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RAPID REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF STEROID PRODUCTS

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SUMMARY

The optimisation of conditions for the application of reversed-phase high-performance liquid chromatography (HPLC) to the analysis of steroid products is discussed. The influence of temperature and flow-rate on column efficiency are described. At 60°, the reduced velocity required to give maximum efficiency, is approximately twice that found at room temperature, allowing faster through-put. (h v) curves are reported for 5-μm Spherisorb-ODS and Hypersil-ODS material for corticosteroids at 60°. On-column injection techniques are discussed and data presented, showing that for corticosteroids on-column loop injection can be efficient as septum injection using the techniques described.

INTRODUCTION

High-performance liquid chromatography (HPLC) has revolutionised the analysis of pharmaceutical products. The versatility of reversed-phase operation has been widely demonstrated, and the present upsurge of interest is a consequence of the commercial availability of permanently bonded phases on microparticulate supports[1–3]. The design and performance characteristics of these new phases have been discussed by Knox and Pryde[4].

It is now accepted practice to define the performance of these materials in terms of three parameters: the reduced plate height (h), the reduced velocity (v) and the column resistance parameter (φ')[5,6]. These parameters are defined in the following three equations:

$$h = \frac{H}{d_p}$$

$$v = \frac{U d_p}{D_m}$$

$$\varphi' = \frac{\Delta p \, d_p^2}{U \eta L}$$

where H = plate height, d_p = mean particle diameter, U = linear velocity of the mobile phase, D_m = molar diffusion coefficient of the solute in the mobile phase, η =

viscosity of the mobile phase, L = column length and Δp = pressure drop across the column. Methods for calculating these parameters from experimental data were given by Bristow[7].

Knox and Pryde[4] showed that, for 5–10-μm materials packed in "infinite-diameter" columns[8], a range of values could be assigned to these parameters that would indicate near ideality. For kinetically model compounds, h values of 1.5–3 at $\nu \approx 6$ and $\varphi' = 500$–1000 are considered to be optimal for 100–200-mm columns at ambient temperature.

Very little has been published on the reduced plate heights that are attainable for larger molecules such as steroids, and the determination of these parameters at elevated temperatures has received even less attention. The purpose of this work was to examine the effects of temperature, column geometry and injection mode on the efficiency of separation of some steroids and criteria for optimising the HPLC analyses of some steroid dosage forms are discussed, with examples.

EXPERIMENTAL

Equipment

The chromatographs used were a Model 601 pump/oven with an LC55 variable-wavelength UV detector (Perkin-Elmer, Beaconsfield, Great Britain) and a modular instrument comprising a Model 740B pump (Spectra-Physics, St. Albans, Great Britain), an ACS 750 forced-air oven (Applied Chromatography Systems, Luton, Great Britain) and a Cecil 212 extended-range UV monitor (Cecil Instruments, Cambridge, Great Britain).

The columns were packed by a dilute slurry method in methanol–water or iso-octane–chloroform using a Haskel MCP-71C pneumatic amplifier pump (Olin Energy Systems, Sunderland, Great Britain) at pressures of 5000–6000 p.s.i.g.

On-column injection was achieved by using either a MACC-II LC Septum Injector and a Hamilton HP 1805 syringe (Phase Separations, Queensferry, Great Britain) or a Rheodyne 7120 injection valve (Magnus Scientific, Alsager, Stoke-on-Trent, Great Britain).

The columns were either 5 or 8 mm I.D. Appolo 316 stainless-steel tubes (Magnus Scientific), with conventional Swagelok fittings or complete units with an injector head (Shandon Southern, Runcorn, Great Britain).

Bonded phases

Spherisorb-ODS (5 μm) (Phase Separations) and Hypersil-ODS (5 μm) (Shandon Southern) were chosen as representative of readily available commercial C_{18} reversed-phase microparticulate packings.

RESULTS AND DISCUSSION

The performance characteristics of the two reversed-phase packings in 100 × 5 mm columns at 60° are shown in Figs. 1 and 2 for the four corticosteroids beta-methasone 17-valerate, clobetasol 17-propionate, clobetasone 17-butyrate and beclomethasone dipropionate (Fig. 3).

The h *versus* ν plots are similar in shape to those found previously at ambient

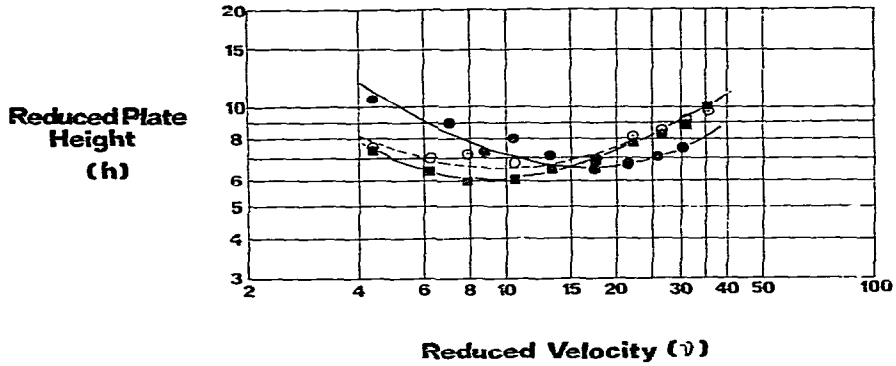


Fig. 1. Results with Spherisorb-ODS (5 μm). Mobile phase: methanol–water (60:40) at 60°. Compounds: steroids I-IV. ●, 5-μl syringe injection; ■, 5-μl loop injection, PTFE plug; ○, 5-μl loop injection, curtain flow.

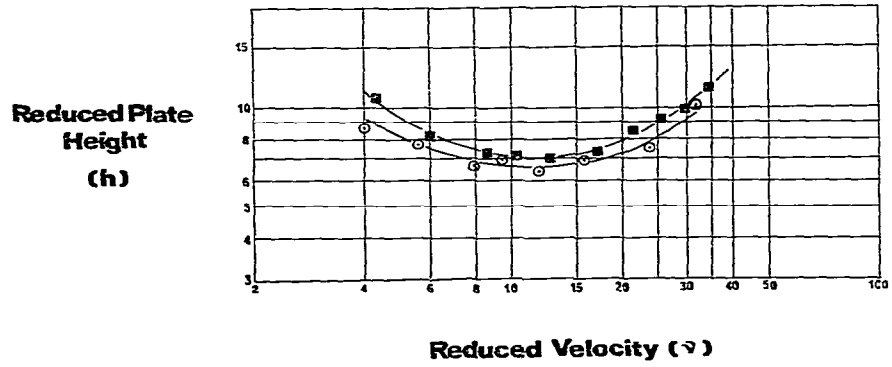


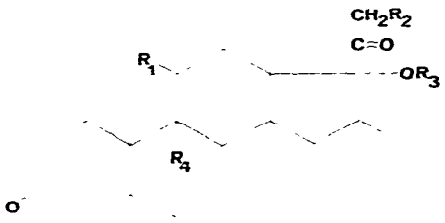
Fig. 2. Results with Hypersil-ODS (5 μm). Mobile phase: methanol–water (65:35) at 60°. Compounds: steroids I-IV. ■, 5-μl loop injection, PTFE plug; ○, 5-μl loop injection, curtain flow.

temperature[4,9]. The molar volumes of these compounds are sufficientiy similar to have little effect on the molar diffusion coefficient, D_m, calculated from the Wilkie–Chang equation[10]. A mean value of $7.5 \cdot 10^{-10}$ m^2·sec^{-1} was calculated for the four corticosteroids in methanol–water (60:40) as the mobile phase at 60°. A value of 0.7 cP was adopted for the viscosity of the mobile phase at 60°.

On inspection of these curves, two differences from model compounds at ambient temperature are apparent. Firstly, $h \approx 6.5$ at the minimum, which is approximately twice that expected for kinetically model compounds. Secondly, the minimum occurs at $\nu \approx 11$, which again is double the value found at ambient temperature[4,9] and, because of better mass transfer at 60°, the minimum of the Knox plot occurs at a higher linear velocity. This is predictable in that D_m increases by a factor of almost 2.5 between 20° and 60°. In practical terms, this means that, for a given column geometry, the optimal flow-rate can be doubled without loss of efficiency by operating at 60°, hence halving the analysis time.

The column resistance parameter, φ', for both materials was found to be *ca.* 700. This is similar to the value of 500 found for a 7-μm spherical ODS-silica[4].

Having recognized the advantages of using an elevated temperature to reduce



	R_1	R_2	R_3	R_4
I Betamethasone 17 valerate	OH	OH	COC_4H_9	F
II Clobetasol 17 propionate	OH	Cl	COC_2H_5	F
III Clobetasone 17 butyrate	O	Cl	COC_3H_7	F
IV Beclomethasone dipropionate	OH	$OCC_2H_5COC_2H_5$ (O)		Cl
V Betamethasone 21 disodiumphosphate	OH	PO_4Na_2	H	F
VI Betamethasone	OH	OH	H	F

Fig. 3. Structures of compounds used.

the analysis time without a decrease in efficiency, the possibility of reducing the void volume was considered. The concept of short columns is attractive because the void volume is reduced and the pressure drop is much smaller. However, with corticosteroids, 50 and 25 × 5 mm columns yielded minimal values for h of 11.3 and 12.5, respectively. A 50 × 8 mm column gave results very similar to those shown in Fig. 1, a minimal value of $h = 7.8$ being found. Thus, for the compounds under consideration, shorter columns lead to poorer efficiencies.

Practical constraints in applying HPLC to the routine production control of pharmaceutical products are not always reconcilable with the "state of the art" requirements. For example, no attempt was made to minimize the extra-column volume in the tube to the detector. Even so, when 100 × 5 mm columns were used, the efficiencies for the four compounds with $k' = 3$–6 were reasonably constant. The efficiencies for compounds with $k' < 2$ were lower, which can be ascribed to extra-column broadening. Practical considerations indicate that in order to prevent interferences from excipients or vehicles, chromatographic conditions should be chosen so as to ensure whenever possible that the k' values of the compounds of interest are greater than 2.

On-column injection is a vital factor in HPLC and one of the most difficult to achieve in practice. In "state of the art" determinations, reliance is usually placed on septum injection[11]. A typical arrangement using a MACC-II LC injector and a column topping procedure is shown in Fig. 4. In routine work, this column topping is not suitable for stable long-term operation. Disturbance of the ballotini bead layer, distortion of the mesh disc and ingress of septum debris are caused by repeated injection. Syringe injection is also a technique that requires considerable practical experience in order to obtain reproducible results. Thus septum injection is not recommended for routine work with relatively unskilled operators. Two methods of on-column loop injection were studied (Fig. 5). The contents of the loop are injected, by stream switching,

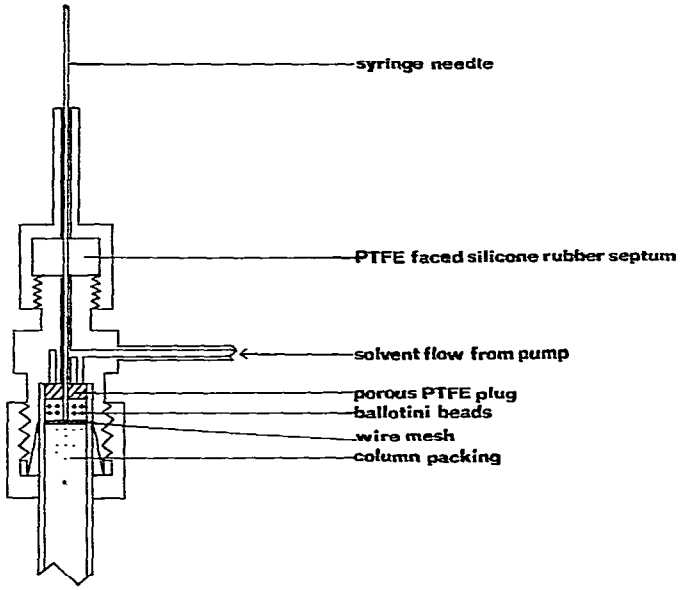


Fig. 4. Arrangement for on-column syringe injection.

directly on to a thin 35-μm porous PTFE plug 1–2 mm thick. The results are shown in Figs. 1 and 2. The shape and minimum position for syringe injection on Spherisorb-ODS were found to differ from those for loop injection. Further work is required in order to establish whether this is an artifact of the system used or a phenomenon as-

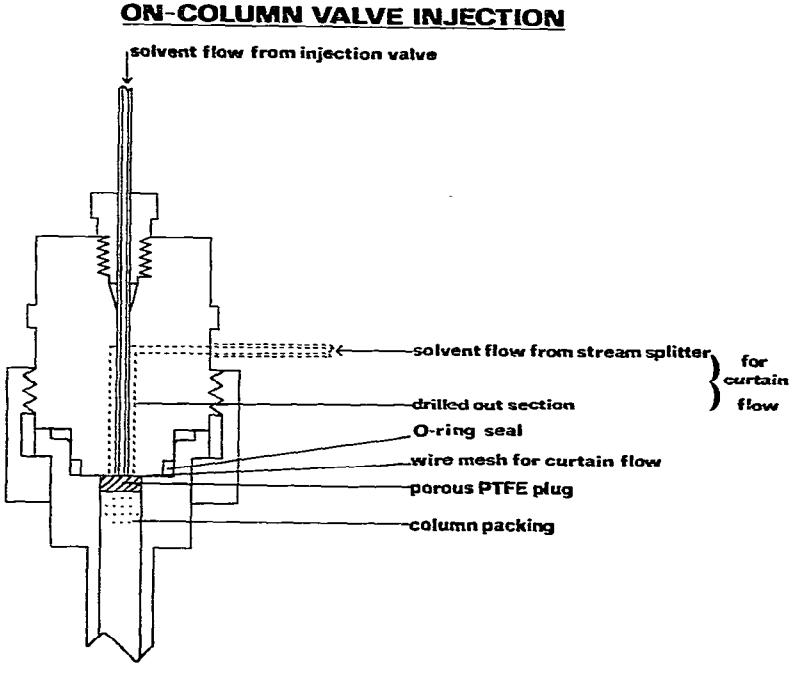


Fig. 5. Arrangement for on-column valve injection.

sociated with the material. Only marginally worse efficiencies, in comparison with syringe injection, were noted when using Spherisorb-ODS, and slightly better efficiencies were obtained with Hypersil-ODS. The use of conventional toppings with a mesh disc caused fronting owing to a "bounce-back" effect. However, using curtain or split flow[11], illustrated in Fig. 5, this effect was obviated. In our model the splitting ratio was 40:60 (curtain:loop). The results obtained were comparable with those of the septum injection and PTFE plug methods.

The use of direct on-column injection without curtain flow or a PTFE plug resulted in cavitation problems and a decrease in efficiency. No difference was noted between the use of a PTFE plug with or without curtain flow. At pressures greater than 1000 p.s.i., however, the PTFE plug tends to collapse. If such pressures are encountered, then the curtain flow method is recommended. The following set of conditions was found to be optimal for the routine chromatography of corticosteroids and related compounds: column, 100 × 5 mm 5-μm silica-ODS; mobile phase, methanol–water; flow-rate, 2.0–3.0 ml·min^{-1}; temperature, 60°; injection technique, on-column loop injection with a PTFE plug or curtain flow; injection volume, 5 μl.

Applications to the analysis of dosage forms

The examples cited are three topical preparations (two creams and an ointment), two tablets and an injection. In all instances, the sample preparation was simple and the internal standards used were usually related compounds. The structures of the active steroid ingredients are given in Fig. 3.

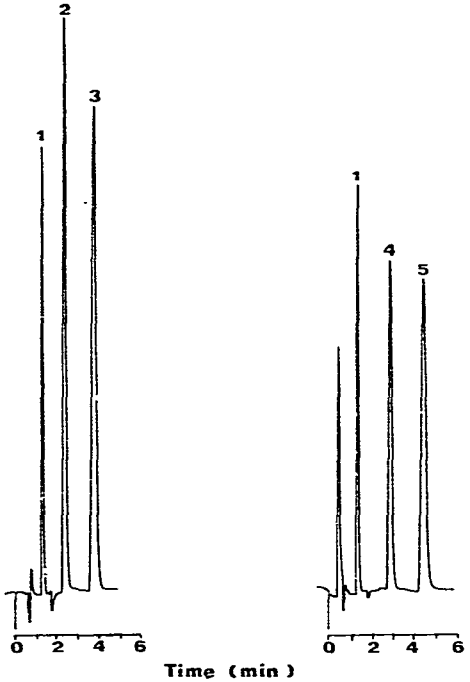


Fig. 6. Chromatograms obtained with steroid creams. Mobile phase: methanol–water (57.5:42.5). Detection at 236 nm (0.2 a.u.f.s.). Peaks: 1 = chlorocresol; 2 = steroid intermediate; 3 = I; 4 = II; 5 = III.

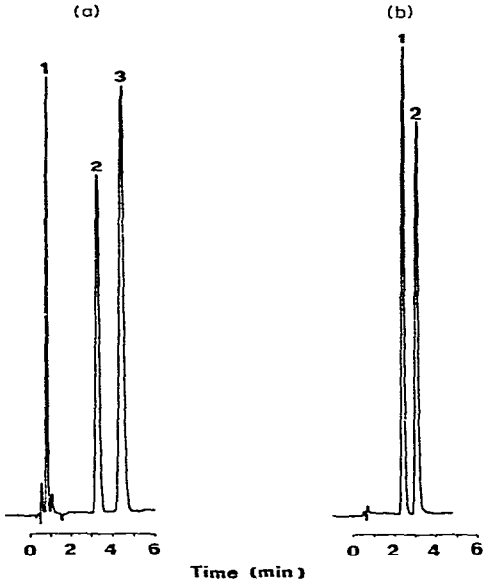


Fig. 7. Chromatograms obtained with steroid ointment and tablet. (a) Mobile phase: methanol–water (57.5:42.5). Detection at 239 nm (0.05 a.u.f.s.). Peaks: 1 = chlorphenesin; 2 = I; 3 = IV. (b) Mobile phase: methanol–water (45:55). Detection at 240 nm (0.05 a.u.f.s.). Peaks: 1 = hydrocortisone; 2 = IV.

Fig. 8. Chromatograms obtained with steroid injection and tablet. Mobile phase: methanol–pH 5.0 McIlvaines' buffer (45:55). Detection at 240 nm (0.5 and 0.2 a.u.f.s.). Peaks: 1 = benzyl alcohol; 2 = sodium benzoate and saccharin; 3 = V; 4 = VI; 5 = *n*-butyl-*p*-hydroxybenzoate.

Creams and ointments were dispersed in hot methanol–water or acetonitrile containing an internal standard. Usually, 1 g of sample was dispersed in 10 ml of extractant. Recoveries from standard additions to base material were 99–101%. The tablets were extracted with hot methanol or dissolved in methanolic buffer, an example of the latter being the betamethasone 21-disodium phosphate tablet. The injection was diluted with the appropriate internal standard solution.

The chromatograms obtained, using a Perkin-Elmer 601 chromatograph with a 100 × 5 mm 5-μl loop injection and a flow-rate of 2 ml·min^{-1}, are given in Figs. 6–8. Similar results were obtained using 5-μm Hypersil-ODS columns. The chlorocresol in the creams, chlorphenesin in the ointment and benzyl alcohol in the injection can be determined at the same time as the active steroid. Electronic integration is the preferred technique for the quantitation of each component. In all examples, the time required for chromatography of a sample is less than 5 min per injection. The standard deviations on replicate injections were *ca.* 1.0% for all components.

CONCLUSIONS

The effects of elevated temperature, column geometry and injection technique on rapid reversed-phase HPLC methods for some corticosteroids have been investigated. Operation of 100 × 5 mm 5-μm silica-ODS columns at 60° has been shown to be optimal. Loop injection systems of the on-column type have been shown to be as efficient as conventional injection techniques.

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REFERENCES

1 A. Pryde, *J. Chromatogr. Sci.*, 12 (1974) 486.
2 J. S. Wragg and G. W. Johnson, *Pharm. J.*, (1974) 601.
3 C. Burgess, *Perkin-Elmer Anal. News*, No. 14 (1977) 1.
4 J. H. Knox and A. Pryde, *J. Chromatogr.*, 112 (1975) 171.
5 G. R. Laird, J. Jurand and J. H. Knox, *Proc. Soc. Anal. Chem.*, (1974) 310.
6 J. C. Giddings, *Dynamics of Chromatography, Part I*, Marcel Dekker, New York, 1965.
7 P. A. Bristow, *Liquid Chromatography in Practice*, HETP, Wilmslow, 1977.
8 J. H. Knox and J. F. Parcher, *Anal. Chem.*, 41 (1969) 1599.
9 D. Aslin, *Int. Lab.*, July/August (1977) 59.
10 P. Chang and C. R. Wilkie, *J. Phys. Chem.*, 59 (1955) 592.
11 T. J. N. Webber and E. H. McKerrell, *J. Chromatogr.*, 122 (1976) 243.