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CHROMATOGRAPHY OF PROGESTERONE AND ITS MAJOR METABOLITES IN RAT PLASMA USING MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COLUMNS WITH CONVENTIONAL INJECTION AND DETECTION SYSTEMS

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SUMMARY

A separation of progesterone and its metabolites 17α-hydroxyprogesterone, 20α-hydroxy-4-pregnen-3-one, androstenedione and testosterone by high-performance liquid chromatography with a ternary solvent system is demonstrated. It is found that using a 100 × 1 mm microbore column, higher sensitivity is obtained in the ultraviolet detection of these compounds by using conventionally sized flow cells despite the higher efficiency resulting from the use of a micro-flowcell. It is also shown that, when a non-eluting solvent is used for injection, large injection volumes do not reduce column efficiency, and a 14-fold increase in sensitivity is obtained with a 1-mm column in place of one 4.6 mm in diameter. Using solid-phase extraction and concentration of the sample as pretreatment, progesterone and its hydroxylated metabolites are determined in 0.5-ml samples of rat plasma. The progesterone levels are compared with those obtained by radioimmunoassay.

INTRODUCTION

The numerous reports in the literature concerning the theory and operation of microbore high-performance liquid chromatography (HPLC) columns, *i.e.* those *ca.* 1 mm in diameter have not resulted in the extensive application of these columns to practical analytical problems which might have been expected. This has been pointed out very recently[1,2] and various reasons have been suggested. These reasons include conflicting reports on the best methods for packing such columns and also some disparity of opinion as to their putative advantages. The majority of the literature on such columns has been concerned with the achievement of either very high plate numbers[3,4] or rapid analysis by using high linear solvent flow-rates[5].

It has been amply demonstrated that, to realise the maximum theoretical plate number of any column, micro-flowcells and minimum connecting volumes are required[6,7]. With photometric detectors, sub-microlitre flowcell volumes are obtained

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by reducing the optical path length. This limits absorbance and, thus, the concentration sensitivity of such systems. Also, it has been shown that injector variance must be reduced, and this has meant that injection volumes for microbore columns have been limited to sub-microlitre levels[3,8]. In addition, the dilution factor of a column has been shown to depend inversely on the volume of injection[9] so that the requirement of small sample injections to maintain efficiency further increases the minimum detectable concentration of microbore systems compared to large-diameter columns. The possibility of preconcentrating any sample volume to sub-microlitre levels is unlikely. It has been suggested that a minimum volume of 50 μl is required for redissolving a solute after extraction and drying[10]. The impression is given, therefore, that microbore columns have sensitivity advantages when only microlitre volumes of sample are available[2].

It has been shown that conventional columns are capable of accommodating very large volumes of injected sample[10,11] if the solute is dissolved in what has been termed a non-eluting solvent. It has also been demonstrated that a higher concentration response is achieved with a flowcell of conventional geometry than with a short-path length microcell when using a microbore column[2]. To our knowledge, no evaluation has been made of the effect of applying large volumes in a non-eluting solvent and using conventional geometries of flowcell on the overall chromatographic sensitivity and efficiency of such microbore columns. In principle, it should be possible to sacrifice efficiency in order to achieve a decrease in minimum detectable sample concentration, provided adequate resolution can be obtained. This is perhaps the major requirement in biomedical application of HPLC, and any advantage realised in reduced minimum detectable sample concentration can be converted to reduced initial sample size requirements[12].

In the present work, the effects of injection volume and detector flowcell dimensions are investigated on the column plate number and sample sensitivity for a set of aqueous standards, consisting of progesterone (P), 17α-hydroxyprogesterone (17P), 20α-hydroxy-4-pregnen-3-one (20P), androstenedione (A), testosterone (T) and norethindrone (N), with columns of different diameter. The information gained is used to determine the concentration of P and its hydroxylated metabolite 20P in a 0.5-ml sample of rat plasma, using N as internal standard and employing conventional injection and detection systems. The only microbore requirement for the 100 $\times$ 1 mm column used is a pumping system capable of producing low volumetric flow-rates (< 100 μl/min).

This work is of interest, since the radioimmunoassay method generally used to determine P is subject to cross-reaction with corticosteroids and also yields no information on metabolite concentration. This is a disadvantage, since the 20P compound has been shown to be present at levels comparable with P[13,14] and to have progestational activity[15,16]. The resolution of all accepted metabolites and the quantitation of P and the major hydroxylated species at the levels found in plasma from a fairly small initial sample size represents, therefore, a relevant test of the utility of the proposed method of using microbore HPLC columns.

EXPERIMENTAL

LKB (Stockholm, Sweden) Model 2150 and modified Waters Assoc. (North-

wich, U.K.) M6000A pumping systems were used, each capable of producing volumetric flow-rates of 10 μl/min. Detection systems used were a Waters 440 ultraviolet detector, having a flowcell of 10 mm pathlength and a nominal volume of 18 μl, and a Shimadzu (Kyoto, Japan) Model SPD-6A variable-wavelength detector which had a 3-mm pathlength and 0.6-μl volume. Materials to prepare and pack columns were obtained from HETP (Macclesfield, U.K.) as was the Hypersil ODS (5 μm) stationary phase. Bondelut cartridges (C_{18}) used for plasma extraction were obtained from Analytichem (Harbor City, CA, U.S.A.). Methanol and acetonitrile were supplied by Rathburn (Walkerburn, U.K.) and J. T. Baker (Phillipsburg, NJ, U.S.A.). Water was purified by a Millipore (Harrow, U.K.) Milli-Q system. All steroid samples were obtained from Sigma (St. Louis, MO, U.S.A.) and used as supplied.

The 100 × 1 mm chromatographic columns were prepared by sandwiching a 1/16-in. diameter 2-μm porosity stainless-steel gauze between the 1 mm I.D. × 1/16 in. O.D. unpolished stainless-steel tubing used as the 5-cm lengths of 0.125 mm I.D. × 1/16 in. O.D. tubing at each end with zero dead-volume fittings. The columns were slurry-packed at a constant pressure of approximately 550 bar by a procedure described previously[17]. The 2-mm I.D. and 4.6-mm I.D. columns were prepared and packed by conventional slurry methods.

RESULTS AND DISCUSSION

Separation of steroids

It was not possible to separate the steroid mixture with any concentration of methanol in water. A typical chromatogram of aqueous standards with methanol–water eluents is shown in Fig. 1B. Using acetonitrile as the organic modifier, resolution among the six steroids could be achieved, but, as can be seen from Fig. 1A, the order of elution is not optimal for quantitation of 17P. A ternary solvent, consisting of equal proportions of each organic solvent produced the desired order of elution with adequate resolution between the critical pair A and T. A typical chromatogram is shown in Fig. 1C. The solvent system was also sufficiently selective to separate a decomposition product of progesterone which was eluted between 17P and 20P. With this eluent hydrocortisone and cortisone were eluted before A. The results demonstrated in Fig. 1 could also be obtained with 2- and 4.6-mm diameter columns, 100 mm in length.

Sample injection volume

The well established practice of injecting a solute in a solvent chromatographically different from the eluting solvent has been used[16] with column switching procedures as a means of preconcentration. It does not appear to have been used to minimise or overcome the need for micro-injection systems, although the possibility has been suggested[1]. In the present work, the variation in peak volume was measured as a function of injection volume for the first-eluted compound, A, using the 100 × 1 mm column and the 0.6-μl flowcell in order to eliminate as far as possible detector variance. The previously used procedure[8] of fitting a conventional valve (Rheodyne 7125) with a 100-μl sample loop and operating the valve for limited time periods was employed with the steroid dissolved in water. Table I shows the effect of injection volume on the peak width obtained and on the absorbance measured.

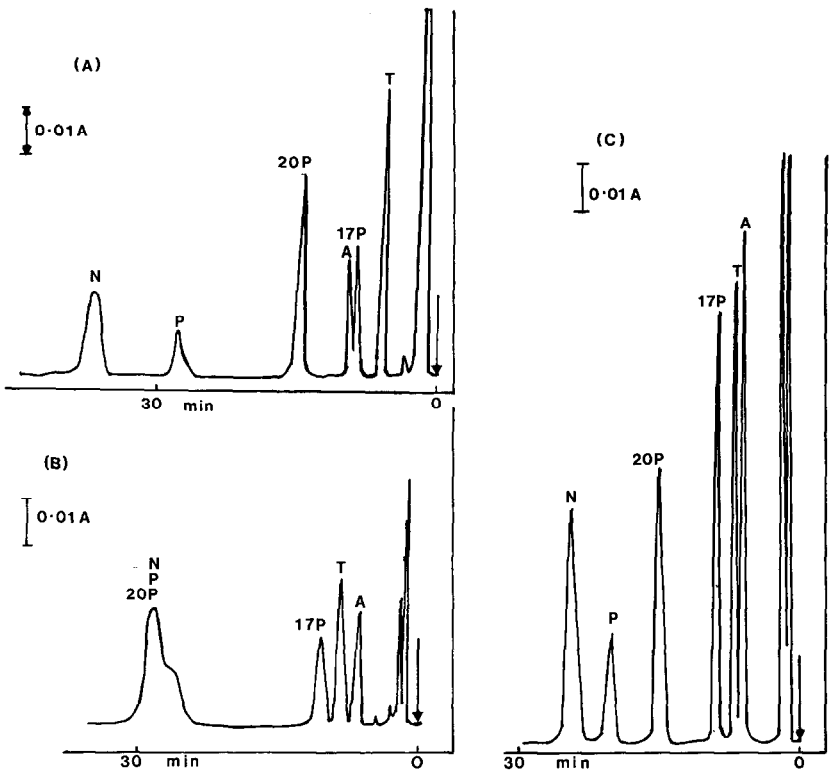

Fig. 1. Representative chromatograms of androstenedione (A) testosterone (T), 17α-hydroxyprogesterone (17P), 20α-hydroxy-4-pregnen-3-one (20P), progesterone (P) and norethindrone (N) in different chromatographic solvents. Column 100 × 1 mm, Hypersil ODS, 5 μm; flow-rate, 100 μl/min; concentration, each steroid 2 μg/ml; injection volume, 20 μl in water. Eluents: (A) acetonitrile–water (35:65); (B) methanol–water (50:50); (C) acetonitrile–methanol–water (25:25:50).

Peak widths are reported rather than the apparent column efficiences, since the system is, in effect, changing from isocratic to gradient as the volume of injection solvent is increased. It is apparent from the results that over a very large range of injection volumes, negligible peak broadening is occurring. Also, the absorbance values obtained increase linearly with volume injected. This verified further the absence of band-broadening due to volume of injection. For subsequent measurements an injection loop of 20 μl was fitted, since this represents the maximum volume that can be injected in duplicate from the previously suggested minimum of 50 μl which can be obtained following reconstitution of an extracted sample[10].

Detector cell volume

The efficiency and sensitivity, in terms of absorbance obtained from a given concentration injected, of the 100 × 1 mm column were determined as follows. A 100 × 1 mm column was coupled directly, either to a Waters 440 (18-μl flowcell) or to a Shimadzu (0.6-μl flowcell) and to the injection valve by using 0.125-mm I.D. tubing of minimum length. The mixture, approximately 2 μg of each steroid per ml

TABLE I

EFFECT OF INJECTION VOLUME ON ABSORBANCE AND PEAK WIDTH FOR ANDROSTENEDIONE

A 100 × 1 mm column is coupled with a 0.6-μl flowcell. Conditions as in Fig. 1C with flow-rate 50 μl/min.

	Volume injected (μl)					
	0.83	*1.7*	*8.3*	*17*	*24.9*	*100*
Peak width (μl)	32.2	33.2	34.0	34.0	33.7	34.5
Absorbance × 10^2	0.152	0.256	1.26	2.48	4.01	16.0

water, was injected (20 μl). The results are shown in Table II. The 0.6-μl flowcell results in plate numbers approximately double those of the 18-ml cell for the rapidly eluted peaks. At higher capacity factors the difference in plate number is not so marked. The results with the 0.6-μl flow-cell indicate an average plate number of 5800, which compares favourably with the plate counts reported recently[1] for a 300 × 1 mm column. The absorbance values quoted show that the increased peak-broadening apparent at the lower efficiences, obtained with the 18-μl cell, is more than compensated by the increased optical pathlength. In real terms, the advantages in absorbance resulting from use of the larger flowcell range from 2.1 to 3.1, depending on the relative efficiences for a particular solute. It was also noted during these measurements that the 0.6-μl flowcell had an appreciably higher noise level than the 18-μl cell. This would indicate that the possible sensitivity advantage of increased signal-to-noise ratio previously suggested[18] is not operative when the flowcell diameter is reduced.

The experiments described above indicated that 1-mm diameter columns having plate counts of about 6000 per 100 mm can accommodate 20 μl of sample solution and that the effect of decreasing flowcell volume results in reduced concentration sensitivity in spite of reduced band-broadening. It appeared appropriate, therefore, to make a direct estimate of the concentration sensitivity increase that can be achieved practically as a result of decreasing the internal diameter of otherwise identical columns with standard injection and detection systems.

TABLE II

COMPARISON OF DIFFERENT DETECTOR FLOWCELL VOLUMES AND PATHLENGTHS ON THE COLUMN EFFICIENCY AND ABSORBANCE OF DIFFERENT STEROIDS WHEN COUPLED TO A 100 × 1 mm COLUMN

Conditions as in Fig. 1C with flow-rate of 30 μl/min.

Flowcell volume (μl)	*Flowcell pathlength (mm)*	*Plate number for each solute*						*Absorbance (× 10^2) for each solute*					
		A	*T*	*17P*	*20P*	*P*	*N*	*A*	*T*	*17P*	*20P*	*P*	*N*
18	10	2267	2478	3587	3703	4469	3948	11.2	9.9	5.1	8.3	6.2	9.0
0.6	3	5632	5098	5433	6923	6776	5207	5.2	4.4	2.1	3.0	2.0	3.2

Column diameter

Injections of aqueous solutions (20 μl) of the six steroids were made into 4.6-, 2.0- and 1.0-mm columns, incorporated sequentially into the same HPLC equipment with the 20-μl loop injection system. A flow-rate of 1.0 ml/min was chosen for the 4.6-mm column as being approximately that commonly used for such columns and which resulted in 0.2 ml/min and 0.05 ml/min for the 2- and 1-mm columns, respectively, to yield the corresponding linear flow-rates. Table III shows the efficiency and absorbance values obtained. Also shown in Table III are the retention times for the various solutes on the different columns. In all columns there is a noticeable increase in effect of extra-column band-broadening shown by the decreased plate count for early-eluted peaks. However, there is a corresponding increase in sensitivity for each compound as column diameter is reduced, the average difference in absorbance between the 1- and 4.6-mm columns being about 14. This increase is accompanied by an increase in retention time, due to the gradient-generating effect of the relatively large injection volume on the 1-mm column.

This increase in absorbance is appreciable, and the comparison has been made under conditions where different diameters have been used with normal injection volumes and detector geometries. It would appear from these measurements that microbore columns will yield higher sensitivity under these conditions than wide-diameter columns by more than one order of magnitude, taking into account the chromatographic system as a whole, including the injection volume. While it has been shown that an increase in mass sensitivity should result which varies inversely as the square of the column diameter[2,18], a more complete description of the dilution factor (*DF*) produced by a column of volume V_{col}, plate number N, and porosity ε for a solute having a capacity factor k' and resulting from an injection volume V_{inj} is[9]

$$DF = \frac{1.41\ \varepsilon(1 + k')\ V_{col}}{V_{inj}\ N^{\frac{1}{2}}} \quad (1)$$

TABLE III

COMPARISON OF COLUMN EFFICIENCY AND SOLUTE ABSORBANCE VALUES FOR COLUMNS OF DIFFERENT DIAMETERS, EACH 100 mm IN LENGTH, FOR AN 18-μl, 10-mm PATH-LENGTH FLOWCELL

Conditions as in Fig. 1C with flow-rates for the 4.6-, 2- and 1-mm I.D. columns of 1.00, 0.21, and 0.05 ml/min, respectively.

Steroid	*Absorbance × 10²*			*Retention time (min)*			*Plate number*		
	Column I.D. (mm)			*Column I.D. (mm)*			*Column I.D. (mm)*		
	1	*2*	*4.6*	*1*	*2*	*4.6*	*1*	*2*	*4.6*
A	6.90	2.80	0.60	7.84	7.24	6.29	1431	2672	2898
T	6.08	2.40	0.55	9.0	8.2	6.6	1887	3446	3250
17P	4.69	1.87	0.40	11.5	10.5	8.4	2034	2948	4411
20P	4.67	1.77	0.30	19.7	18.8	17.2	2913	4019	5121
P	2.45	0.85	0.13	26.7	25.5	21.9	3464	4905	5290
N	2.95	1.10	0.21	31.9	29.8	24.6	3160	5208	6939

Eqn. 1 can be applied to the extremes of the columns used in the present work and written as a relative sensitivity, which will be more meaningful than the direct comparison of column diameters when other variables may not be constant. If the mass sensitivity, S, is inversely related to the dilution factor for a given column,

$$\frac{S_1}{S_{4.6}} = 21.2\left(\frac{\varepsilon_{4.6}}{\varepsilon_1}\right)\left(\frac{V_{\mathrm{inj}_1}}{V_{\mathrm{inj}_{4.6}}}\right)\left(\frac{1 + k'_{4.6}}{1 + k'_1}\right)\left(\frac{N_1}{N_{4.6}}\right)^{\frac{1}{2}} \quad (2)$$

This relationship is reasonably consistent with the results obtained in the present investigation. The ε terms will be the same, since the same material is used as the stationary phase, and packing density appears adequate from the high efficiencies observed under ideal conditions. The capacity factors, although different, due to the unavoidable gradient operation of the microcolumn, are both large enough for the complete term $(1 + k'_{4.6})/(1 + k'_1)$ to be close to unity. Also, the V_{inj} value in these experiments has been made the same for each column. Thus, the only term which may reduce the sensitivity ratio is the plate number term. The results indicate that, conservatively, the 1-mm column is about half as efficient as the 4.6-mm diameter column, due to the extra-column variance of the large flowcell. The real advantage, therefore, to be expected from the microbore system operated under these conditions is $21.2/\sqrt{2} = 15.0$ which is in good agreement with the average value of 14 obtained for this set of compounds.

Biological measurements

A 0.5-ml plasma sample was pretreated by solid-phase extraction with Bondelut (C_{18}) cartridges in the following way. After wetting the cartridge with methanol and water, 0.5 ml plasma was applied. This was washed successively with 2 ml water and 2 ml of 20% methanol in water. The steroids were then eluted by 2 × 0.5 ml volumes of methanol. The extract was reduced to dryness in a stream of nitrogen, and the extracted material was redissolved in 50 µl of 20% aq. methanol, resulting in a ten-fold increase in concentration. Fig. 2 shows the resultant chromatograms, obtained on subjecting plasma, spiked with 50 ng/ml of each steroid (Fig. 2C) and blank pooled plasma (Fig. 2B) to this treatment. Fig. 2A shows the direct injection of an aqueous solution containing 400 ng/ml of each steroid.

Male rat plasma was spiked with four concentrations of P and 20P over the range 10–50 ng/ml. These were subjected to the above pretreatment and chromatography, and calibration curves were prepared. The regression equations of these lines were

$$\text{P: Peak height ratio P/N} = 0.0176\ C_{\mathrm{P}} + 0.00192,\ R^2 = 0.996$$
$$\text{20P: Peak height ratio 20P/N} = 0.0222\ C_{\mathrm{20P}} + 0.0059,\ R^2 = 0.997$$

where C represents the concentration of each steroid in ng/ml. It was estimated that the detection limit of these steroids was in the region of 2 ng/ml in plasma at a signal-to-noise ratio of 3. The precision of the method based on a pooled plasma sample was 4% relative S.D. ($n = 6$).

In order to compare this determination with the generally used radioimmu-

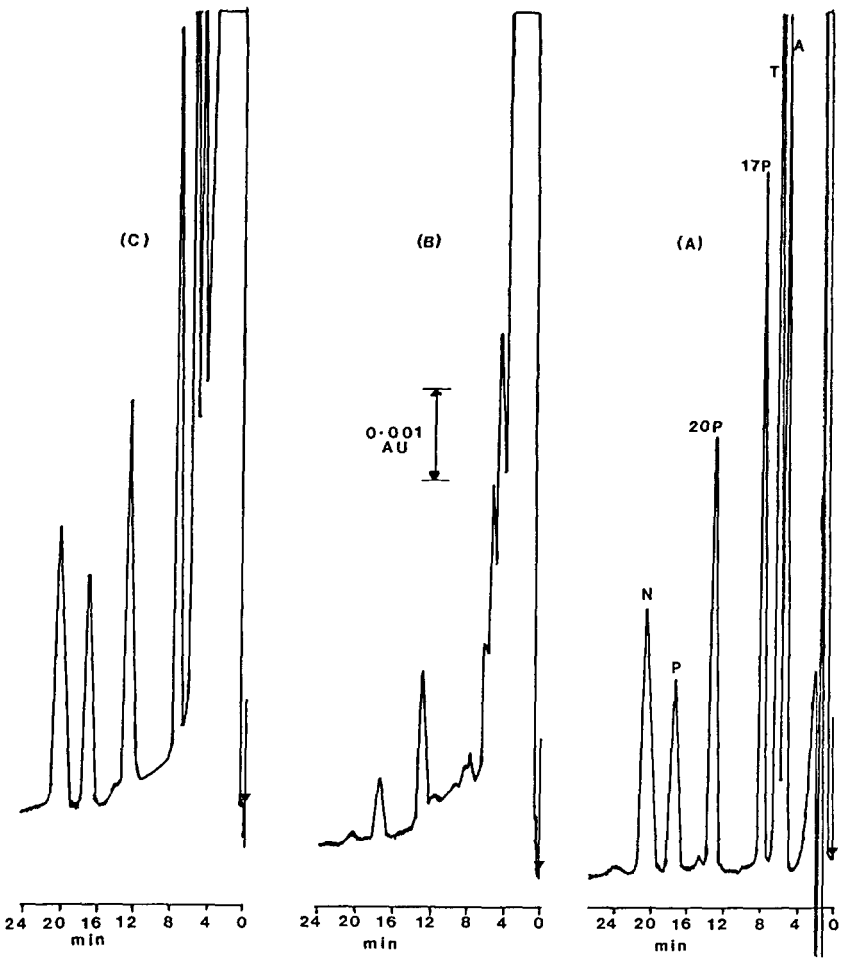

Fig. 2. Representative chromatograms of aqueous steroid standards 400 ng/ml (A); blank pooled plasma (B), and plasma spiked with 50 ng/ml of each steroid after extraction and concentration (C). Compound identification and chromatographic conditions as in Fig. 1C.

noassay method for progesterone, six plasma samples were assayed for progesterone by the above HPLC method and also by radioimmunoassay, using a kit supplied by Radioassay Systems Labs. (Carson, CA, U.S.A.) with tritium as the label. The HPLC method also yielded values for the putative active metabolite 20P. The results of these measurements are shown in Table IV. It is seen that good agreement is obtained for the progesterone values and that, while appreciable levels of the 20P are present, they correspond to roughly half the value of the progesterone concentration in corresponding samples.

TABLE IV

CONCENTRATIONS OF PROGESTERONE (P) AND 20α-HYDROXY-4-PREGNEN-3-ONE (20P) BY HPLC AND RADIOIMMUNOASSAY (RIA) IN SIX PLASMA SAMPLES

Chromatographic results represent the mean of duplicate determinations.

Sample No.	*Concentration ± std. error (ng/ml)*		
	P		*20P, HPLC*
	RIA	*HPLC*	
1	13.9	16.1 ± 1.1	6.34 ± 1.1
2	21.5	18.9 ± 1.1	7.10 ± 1.1
3	16.8	17.6 ± 1.1	6.80 ± 1.1
4	6.4	6.7 ± 1.2	4.63 ± 1.1
5	15.3	12.4 ± 1.2	5.70 ± 1.1
6	9.7	9.8 ± 1.5	4.70 ± 1.4

CONCLUSIONS

This work indicates that short microbore columns can be packed by relatively conventional means without the need for elaborate fittings or excessively high pressure and that such columns yield plate counts in the region of 6000 per 100 mm when operated with a micro-flowcell and conventional injection systems under conditions where on-column preconcentration of solute is possible.

The unorthodox approach of utilising a large-volume flowcell rather than one of sub-microlitre dimensions results in a degree of band-broadening, but this is offset by maintenance of higher absorbance. This, coupled with the observed lack of peak-broadening due to what has hitherto been considered an unacceptable injection volume, *i.e.* 20 μl, allows a real increase in overall mass sensitivity to be realised on reducing the column diameter.

These findings in no way contradict the theoretical ideas proposed by other researchers. It is realised that such increases in sensitivity will only be obtainable under certain conditions. Firstly, the capacity factors must be large enough so that the peaks will be relatively broad, but this will very often be the case with any diameter of column in order to ensure freedom from interference by large concentrations of reagents and early-eluted endogenous compounds. Also, the selectivity must be high. Resolution must be achieved on thermodynamic rather than kinetic grounds so that specificity of analysis is preserved. Modern developments involving the use of multicomponent solvent systems to improve selectivity for neutral solutes[19] and ion-pairing strategies for mixtures of ionogenic compounds[20,21] have tended to favour this approach to resolution. The compromise which is being made in the present work is that theoretical plate number and thus column peak capacity and also, to some extent, time of analysis are being sacrificed to obtain high mass sensitivity from the chromatographic analysis.

A large number of published chromatographic analyses involve the quantitation of relatively few individual compounds, and often the main criterion is to measure very low detection limits when appreciable sample volume is available, or to

improve detection limits when the amount of sample available is limited. The present work illustrates an alternative strategy of employing microbore columns with real biological samples, which may be attractive to other analysts with similar criteria allowing the use, as it does, of existing detection and injection equipment.

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