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MICRO-BORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF PHARMACEUTICAL COMPOUNDS

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SUMMARY

A micro-bore high-performance liquid chromatographic (HPLC) system has been constructed using commercially available instruments and pre-packed micro-bore columns. The flow-rates to obtain the highest efficiency for a reversed-phase column and for a silica column are *ca.* 20 μl/min and 5 μl/min, respectively. The micro-bore HPLC system is sixteen times more sensitive than a conventional HPLC system for detection of a compound. With little or no modification in the composition of mobile phases used in conventional HPLC, micro-bore HPLC attains high column efficiency and peak resolution for the analysis of antibiotics, steroids, ibuprofen and sulfonylurea. Micro-bore HPLC has been demonstrated to be capable of performing high speed analysis with a relative standard deviation of *ca.* 1%.

INTRODUCTION

Due to its speed, accuracy, precision, specificity and high sensitivity, high-performance liquid chromatography (HPLC) has become an indispensable tool for analysis of and monitoring of the efficacy of therapeutic agents[1]. One outstanding addition to HPLC technology is the development by Ishii *et al.*[2] of the micro-bore packed column and associated instrumentation. The technique involves miniaturization of LC equipment by use of a short, micro-bore, packed column, a low dead-volume flow cell and a pump to deliver mobile phase at a low flow-rate of *ca.* 10 μl/min. Recently, micro-bore instruments and packed micro-bore columns became available from various commercial sources. The micro-bore packed HPLC system promises to offer several advantages over conventional HPLC systems by realizing considerable savings of solvent and by attaining very high column efficiency[2–5].

This paper describes applications of a commercially available micro-bore HPLC system for the analysis of pharmaceutical compounds.

EXPERIMENTAL

Instruments

A Waters M6000A pump (Waters Assoc., Milford, MA, U.S.A.) was modified

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by installation of a switching device to allow full flexibility of solvent delivery rate. The dynamic range of the modified pump was 1 μl/min to 9.9 ml/min. A Rheodyne 7410 injector with an internal loop of 0.5 μl (Rheodyne, Berkeley, CA, U.S.A.) was used to inject sample solutions on micro-bore packed columns. Micro-bore columns packed with 10-μm particle size silica (50 cm × 1 mm I.D., microsphere silica; Alltech, Deerfield, IL, U.S.A.) and RP-8 (25 cm × 1 mm I.D., Partisil 10 CCS/C8; Whatman, Clifton, NJ, U.S.A.) were used. The column effluent was monitored at 254 nm, 340 nm or 190 nm using a JASCO UVIDEC 100-III multi-wavelength detector with a micro-flow cell of 0.5-mm light path (dead volume 0.3 μl) or a 5-mm light path flow cell (dead volume 1 μl) (Japan Spectroscopic, Tokyo, Japan) at an attenuation setting of 0.01 absorbance unit full scale (a.u.f.s.).

Reagents

All the solvents used were of UV grade, distilled in glass, obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Ammonium acetate, ammonium phosphate and sodium phosphate were all analytical-reagent grade obtained from Mallinckrodt (Paris, KY, U.S.A.) or J. T. Baker (Phillipsburgh, NJ, U.S.A.). 1-Fluoro-2,4-dinitrobenzene (DNFB) used to derivatize neomycin was obtained from Aldrich (Milwaukee, WI, U.S.A.).

The derivatization procedure for the analysis of neomycin was as described by Tsuji *et al.*[6].

Mobile phases

The silica packed micro-bore column was used to chromatograph the following pharmaceutical compounds.

Steroids. For the analysis of cortisone acetate, fluorometholone, hydrocortisone, hydrocortisone hemisuccinate, methylprednisolone, prednisolone and prednisone, the mobile phase comprised water-saturated butyl chloride–butyl chloride–tetrahydrofuran (THF)–methanol–glacial acetic acid (450:450:105:53:44)[7].

Sulfonylurea. A mobile phase comprising water-saturated hexane–hexane–THF–ethanol–glacial acetic acid (475:475:20:15:9) was used for chromatography of tolbutamide and tolazamide.

Antibiotics. The mobile phase used for chromatography of neomycin was chloroform–THF–water–glacial acetic acid (590:400:8:2)[6]. For novobiocin, the mobile phase was water-saturated butyl chloride–butyl chloride–THF–methanol–glacial acetic acid (44:44:5:4:3)[8].

The RP-8 packed micro-bore column was used to chromatograph the following compounds.

Ibuprofen. The mobile phase comprised acetonitrile–water containing 1% monochloroacetic acid (65:35) at pH 3.0.

Antibiotics. For chromatography of ampicillin the mobile phase was acetonitrile–water–0.2 *M* ammonium acetate buffer (115:85:10) at pH 6.0[9].

RESULTS AND DISCUSSION

Chromatographic parameters

During the chromatography of ibuprofen using an RP-8 micro-bore column,

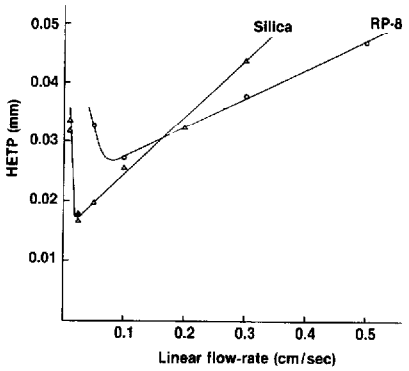

Fig. 1. HETP curves for silica and RP-8 packed micro-bore columns.

the flow-rate of the mobile phase was varied from 20 to 100 μl/min (linear flow-rate: 0.025–0.5 cm/sec) and the height equivalent to a theoretical plate (HETP) of the eluting ibuprofen peak was calculated at each flow-rate condition. As shown in Fig. 1, the flow-rate for the maximum performance of an RP-8 column is *ca.* 20 μl/min or a linear flow-rate of 0.1 cm/sec. At this flow-rate, a plate number, N, of 36,800 per meter was obtained. On the other hand, the optimum flow-rate for the maximum performance of a silica packed micro-bore column for the analysis of steroids was 5 μl/min or a linear flow-rate of 0.025 cm/sec (Fig. 1). At this flow-rate the N value was 60,000 per meter. This high column efficiency would not be a luxury but would be essential for the analysis of complex pharmaceutical compounds of biological origin. At flow-rates of 5–20 μl/min, micro-bore HPLC realizes a saving of solvent by nearly 99% over that of conventional HPLC.

Varying the flow-rate from 10 μl/min to 60 μl/min did not significantly affect peak resolution, R_s. The R_s between flurometholone and prednisolone peaks was 1.1 regardless of the flow-rate.

If N, the capacity factor, k', and the light path length of a flow cell are identical,

TABLE I

PEAK DETECTION SENSITIVITY BETWEEN MICRO-BORE HPLC AND CONVENTIONAL HPLC

HPLC	*Column*	V_0 *(μl)*	N (m^{-1})	*Peak volume,* V_m		*Relative mass sensitivity, micro/ conventional*
				$k' = 1$	$k' = 2$	
*Theoretical**						
Conventional	25 cm × 4.6 mm	1662**	25,000	84	126	10.6/1
Micro-bore	50 cm × 1 mm	157**	25,000	7.9	11.9	
*Real****						
Conventional	25 cm × 4.6 mm	5800	25,000	293	440	16.5/1
Micro-bore	50 cm × 1 mm	350	25,000	17.7	26.6	

* Theoretical peak volume, V_m, was calculated using the equation: $V_m = 4\ V_0\ (1 + k')/N$.

** Theoretical void volume, V_0, was calculated from column packing efficiency of 60/40 (particle/vacancies) (ref. 2).

*** Real peak: prednisone ($k' = 1$); hydrocortisone ($k' = 2$).

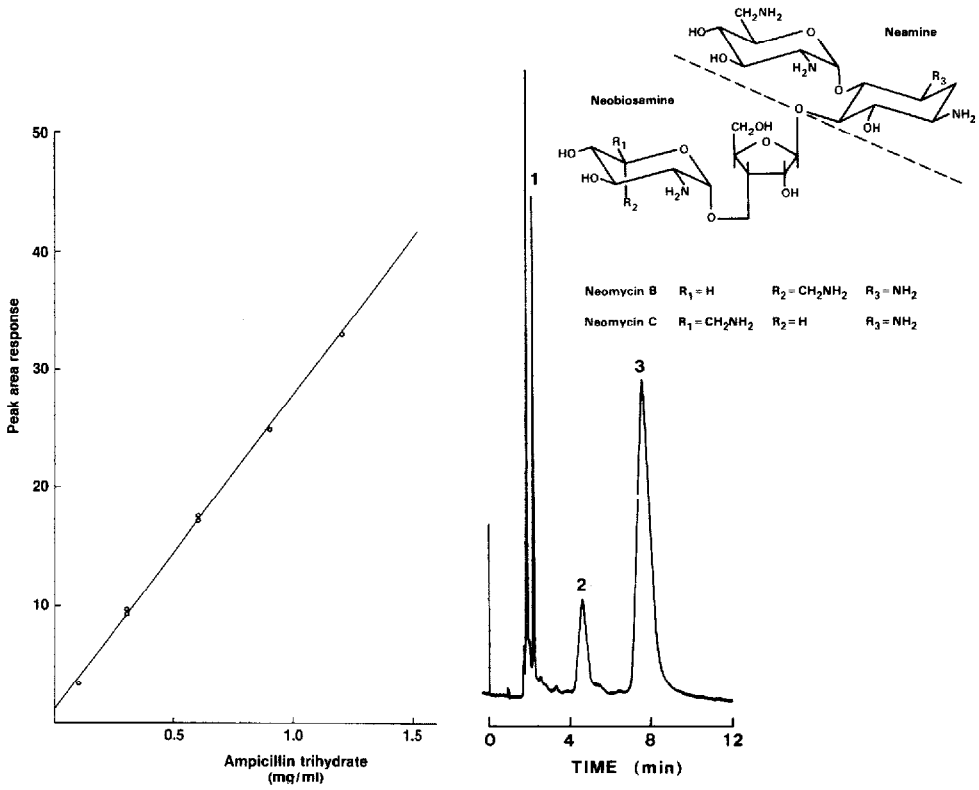

Fig. 2. Ampicillin standard curve using a RP-8 micro-bore HPLC column.

Fig. 3. A silica-packed micro-bore HPLC chromatogram indicating separation of neomycins B and C. Mobile phase: chloroform–THF–water–glacial acetic acid (590:400:8:2) at a flow-rate of 200 μl/min. Peaks: 1 = dinitrophenol; 2 = neomycin C; 3 = neomycin B.

the peak volume, V_m, may be used to compare the sensitivity for detection of a compound between two chromatographic systems[10]. The theoretical mass sensitivity of the micro-bore HPLC system was calculated to be ten times that of the conventional HPLC system for detection of a compound (Table I). A relative mass sensitivity of 21 times was calculated by Scott and Kucera[11]. In reality, however, extra-column effects enter into HPLC systems and significantly affect peak detection sensitivity. The micro-bore HPLC system constructed is actually sixteen times more sensitive than the conventional HPLC system routinely used for the detection of steroids (Table I).

The linear range of the micro-bore HPLC system was examined by constructing a standard curve using ampicillin trihydrate. The standard curve was linear at ampicillin trihydrate concentrations from 0.1 to 1.5 mg/ml with r^2 of 0.9993 (Fig. 2), but exhibited curvature beyond 2 mg/ml.

Chromatography of pharmaceutical compounds

Antibiotics. A normal-phase micro-bore HPLC chromatogram for the analysis of neomycin indicating separation of neomycins B and C is shown in Fig. 3. Elution

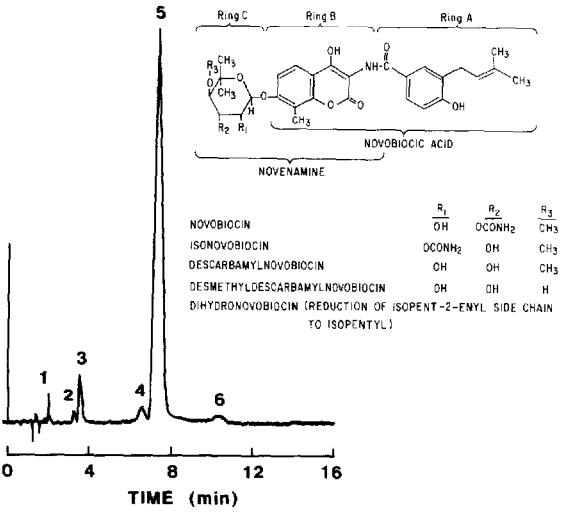

Fig. 4. A normal-phase micro-bore HPLC chromatogram indicating separation of novobiocin from its impurities. Mobile phase: water-saturated butyl chloride–butyl chloride–THF–methanol–glacial acetic acid (44:44:5:4:3). Flow-rate: 300 μl/min. Peaks: 1 = ring B; 2 = ring A amide; 3 = novobiocic acid; 4 = isonovobiocin; 5 = novobiocin; 6 = descarbamylnovobiocin.

of dinitrophenol was brought closer to the solvent front by inclusion of acetic acid in the mobile phase. A normal-phase micro-bore chromatogram of novobiocin is shown in Fig. 4. Novobiocin is very well separated from isomers, impurities and degradation compounds. A reversed-phase chromatogram of intentionally degraded ampicillin using an RP-8 column is presented in Fig. 5. Degradation peaks are very well separated.

Ibuprofen. A reversed-phase RP-8 micro-bore HPLC chromatogram of ibuprofen with an internal standard, valerophenone, is shown in Fig. 6.

Sulfonylurea. A normal phase micro-bore chromatogram of tolbutamide and tolazamide is shown in Fig. 7.

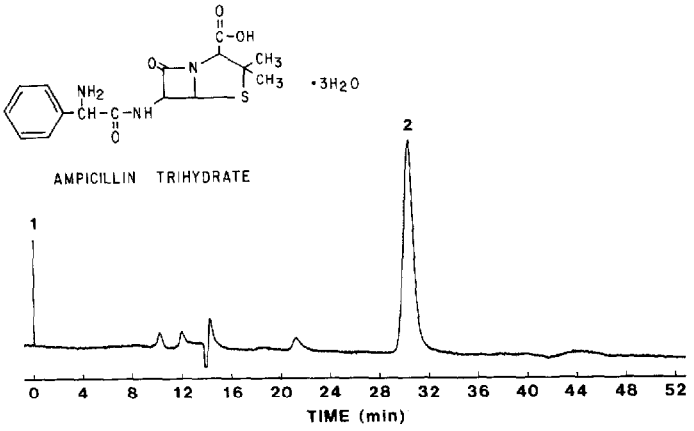

Fig. 5. Micro-bore HPLC chromatogram of intentionally degraded ampicillin using a RP-8 column. Mobile phase: acetonitrile–0.2 *M* ammonium acetate–water (115:85:10), pH 6.0. Flow-rate: 20 μl/min. Peaks: 1 = injection; 2 = ampicillin.

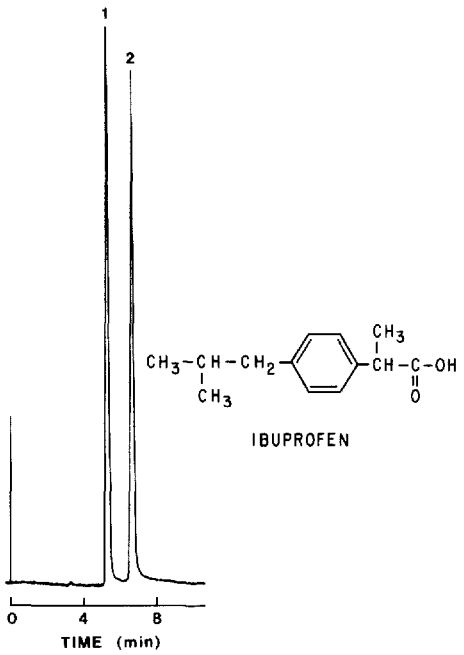

Fig. 6. A chromatogram of ibuprofen (1) with an internal standard, valerophenone (2), using an RP-8 packed micro-bore column. Mobile phase: acetonitrile–water (with 1% chloroacetic acid) (65:35) at pH 3.0. Flow-rate: 80 μl/min.

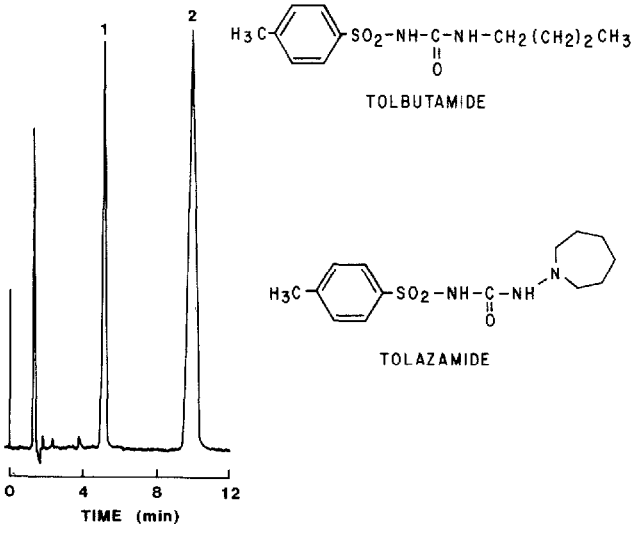

Fig. 7. A normal phase micro-bore HPLC chromatogram of sulfonylurea. Mobile phase: water-saturated hexane–hexane–THF–ethanol–glacial acetic acid (475:475:20:15:9). Flow-rate: 300 μl/min. Peaks: 1 = tolbutamide; 2 = tolazamide.

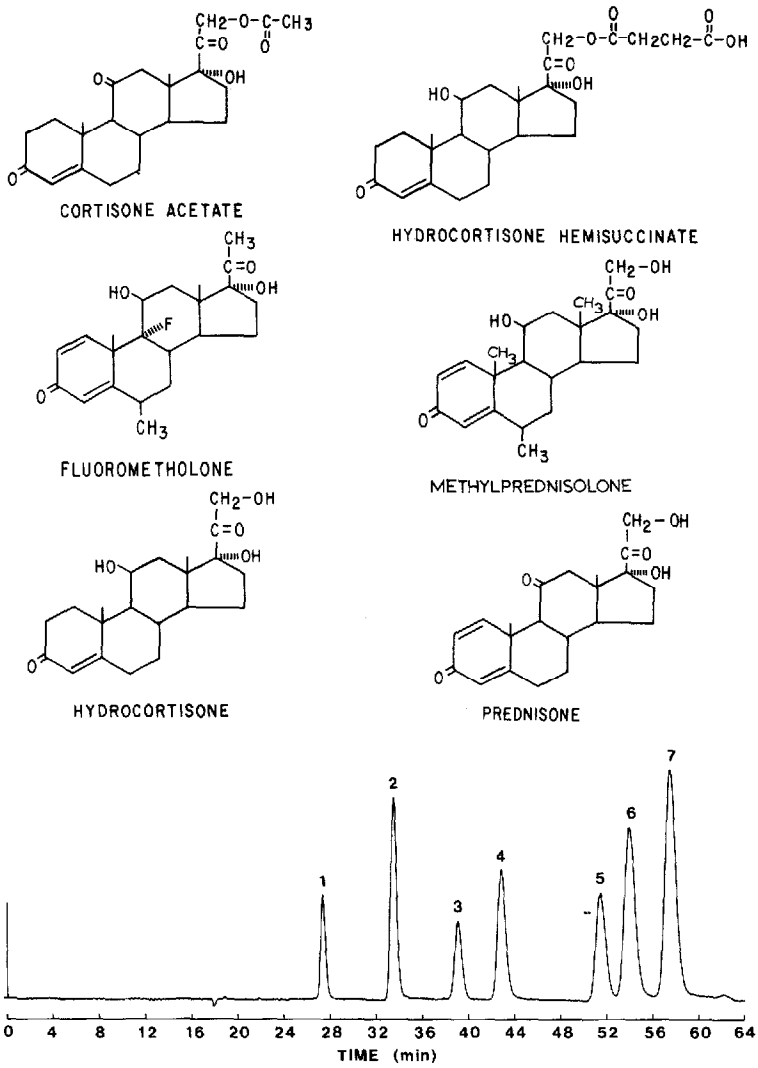

Fig. 8. A normal-phase micro-bore chromatogram indicating high column efficiency separation of cortisone acetate (1), fluorometholone (2), hydrocortisone (3), hydrocortisone hemisuccinate (4), methylprednisolone (5), prednisolone (6) and prednisone (7). Mobile phase: water-saturated butyl chloride–butyl chloride–THF–methanol–glacial acetic acid (450:450:105:53:44).

Steroids. High column efficiency and resolution for steroid peaks are demonstrated in Fig. 8. The peaks of cortisone acetate, prednisone, hydrocortisone hemisuccinate, hydrocortisone, methylprednisolone, prednisolone and fluorometholone are well separated. In contrast, separations of methylprednisolone, prednisolone and fluorometholone by conventional HPLC using a 5-μm particle size silica column (25 cm $\times$ 4.6 mm) are not ideal (Fig. 9).

The micro-bore HPLC chromatography of these pharmaceutical compounds was performed using mobile phases quite similar or identical compounds was per-

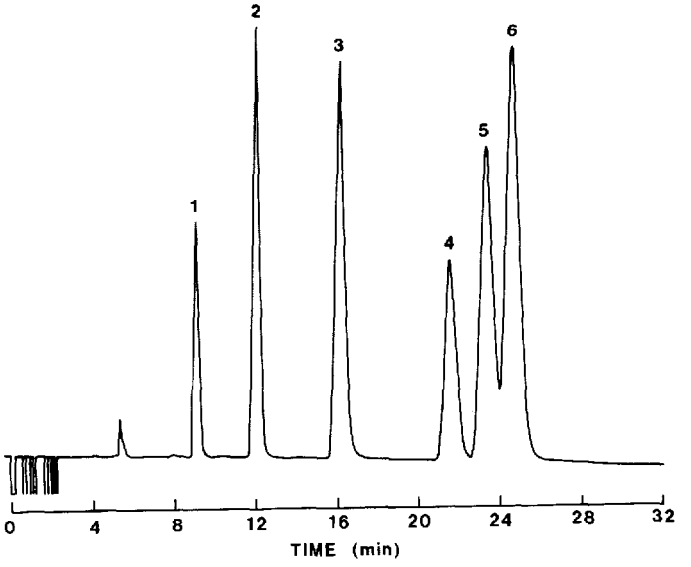

Fig. 9. Conventional 5-μm particle size packed normal-phase HPLC (25 cm × 4.6 mm) chromatogram of cortisone acetate (1), prednisone (2), hydrocortisone (3), methylprednisolone (4), prednisolone (5) and fluorometholone (6). Mobile phase: water-saturated butyl chloride–butyl chloride–THF–methanol–glacial acetic acid (450:450:70:35:30). Flow-rate: 1 ml/min.

formed using mobile phases quite similar or identical in composition to those used in conventional HPLC[1,6–9]. No extensive development time was needed to perform the micro-bore HPLC analysis.

High-speed micro-bore HPLC

As shown in Fig. 10A, a conventional HPLC system takes *ca.* 20 min to complete chromatography of prednisone, used as an internal standard for the analysis of methylprednisolone acetate. If a flow-rate of 10 μl/min were used, the micro-bore HPLC would have taken 35 min to elute a non-retained peak (V_0 = 350 μl, *cf.*, Table I). On the other hand, when a high flow-rate of 300 μl/min is used, it takes less than 1.2 min to elute a non-retained peak. The possibility of performing high speed analysis of steroids by a commercially available micro-bore HPLC system was demonstrated using a flow-rate of 300 μl/min. Chromatography of methylprednisolone acetate and prednisone can be completed in 3.5 min (Fig. 10B).

Applications of micro-bore HPLC using a specialized short column for high-speed analysis of benzene and diazepam derivatives has been reported[10,12]. However, no quantitative data are available. The precision of the high-speed micro-bore HPLC system for the analysis of methylprednisolone acetate using prednisone as an internal standard was determined using a commercially available 10-μm particle size silica packed micro-bore column (50 cm × 1 mm I.D.) with a 0.5-μl loop injector. A Hewlett-Packard 3390A reporting integrator was used to quantitate the peak areas of steroids. With a flow-rate of 300 μl/min, a steroid sample was repeatedly injected every 3.8 min. A chromatogram showing the high speed analysis of steroids is given in Fig. 11. The relative standard deviation for methylprednisolone acetate was calculated to be 0.54% and 1.14% at the flow-rates of 200 μl/min and 300 μl/min, respec-

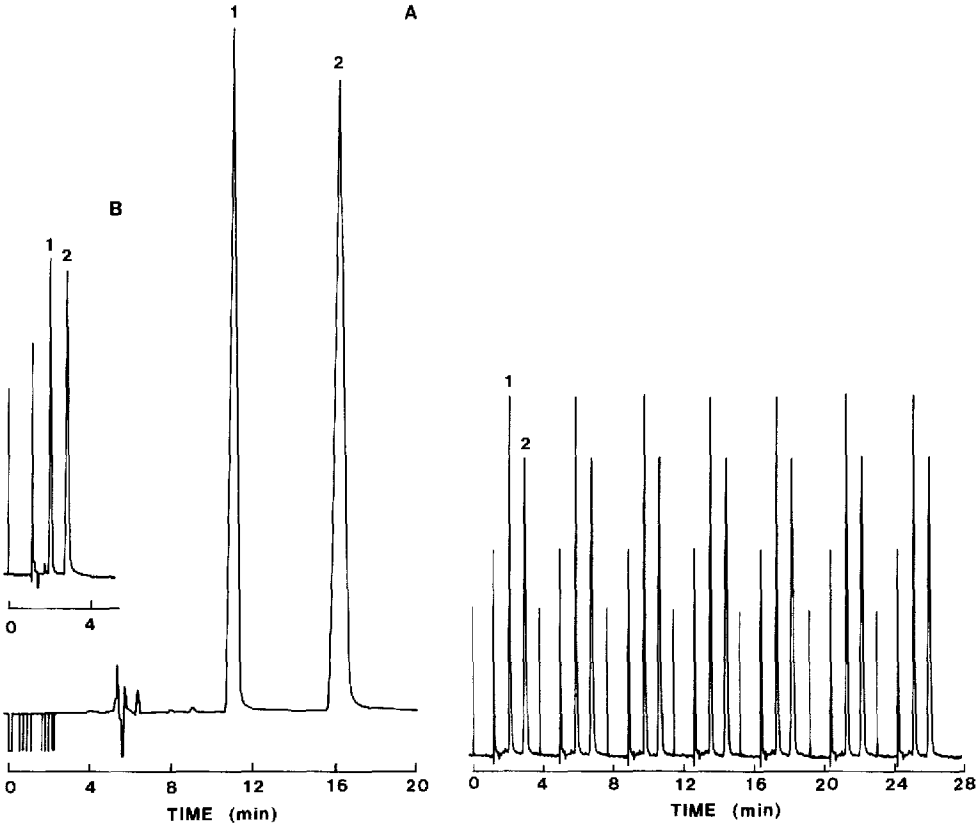

Fig. 10. Normal-phase chromatograms for the analysis of methylprednisolone acetate (1) with prednisone (2) used as an internal standard. A, Conventional HPLC: mobile phase, water-saturated butyl chloride–butyl chloride–THF–methanol–glacial acetic acid (475:475:70:35:30); flow-rate, 1 ml/min. B, High-speed micro-bore packed LC: mobile phase, as in A but 450:450:105:53:44; flow-rate, 300 μl/min.

Fig. 11. High-speed quantitation of methylprednisolone acetate using a silica-packed micro-bore column at a flow-rate of 300 μl/min. Sample size: 0.5 μl. Mobile phase: water-saturated butyl chloride–butyl chloride–THF–methanol–glacial acetic acid (450:450:105:53:44). Peaks: 1 = methylprednisolone acetate; 2 = prednisone.

tively (Tables II and III). Even at the high flow-rate of 300 μl/min, solvent usage of the high speed micro-bore HPLC is approximately 1/20 that of the conventional HPLC (4 min × 300 μl/min *vs.* 20 min × 1000 μl/min). Use of a shorter micro-bore column could make the analytical time even shorter.

Micro-bore HPLC has been demonstrated to be a practical tool with the following advantages: (1) considerable savings in solvent usage by 90–99%; (2) increased mass detection sensitivity by a factor of 10–16 making the system ideal for trace impurity studies; (3) high column efficiency and peak resolution; (4) high speed analysis and (5) only small volumes of sample are required. In order to make the micro-bore HPLC widely acceptable to pharmaceutical industry, however, the development of an automated sample injection apparatus would be needed. The authors fully concur with Scott[12] who stated that it is likely that they (micro-bore columns) will become the analytical columns of the future".

TABLE II

PRECISION OF HIGH SPEED MICRO-BORE HPLC FOR THE ANALYSIS OF METHYLPREDNISOLONE ACETATE

A silica packed micro-bore column was used with a 0.5-μl loop injector. Flow-rate: 200 μl/min.

Expt. No.	*Peak area*		*Peak area ratio*
	Methylprednisolone acetate	*Internal std. (prednisone)*	
1	750,840	933,810	0.80406
2	744,310	917,280	0.81143
3	744,630	917,690	0.81142
4	735,680	906,630	0.81144
5	741,580	912,940	0.81230
6	733,900	914,620	0.80241
	Relative S.D. = 0.54%		

TABLE III

PRECISION OF HIGH SPEED MICRO-BORE HPLC FOR THE ANALYSIS OF METHYLPREDNISOLONE ACETATE

Flow-rate: 300 μl/min.

Expt. No.	*Peak area*		*Peak area ratio*
	Methylprednisolone acetate	*Internal std. (prednisone)*	
1	666,280	808,280	0.82432
2	666,360	808,230	0.82447
3	654,740	819,370	0.79908
4	655,980	809,380	0.81047
5	656,900	800,910	0.82019
6	644,850	800,910	0.80825
7	649,100	797,300	0.81412
	Relative S.D. = 1.14%		

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