatographic peaks corresponding to two of the steroids assayed and the composition of the mobile phase since the resolution and the capacity factor are related through the well known equation:

$$R_s = \frac{1}{4}\sqrt{N}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2'}{1 + k_2'}\right) \tag{2}$$

where N is assumed to be constant for all compositions of the mobile phase and was estimated to be 4000 theoretical plates from several experimental determinations. By plotting the minimal resolution between FPA and each related impurity as a function of the composition of the ternary mobile phase a three-dimensional window diagram is obtained.

A window diagram features two essential parameters. One is the minimal resolution, which must be greater than 1.5 (*viz.* the limit for correct resolution of components present in a ratio of 1000:1). The other is the analysis time, which is taken as the time required for the last steroid to be eluted and should therefore be as short as possible.

We obtained the chromatograms of the mobile phases whose compositions and positions in the diagram are reflected in Fig. 2. Each composition yielded a chromatogram in which retention data obtained for each compound provided points for the experimental design and were used to calculate the coefficients of eqn. 1 by multiple regression analysis.

For a given water content in the mixture, the analysis time increases with decreasing proportion of ethyl acetate in the mixture. This is quite logical because ethyl acetate is less polar than methanol and hence has a higher reversed-phase eluting power. As regards the variation in the selectivity of the mobile phase with its composition, it occurs at peak overlapping. For a water content of 50%, 9α-bromoprednisolone acetate (BPA) is eluted before 9β,11β-epoxy-17α-hydroxypregne-1,4-dien-3,20-dione 21-acetate (EPA) at low methyl acetate contents then both peaks converge until co-elution, and EPA is eluted before BPA at high ethyl acetate content. Even more interesting is the variation pattern of the selectivity for the mobile phases containing 60% water since these involve the compound of interest (FPA). We studied no mobile phases containing 40% methanol and 60% water because they resulted in rather long retention times; however, in a previous work [7] we found that FPA is eluted before FDA, prednisolone acetate (PLA) and prednisone acetate (PNA) under these conditions. On increasing the proportion of ethyl acetate at the expense of methanol, the PLA and PNA peaks approach that of FPA to the point of co-elution and later both PLA and PNA are eluted before the steroid of interest.

From the experimental data gathered one can conclude that a 2:58:40 (v/v/v) ethyl acetate–methanol–water mixture provides a minimal resolution of 1.5 (estimated value, 1.6) between FPA and the other compounds in the shortest possible time (6.8 min) Fig. 3 shows the variation in the expected minimal resolution with the composition of the mobile phase. The chromatogram obtained with this mobile phase is shown in Fig. 4. As can be seen, the resolution of FPA is quite rapid and complete, at the expense of more extensive overlap between the other components.

Table I lists the estimated and experimental analysis times and minimal resolutions obtained with this mobile phase. As can be seen, the two sets of data are quite consistent, particularly at the
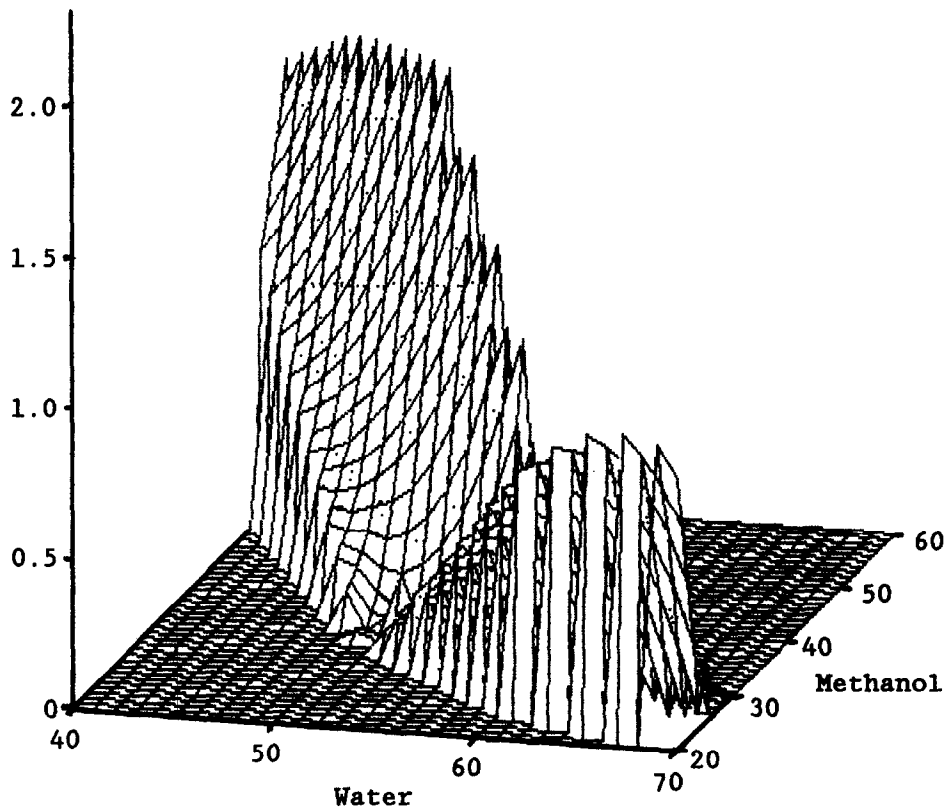
Minimum

Resolution

Fig. 3. Variation in the minimal resolution between FPA and its impurities as a function of the mobile phase composition for the ethyl acetate–methanol–water system. Water and methanol scales in %(v/v).

shorter analysis times. The divergences observed at very low resolution can be disregarded since resolution smaller than 0.5 result in a thorough peak overlap and hence prevent experimental determination of the minimal resolution.

Study of the methyl acetate–methanol–water mobile phase

The procedure used was identical to that followed for the previous phase. Like ethyl acetate, methyl acetate has reversed-phase eluting power that is slightly higher than that of methanol and slightly lower than that of the ethyl ester because of the higher polar character endowed by the methyl group compared with the ethyl group, which is somewhat

bulkier. As far as the selectivity is concerned, peak overlap is similar, though less marked than that encountered with ethyl acetate, as one would expect from the chemical similarity between the two esters.

The optimal composition for this mobile phase was methyl acetate–methanol–water (3:57:40, v/v/v). This yielded a minimal resolution of 1.5 and an expected chromatographic analysis time of 6.6 min.

CONCLUSIONS

By including acetic esters as modifiers of mobile phases used in reversed-phase liquid chromatography, the analysis times can be substantially shortened, particularly if ethyl acetate is used. Also, they
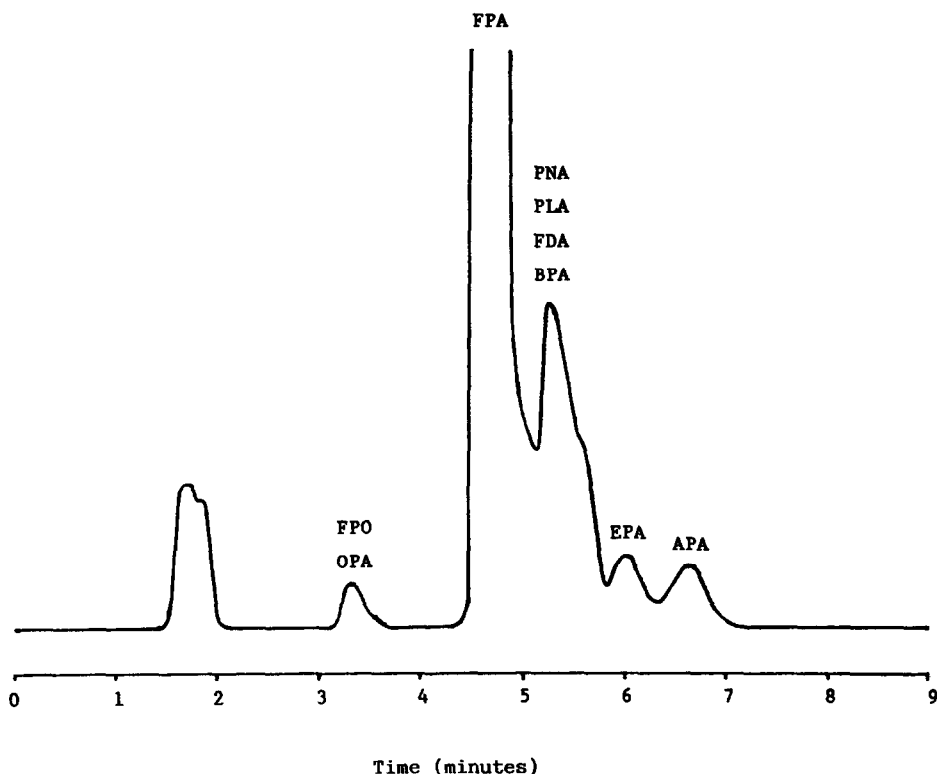
Fig. 4. Chromatogram obtained from a mixture of standards at 1000 mg/l in FPA and 1 mg/l in the other components (except OPA 0.1 mg/l). Mobile phase: ethyl acetate–methanol–water (2:58:40, v/v/v).

TABLE I

RETENTION TIMES AND MINIMUM RESOLUTION OB-
SERVED AND PREDICTED WITH THE MODEL OF
EVERY COMPOSITION SPECIFIED IN FIG. 2

t_{obs} = Observed analysis time; t_{prd} = predicted analysis time with
the model; R_{obs} = observed minimum resolution for FPA;
R_{prd} = predicted minimum resolution with the model for FOA.

Eluent composition (%, v/v/v): ethyl acetate–methanol–water	t_{obs} (min)	t_{prd} (min)	R_{obs}	R_{prd}
00:60:40	8.3	8.8	1.81	1.94
05:55:40	5.2	5.0	1.44	1.19
00:50:50	24.1	23.1	2.15	2.23
05:45:50	10.9	11.2	0.98	0.96
10:40:50	7.5	7.3	0.00	0.21
15:35:50	5.9	6.0	0.00	0.04
05:35:60	26.0	27.6	0.00	0.30
10:30:60	15.7	15.9	0.00	0.49
15:25:60	12.1	12.0	0.89	0.99
05:25:70	69.2	70.2	0.49	0.29
10:20:70	38.3	36.8	1.40	1.22
2:58:40 (optimum)	6.5	6.8	1.88	1.63

modify the selectivity in the mobile phase. This
additional asset was not exploited in our case
because the optimal composition of the mobile
phase involved a very low proportion of the ester
and a high methanol content, which hindered the
attainment of significant selectivity differences.
Nevertheless, this could be of great use in resolving
other types of mixtures.

As far as the isolation of FPA from its accompa-
nying impurities is concerned, the resolution
achieved by using a mobile phase including an acetic
ester by a statistically optimized procedure was quite
good and matched the experimental results. Also,
the separation time was shortened in relation to the
use of unmodified phases, which is of great interest
with a view to improving product quality control
procedures.

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