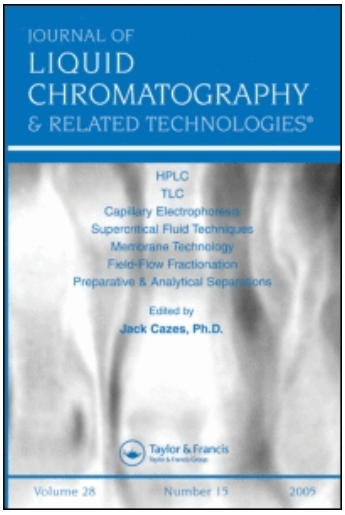

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Practical Microbore Column HPLC: System Development and Drug Applications

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PRACTICAL MICROBORE COLUMN HPLC: SYSTEM DEVELOPMENT AND DRUG APPLICATIONS

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ABSTRACT

Commercially available packed microbore columns have been used to demonstrate the potential of microbore high performance liquid chromatography (micro-LC) systems. The necessity of optimizing the chromatographic system for micro-LC is demonstrated using sulfa drugs and antibiotic standards, and some of the advantages of micro-LC such as high mass sensitivity are shown. Finally micro-LC has been used for the determination of two drugs, reserpine and trichlormethiazide in biological fluids.

INTRODUCTION

There is increasing interest in the use of reduced diameter (microbore) columns for HPLC, and there are a number of reported advantages from these systems [1]. Economy of both solvent and packing material is possible due to the reduced dimensions of the HPLC system. High mass sensitivity and high speed separations can be obtained, and high efficiencies achieved by packing long

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columns or joining columns together [2]. The decreased mobile phase flow rate increases the compatability for interfacing a micro-LC system to a mass spectrometer [3], in addition to other mass sensitive detectors. Much work has been carried out using packed microbore 1mm i.d. columns [1,2].Columns of even smaller i.d. have also been used for HPLC eg open tubular [4] and packed [5,6] capillary columns.

In this work some of the practical considerations are shown for setting up a micro-LC system using commercially available 1mm i.d. packed columns. The apparatus used is based upon commercially available equipment designed for conventional HPLC, i.e. columns of 4.6 mm i.d. and flow rates of $^{1-2}$ mL min^{-1}. However, equipment used for micro-LC must be modified to incorporate low dead volumes. For example, if a flow rate of 40 μL min^{-1} is used a dead volume of 20 μL in the detector would have a marked effect on peak resolution totally independent of the separating power of the column. Thus the injection volume, detector cell volume and connecting tube volumes are all important and must be minimized.

A sulphonamide drug mixture was used to determine the effect of the detector cell volume on the micro-LC system and to demonstrate picogram on-column sensitivity. Mixtures of standard antibiotics were used to

demonstrate the effect of mobile phase flow rate and injection volume on the system. Finally, two biological applications are shown for the determination of reserpine and trichlormethiazide in equine urine.

EXPERIMENTAL

Apparatus

The basic chromatographic system used in this work is similar to that previously described [3,7]. It consists of a modified Waters M-660 solvent programmer [8] and two Waters M-6000A pumps (part no 98470; Waters Associates, Milford, MA), delivering flow rates of 10-999 μL min^{-1}. The output from the two pumps is delivered directly to the inlet port of a valve loop injector. Two valve loop injector designs have been used. The first injector utilized was a Valco submicroliter injector (Part No. AH-CFSV -4-UHPA-N60, Valco Inc., Houston, TX) that was manually actuated. Later improvements included the addition of an air actuator (Part No. AH3-60) and an electronic actuator (Part No. ECF5V.5). The Valco injector equipped with a 0.5 μ L loop did not provide routine reliability with either manual, air or electronic actuation.

A Rheodyne Model 7410 submicroliter injector (Rheodyne Inc., Cotati, CA) equipped with a 0.5 μ L loop and manually actuated was installed in the micro-LC system. This injector has performed reliably for

two years with only routine maintenance. Controlled experiments for reproducibility of injection volume and ghosting were very satisfactory.

A number of packed microbore (1 mm i.d.) reversed-phase HPLC columns were used in this work; a) Alltech Associates, C_{18}, 50 cm, Part No. 1740, Deerfield, IL; b) Chrompack, C_{18}, 50 cm, Part No. 25755, The Netherlands; c) CM Laboratories, C_{18}, 50 cm Nutley, NJ; d) Whatman, ODS-3, 25 cm Part No. 4240-128, Clifton, NJ; e) P. Kucera, Zorbax, 50 cm. Hoffman-LaRoche, Nutley, NJ. In order to minimize dead volume the micro-LC column is connected directly to the outlet port of the injector and detector for columns a,b,c, and e. However, due to incompatability of fittings, a small length of 0.004 in i.d. tubing (Part No. 3031; Alltech Associates, Deerfield, IL) was used for connecting column d to the injector and detector. In all cases 2 μm frits were positioned at the column inlet to prevent column particulates from entering the injector, and at the column outlet to prevent loss of packing material into the detector cell. Dirty or clogged frits cause a gradual increase in pressure in the system. This can be corrected by removing the frit and immersing it in methanol in an ultrasonic bath, or if the frit is part of the column, placing the end of the column in methanol in the ultrasonic bath. This can be

prevented to a certain extent by prefiltration of solvents and sample solution before use.

A Waters Model 440 fixed wavelength (254 nm) UV detector was used for this work equipped with either a regular detector cell (approximately 12 μL volume) or a "micro-cell" (approximately 1 μL volume: Part No. 97212 Waters Assoc.), similar to that previously described by Hermansson [9].

The formation of air bubbles in the micro-LC system can lead to troublesome problems in the UV detector, and they can arise in a number of ways. Any leaks in the HPLC hardware must be eliminated since anywhere that solvent leaks out air can leak in. Dissolved gases in the solvents themselves are minimized by continuous helium sparging [10[. Sometimes bubbles arise upon mixing pure methanol and pure water in the mixing chamber. If this is a problem it can often be overcome by pre-mixing, for example, 10% methanol in the water prior to use. A new microbore column or one which has not been used recently will often have air trapped within it although reversed phase columns should be stored in methanol. It has been found that the best method of eliminating this is to pump pure methanol through the column for a period of time. The pressure drop which occurs after the HPLC effluent leaves the detector is often sufficient to cause formation of bubbles in

the cell. In most cases it is advisable to have some source of back pressure on the cell. This can be accomplished in a number of ways. For example, an old HPLC column connected to the exit of the detector cell will provide sufficient back pressure to preclude outgassing of the eluent while it is passing through the detector cell.

Solvents and Samples

The solvents used in this work were distilled-in-glass methanol and acetronitrile (Burdick and Jackson, Muskegon, MI) and HPLC grade water (J.T. Baker Co., Phillipsburg, NJ). The solvents are suction filtered through a 0.2 μm pore size Millipore filter (Part No. GVMP 0270; Millipore Corp., Bedford, MA) and stored in clean, 500 mL glass bottles. Samples are dissolved in the HPLC eluent whenever possible and filtered through a 0.45 μm disposable filter (Part No. SLHV025NS; Millipore Corp.) before use.

The sulfa drug standards were obtained from commercially available sources. The antibiotic samples were obtained from The Upjohn Co., (Kalamazoo, MI) Trichlormethiazide (Naquasone; Shering Corp., Bloomfield, NJ) was administered orally (400 mg) to a standardbred horse, and urine samples were collected as has been previously described [3]. Extraction and preliminary sample clean up of trichlormethiazide administration

and spiked urine samples were performed as previously described for hydrochlorothiazide [11]. Hydrochlorothiazide (Merck, Sharp and Dohme, West Point, PA) was used as the internal standard and standard trichlormethiazide was obtained by extraction from a pharmaceutical preparation (Naquasone).

Extraction of Reserpine from Equine Plasma

Samples of control equine plasma (4 mL) were spiked with 10-50 ng/mL reserpine (Aldrich Chem. Co., Milwaukee, WI) and extracted with 5 mL of trisolvent (Hexane:dichloromethane:ether, 1:1:1) for 5 min in screw cap test tubes on a Roto Rack at 60 rpm. After centrifugation the organic layer was transferred to a clean test tube and concentrated to dryness in a 65°C water bath under a gentle stream of nitrogen. The residue was redisolved in 50 µL of methanol for micro-LC.

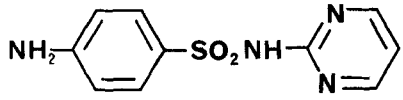
RESULTS AND DISCUSSION

a) Effect of Detector Cell Volume Upon Chromatographic Separations

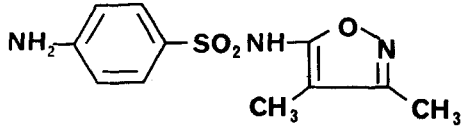
The conventional Waters UV detector is equipped with a detector cell volume of approximately 12 µL. The low flow rates used for micro-LC would generate considerable band broadening from this large cell volume. The results from this conventional LC cell has been compared to that of a "micro-cell" of approximately 1 µL cell volume.

Table 1 Sulfonamide sulfadrugs:sulfadiazine (1), sulfisoxazole (2) and sulfadimethoxine

1. Sulfadiazine



2. Sulfisoxazole



3. Sulfadimethoxine

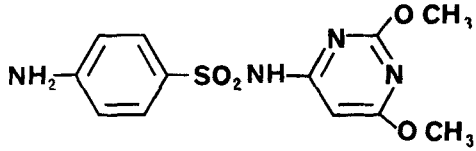


Figure 1 shows the separation of three sulfa drugs (Table 1) sulfadiazine (1), sulfisoxazole (2), and sulfadimethoxine (3), using the 12 μL volume cell (Fig. 1a) and the micro-cell (Fig. 1b). It can be seen that significantly better efficiency is obtained using the micro-cell. Table 2 summarizes the separation parameters obtained using both cells. The peak asymetry (A_s), resolution (R_s) and plate counts ($N_{\frac{1}{2}pkht}$ or N_5 are all increased by using the micro-cell. It was also found that at lower levels eg. approximately 20 pg of sulfa drug injected on column, the first peak, sulfadiazine (1) could not be distinguished from the solvent peaks using the conventional cell, although it was easily observed using the micro cell.

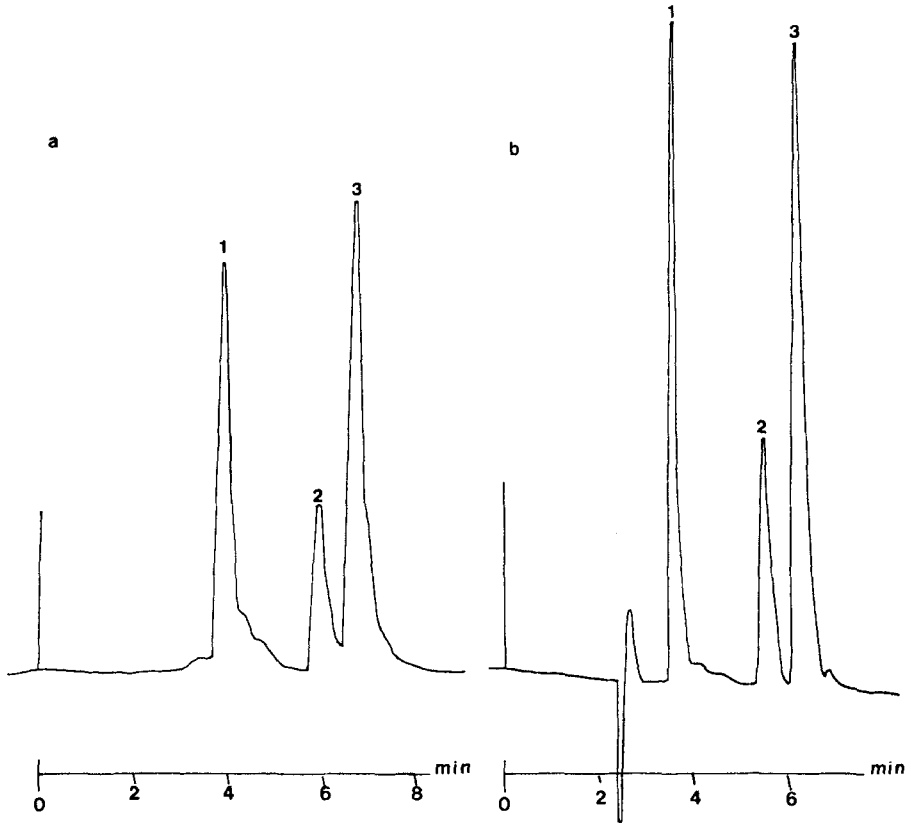


Fig. 1 The separation of three sulfa drugs (1, 2 and 3; 16.5 ng of each), using a) detector cell volume 12 μL and b) detector cell volume 1 μL (micro cell). Conditions: column, Whatman ODS-3 25 cm x 1 mm I.D.; mobile phase, acetonitrile: water, 4:6 (v:v); flow rate, 40 μL min^{-1}; injection volume, 0.5μL.

Table 2 Micro-LC separation parameters for the sulfadrugs in Table 1 using the conventional Waters Model 440 12 µL detector cell and a modified 1 µL micro-LC detector cell. The micro-LC column and conditions were as in Figure 1

	12 µL Cell				1 µL Cell			
	As	$N_{\frac{1}{2}}$	N_5	Rs	As	$N_{\frac{1}{2}}$	N_5	Rs
Sulfadiazine	2.75	1942	1892		2.14	2449	2970	
				2.50				4.04
Sulfisoxazole	2.09	2106	2916		1.40	3219	3540	
				1.09				1.46
Sulfadimethoxine	2.60	2116	2070		1.50	2959	2652	
Av. plates/meter		8467	9168			11503	12216	

As = assymmetry

$N_{\frac{1}{2}}$ = number of plates per column based on peak width measured at half peak height

$N_{5\sigma}$ = number of plates per column based on peak width masured at 4.4% of peak height

Rs = resolution

The plate counts shown in Table 2 are not as high as might be expected from micro-LC columns. This work was carried out on one of the earliest micro-LC columns available in the laboratory, which had already seen significant usage. Also, the sulfa drugs are polyfunctional molecules with a number of active sites and are more likely to have peak tailing than the aromatic mixtures supplied with the column which generate approximately 10,000-20,000 plates per column. Similar results to these have been previously shown using three different sulfadrugs, and these examples also illustrate the benefit of reducing the detector cell volume [9].

b) Sensitivity

High mass sensitivity is one of the advantages of micro-LC. The same sample concentration was used for both micro-LC and conventional LC eg. 1 μ g μL^{-1}. The volume of the eluted peak by conventional LC was approximately 1 mL so that a mass of 0.5 μg can be detected-assuming the concentrations at peak maximum is twice the average peak concentration. If the peak volume eluted using micro-LC is 50 μL, then the mass to be placed on the column to produce a similar detector response need only be 25 ng [1]. It has been proposed by Scott and Kucera [2] that micro-LC is approximately 21 times more sensitive than conventional LC for columns packed under the same conditions. Since sensitivity

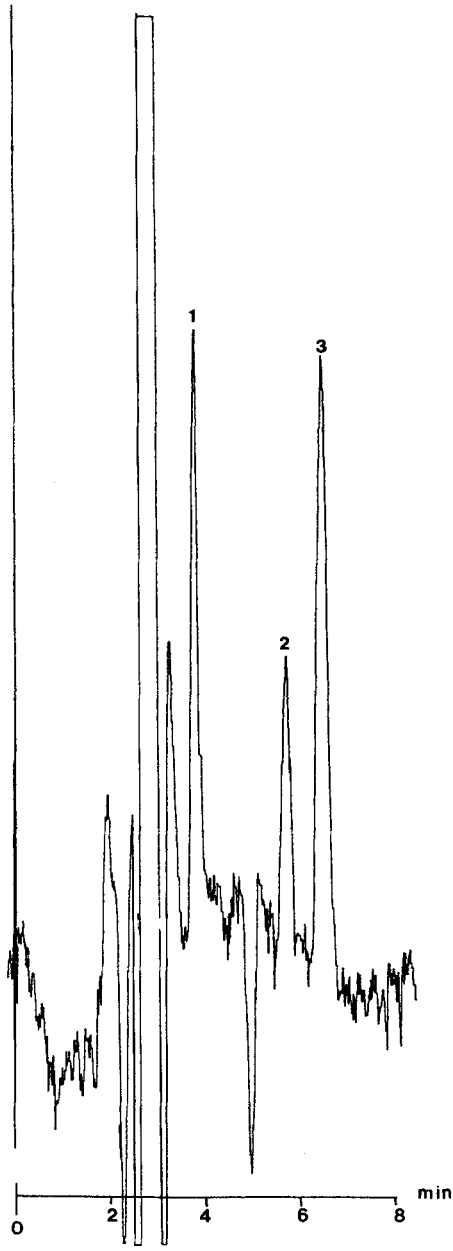


Fig. 2 The separation of 16.5 pg each of the three sulfa drugs (1, 2 and 3). HPLC conditions as Fig. 1; 1 μL volume microcell.

is dependent on the peak volume and thus the column efficiency, very high efficiency columns will provide decreased peak volumes resulting in higher sensitivity. Figure 2 shows the separation of three sulfa drugs with 16.5 pg of each injected on column with a signal to noise ratio of 7:1.

The amount of sample which can be injected onto a microbore column is a limiting factor in this work since sample size greatly affects the efficiency of a micro-LC separation (ref. section d). For example, in Figure 2 the injection volume was 0.5 μL of a 33 ppb solution. If a conventional LC column (4.6 mm i.d.) had been used an injection volume of eg. 10-25 μL could be used. Thus for the same sample 330-825 pg could be injected onto the column. Since at least 20 times more sample can be injected on to the column, this somewhat negates the sensitivity advantage obtained using micro-LC. However, for many applications eg. determination of drugs in biological fluids or tissues, often only a very small sample size is available.

If very dilute samples eg 1 ppb or less, are examined some method of sample pre-concentration is required. In one report using pre-concentration [7], 1 ppb of acetophenone was detected. However, a 10 mL sample was used and this is equivalent to 10 ng of acetophenone on column. Since we have seen appreciable

signal with less than 20 pg of the sulfa drugs injected on column, a similar sample pre-concentration system could provide ppt detection limits.

Another advantage of using some kind of sample pre-concentration system is that it can include a method of eliminating extraneous matter such as proteins and lipids from biological samples before they are introduced onto the microbore column. This eliminates an extraction procedure which should speed analysis time and reduce the possible sources of error in a system.

c) Effect of Flow Rate on Separation

The separation of the three standard antibiotics nodusmicin (4), nargenicin (5) and 18-deoxynargenicin (6), at flow rates of 30 $\mu L min^{-1}$ is shown in Figure 3. Although approximately equal amounts of the three antibiotics were injected on column, a significantly reduced UV response for (4) is seen presumably due to the absence of the large chromphoric R_2 group (ref. Table 3).

The flow rate of the HPLC eluent can markedly affect the plate count (N) of an HPLC column and it has been demonstrated for microbore columns that the HETP value decreases with decreasing mobile phase velocity [2]. Table 4 shows the values of N obtained for the last peak (6) in Figure 3 and the resolution R_s of peaks 5 and 6, obtained at flow rates of 10-110 $\mu L min^{-1}$. It can be seen that both plate count and reso-

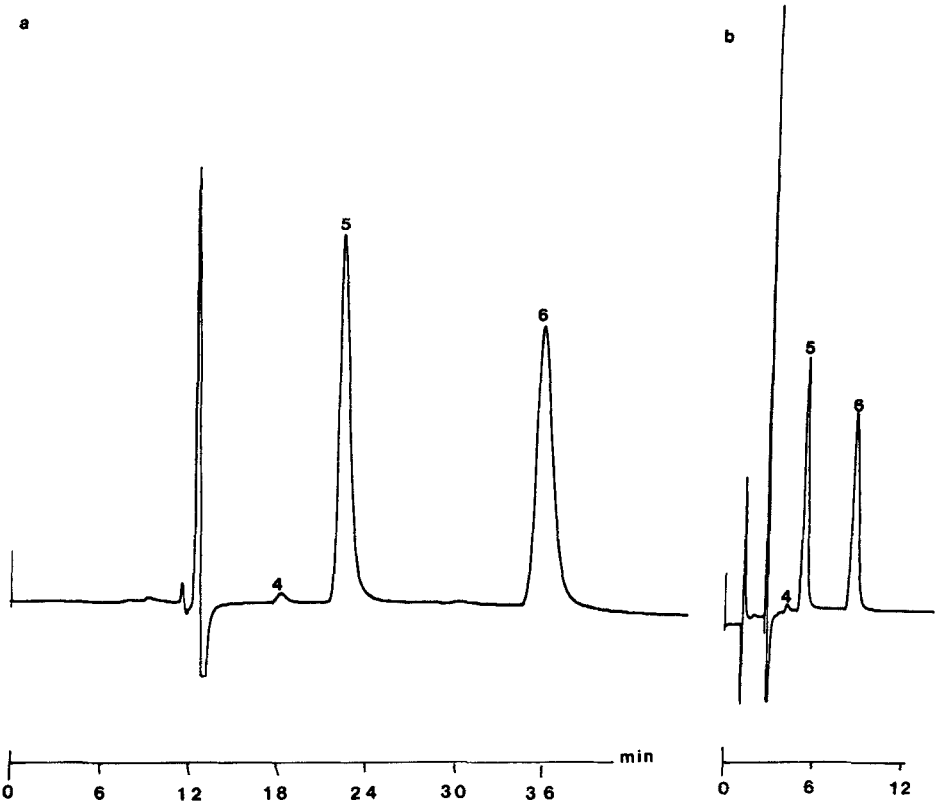
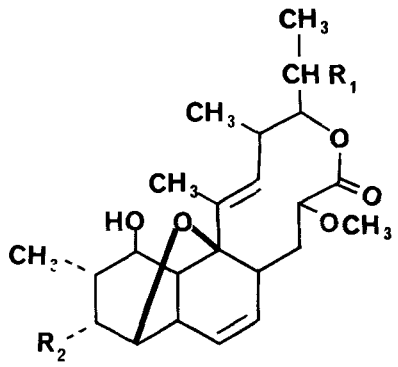
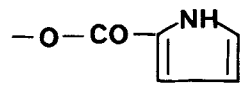


Fig. 3 The separation of three antibiotics (4, 5 and 6; 55 ng of each) at a) 30 µL min^{-1} and b) 90 µL min^{-1}
Conditions: column, Whatman ODS-3 25 cm x 1 mm I.D.; mobile phase, methanol: water, 7:3 (v:v); injection volume 0.5 µL; 1 µL volume micro-cell.

Table 3 Structures of nodusmicin (4), nargenicin (5) and 18-deoxynargenicin



	R_1	R_2
4. Nodusmicin	— OH	— OH
5. Nargenicin	— OH	
6. 18-dexoynargenicin	— H	

lution increase with decreasing flow rate, reaching maximum values at around 30 $\mu L min^{-1}$. The results obtained at 10 $\mu L\ min^{-1}$ show decreased N and R_s. This is possibly due to unreliability of the pump performance at these very slow flow rates rather than column performance.

Thus, for this micro-LC system the optimum flow rate for N and R_s is approximately 30 $\mu L min^{-1}$. In general, 40 $\mu L min^{-1}$ has been used for the majority of the work shown in this paper since this appears to give acceptable plate counts, and HPLC separations generally within 30 min. Also, 40 $\mu L min^{-1}$ of eluent has been found to produce a tolerable pressure for interfacing

Table 4 Plate count (N) and resolution comparison for 18-deoxynargenicin (6) in the mixture shown in Figure 4 at varying eluent flow rates (10-110 μL/min) using a 0.5 μL injector loop volume (a) and at 40 μL/min eluent flow rate using the 5.0 μL injector loop volume (b)

Flow Rate ($\mu L\ min^{-1}$)	N Column (peak 6)	Rs (between 5 and 6)
10[a]	2500	6.71
30[a]	4700	6.14
50[a]	3700	5.2
50[b]	600	1.9
70[a]	2900	4.9
90[a]	2400	4.6
110[a]	1873	3.7

a. 0.5 μL injector loop, Rheodyne Model 7410

b. 5.0 μL injector loop, Rheodyne Model 7410

the micro-LC system to a mass spectrometer for micro LC/MS [3].

d) Effect of Injection Volume on Separations

Figure 4 shows a comparison of the separation of the three standard antibiotics 4, 5 and 6, using a 0.5 μL injection volume (4a) and a 5 μL injection volume (4b). The same mass of sample was injected on column in both cases.

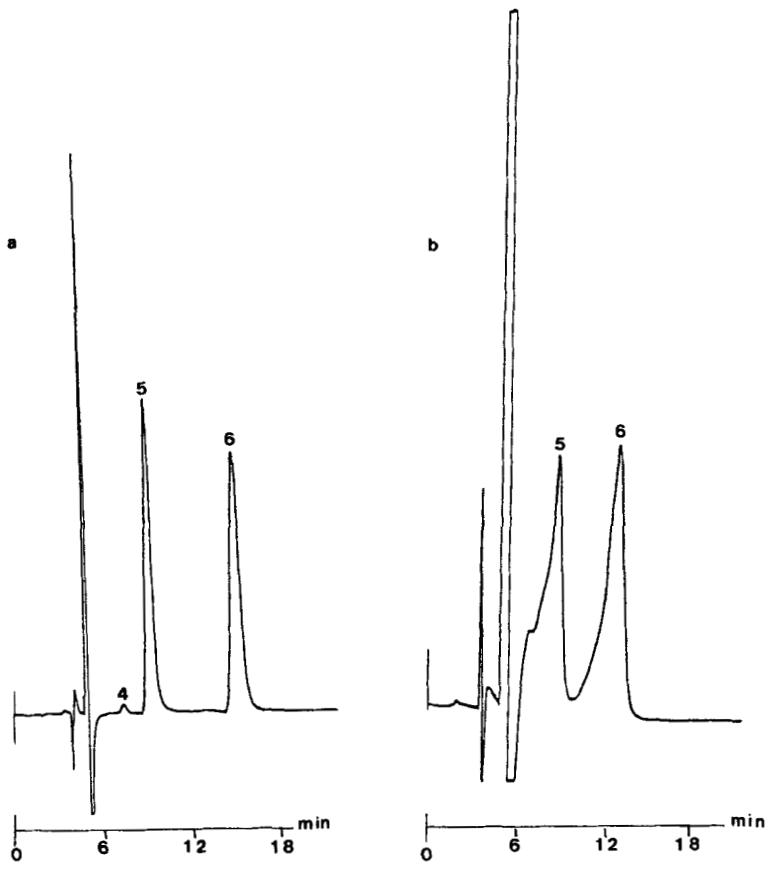
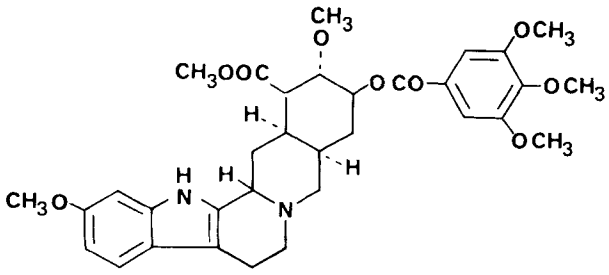


Fig. 4 The separation of three antibiotics (4,5 and 6 Using a) injection volume 0.5 µL of 110 ng/µL of 4,5 and 6, and b) injection volume of 5 µL of 11 ng/µL of 4,5 and 6. HPLC conditions as Fig. 3; flow rate 50 µL min^{-1}.

Table 4 shows a comparison of plate counts for the last peak in Figure 4, 18-deoxynargenicin (6), at flow rates ranging from 10-110 μL/min utilizing the antibiotic mixture and micro-LC conditions shown in Figure 4. Table 4 also shows the resolution of the last two components (5 and 6) as a function of micro-LC flow rate using the 0.5 μL injector loop for flow rates of 10, 30, 50, 70, 90 and 110 μL/min and the analogous data for the 5.0 L injector loop at 50 μ L/min. The corresponding plate count and resolution at 50 μL/min, for example, resulting from the use of the 0.5 and the 5.0 μL injector loop clearly documents the loss of chromatographic performance when the larger sample volume is injected onto the micro-LC column. The maximum sample which can be injected onto a chromatographic column without causing band broadening is proportional to the internal diameter of the column. It has been calculated that for a 1 mm x 250 mm i.d. column with approximately 10,000 plates this volume is approximately 1 μL [8].

e) Determination of Reserpine in Equine Plasma

Reserpine (7) is a potential drug of abuse in the horse because it produces a state of indifference to the environment and thus can be used to calm the animal [12]. Its determination in biological fluids is difficult because a sensitive method is not readily avail-



7. Reserpine

able. Using ion-pairing HPLC techniques with fluorescence monitoring, a detection limit of 100 pg of reserpine per mL of plasma was reported [13]. It was thought that the sensitivity afforded by micro-LC should be advantageous in the determination of reserpine.

Figure 5 shows preliminary micro-LC results for the determination of reserpine in equine plasma. Figure 5a is a UV chromatogram of an extract of a control equine plasma and Fig. 5b shows a UV chromatogram of an extract of the same plasma spiked with 50 ng of reserpine. This is equivalent to 500 pg injected on column. 10 ng of reserpine per mL of plasma (ie 400 pg on column) was easily detected. If the extract is taken up in a smaller sample volume, detection limits of the order of high picogram per mL of plasma should be attainable.

These measurements were made using UV detection at 254 nm which is not an absorbance maximum for re-

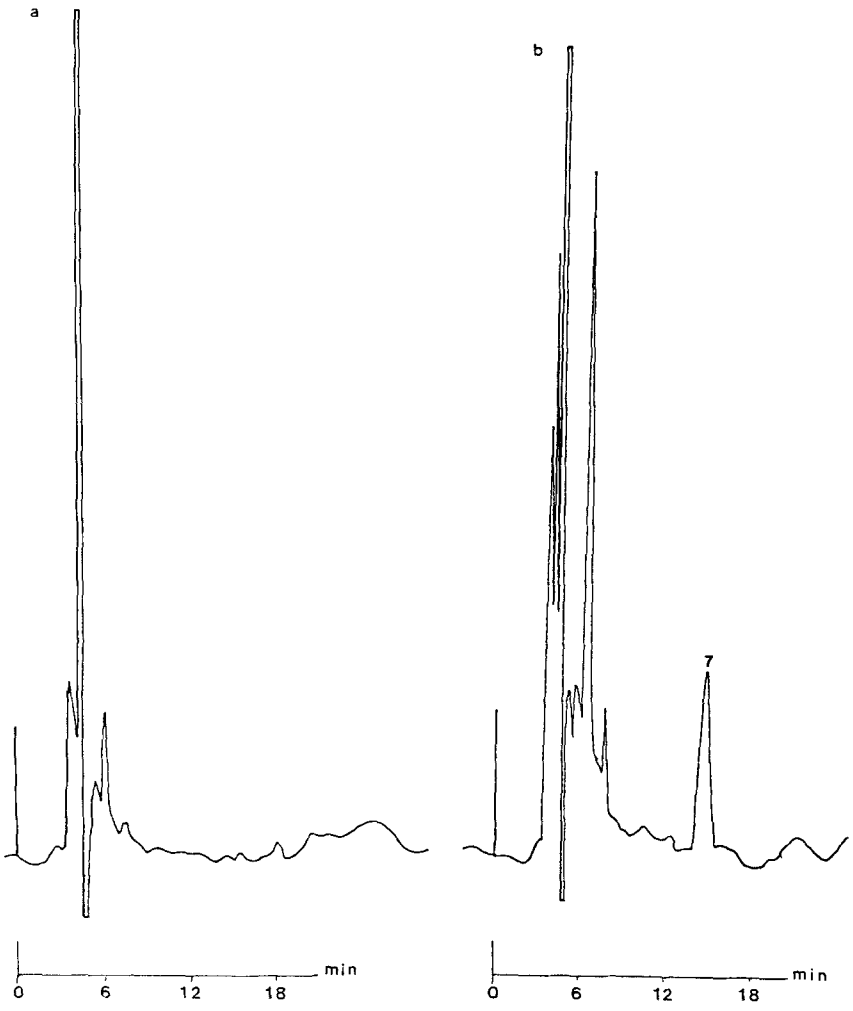
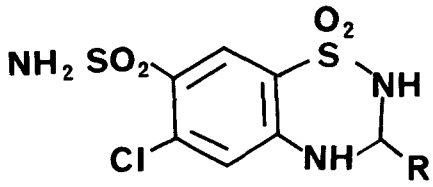


Fig. 5 Micro LC of equine plasma extracts; a) blank extract, and b) spiked with 50 ng reserpine (7) per mL of plasma. Conditions: column, Whatman ODS-3, 30 cm x 1 mm I.D.; mobile phase, methanol: water, 9:1 (v:v); flowrate, 40 μL min^{-1}; injection volume, 0.5μL; 1 μL volume micro-cell.

Table 5 Structures of the diuretics trichlormethiazide (8) and hydrochlorothiazide (9)



	R
8. Trichlormethiazide	- $CHCl_2$
9. Hydrochlorthiazide	- H

serpine (μ_{max} 216, 267, 295 nm; E 617,000; 17,000; 10,200). If a 267 nm filter was used the response would be increased. If a micro fluorescence detector was utilized it is likely that increased sensitivity would be available. These preliminary results demonstrate the potential of the technique and at the present time the detection limits for reserpine in plasma by micro-LC are being assessed.

f) Determination of Trichlormethiazide in Equine Urine

Trichlormethiazide (8, Table 5) is a potent thiazide diuretic. A method for its determination in human plasma and urine has been published [14], and also an HPLC assay of hydrochlorthiazide (9, Table 5), another thiazide diuretic found in equine plasma and urine,

using trichlormethiazide as internal standard [11]. In the latter studies trichlormethiazide was determined using hydrochlorthiazide as internal standard.

Figure 6 shows micro-LC UV chromatograms of equine urine extracts. Figure 6a is from an extract of control equine urine and Figure 6b an extract of control urine spiked with 2.5 μg/mL each of trichlormethiazide and hydrochlorthiazide. A linear cabibration of the trichlormethiazide/hydrochlorthiazide ratio based on peak heights ratios, was obtained over the range of 10 ng - 10 μg of trichlormethiazide per mL of urine. Figure 7 shows a preliminary result obtained from the administration of trichlormethiazide to a horse. The urine sample was collected eight hours after oral administration of 400 mg of trichlormethiazide (Naquasone) and the sample was spiked with 500 ng/mL of hydrochlorthiazide. This example was obtained using a different micro-LC column than that used in Figure 6.

CONCLUSIONS

The results shown illustrate that micro-LC using commercially available columns and equipment is a viable analytical tool. The potential of micro-LC for solving many analytical problems is obvious and there are certain advantages over conventional HPLC, particularly if solvent consumption is important or if one is sample limited. The implementation of these techniques to micro

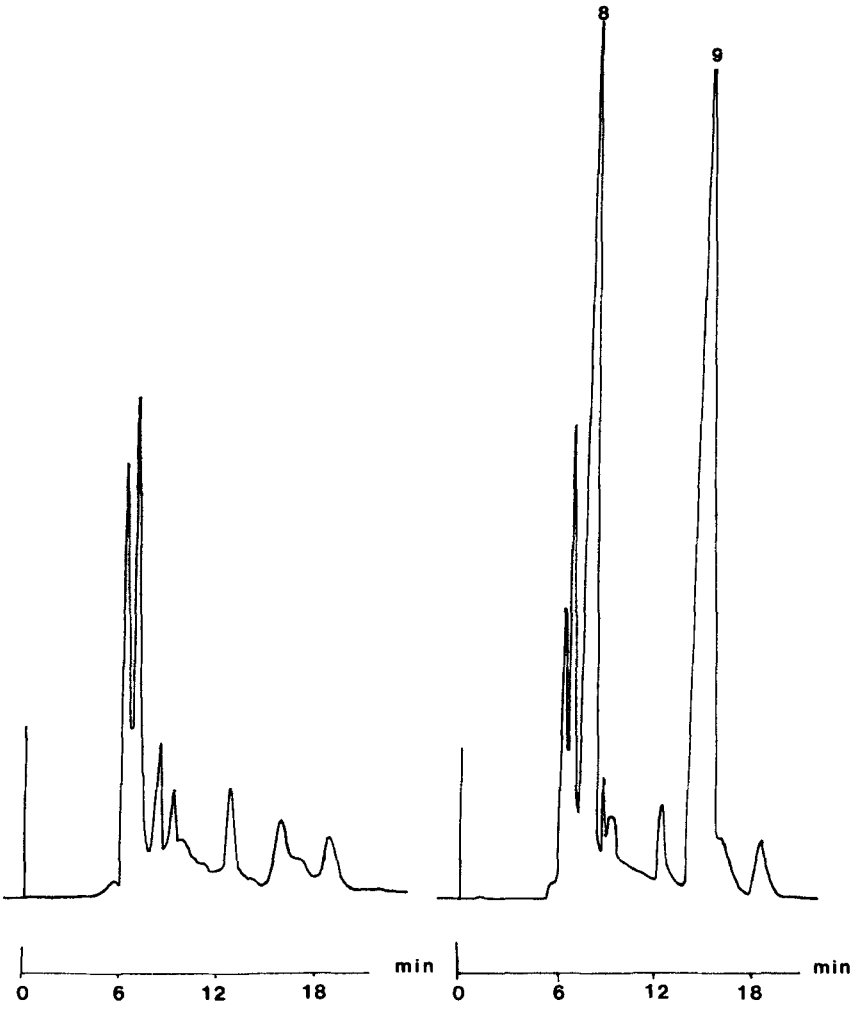


Fig. 6 Micro-LC of equine urine extracts; a) control urine extract and b) extract of control urine spiked with 2.5 μg each of trichlormethiazide (8) and hydrochlormethiazide (9) per mL of urine. Conditions: column, Alltech C_{18}, 50 cm x 1 mm; mobile phase, methanol: water, 4:6 (v:v); flow rate, 40 μL min^{-1}; injection volume, 0.5 μL; micro cell.

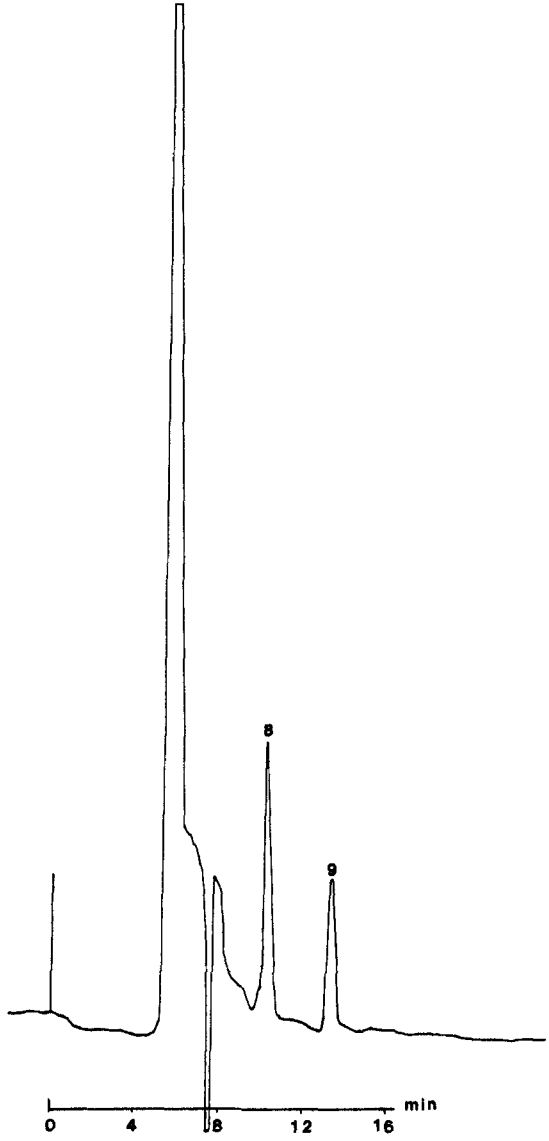


Fig. 7 Extract of equine urine taken 8 hrs after administration of 400 mg of trichlormethiazide (8) to a horse, spiked with 0.5 μg of hydrochlorthiazide per mL of urine. Conditions: column, CM Laboratories, C_{18}, 25 cm x 1 mm i.d.; mobile phase, acetonitrile: water, 35:65 (v:v); flowrate, 40 μL min^{-1}; injection volume, 0.5 μL; 1 μL volume micro-cell.

LC/MS offers the added opportunity for specificity and improved LC/MS sensitivity.

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