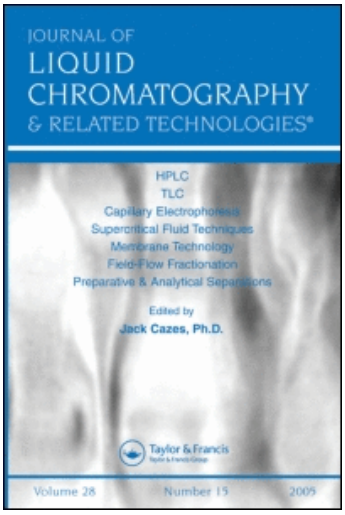

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A Study of Precolumn and Analytical Column Dimensions for On-Line Sample Pretreatment in HPLC

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A STUDY OF PRECOLUMN AND ANALYTICAL COLUMN DIMENSIONS FOR ON-LINE SAMPLE PRETREATMENT IN HPLC

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ABSTRACT

A systematic study has been made of the effect of precolumn size and shape in relationship to the size of analytical column for column switching systems in HPLC. It is found that, even for miniature precolumns (volumes 30 - 500 μl) the volume does not affect the efficiency achieved nor the recovery of a set of six steroids from aqueous solution when 4.6 and 2 mm diameter analytical columns are used. Coupling of these precolumns with 1 mm diameter analytical columns results in decreased efficiency although in the systems tested sensitivity increased as analytical column diameter decreased. Such coupled systems could be applied to analysis of selected steroids in matrices of urine and saliva with little difficulty. Deterioration in both efficiency and recovery were found when extracting from a plasma matrix although linear calibrations were obtained.

INTRODUCTION

Solid phase extraction is most frequently carried out as an off-line procedure and is capable of providing effective removal of endogenous species from biological

samples. Preconcentration of the analyte(s) is also possible which can improve detection limits. On line, the solid extractant is contained in a closed precolumn and switching valves are used for transfer of analytes. The perceived advantages of the latter are simplification of the overall analysis and improvement of detection limits by utilising all of the sample available. The principles of this approach have been reviewed [1]. An advantage often claimed for such on-line column switching methods is the longevity of the column[2,3]. This is perhaps surprising in terms of the contradictory evidence that cartridges, used off-line, incorporating such stationary phases (most frequently C-18) are not reusable[4]. A survey of the literature since the above review showed that a wide variety of precolumn dimensions are in use and also reveals that many such column switching methods incorporate an additional off-line pretreatment procedure. No systematic study has been reported concerning the interrelationship between precolumn and analytical column dimensions. In addition, while many publications report the number of injections possible without adversely affecting the performance of the different columns, it is not always possible to generalise from these data since sample size and prior pretreatment may vary.

Such a systematic study is of particular interest when so called microbore analytical columns are employed. Such columns have been shown to provide advantages in concentration sensitivity when large sample volumes are injected in weak chromatographic solvents[5]. The performance of such columns of 1 and 2 mm diameter may well depend upon the band broadening introduced during the column switching. Relatively few publications have been located which report such data[6,7]. The work of Goewie et al[8] showed that for a 250 x 4.6 mm analytical column, precolumn dimensions were not critical in terms of efficiency of resultant peaks. On the other hand, it has been shown[9] that appreciable band broadening due to extra column effects results when microbore analytical columns are employed in column switching mode.

In the light of the above, the purpose of the present work is to:

1. Make a systematic study of the effect of precolumn/analytical column

dimensions with respect to analyte recovery from aqueous solution, plate number and sensitivity of detection using a set of six steroids as test solutes.

2. Evaluate the practical utility of such systems in the pretreatment of the common biological matrices of plasma, urine and saliva with respect to recovery, ruggedness and effectiveness of sample clean-up and compare the findings with reports in the literature.

EQUIPMENT AND MATERIALS

Chromatographic equipment consisted of Varian 2510 and Shimadzu LC5A pumps coupled with a Jasco 875-UV variable wavelength detector. Analytical columns were stainless steel, 100 mm long of diameters 1,2, and 4.6 mm. The loading valve was a Rheodyne 7125 fitted with a 20 or 1000 μl loop and column switching was via a Rheodyne 7010 valve. The analytical columns were slurry packed in the laboratory using 5 μm ODS Hypersil. Precolumns were constructed as in Figure 1A. having internal diameters of 1, 2,3 and 4 mm with a standard length of 10 mm. This design allowed variation of column length and diameter within a standard connecting holder which facilitated incorporation into the chromatographic system. An additional set of precolumns was made having a standard diameter of 3 mm and lengths of 2.5, 5, 7.5 and 10 mm incorporating suitable PTFE spacers. The precolumns were dry filled using 40 μm octadecyl silica obtained by removing the packing from Bondelut sample pretreatment cartridges. A simplified diagram of the column switching system is shown in Figure 1B. The set of test steroids used were obtained from Sigma and consisted of progesterone (P), 17α-hydroxyprogesterone (17P), 20α-hydroxy-4-pregnen-3-one (20P), androstenedione (A), testosterone (T) and norethindrone (N). These were chosen since the separation of these compounds required a ternary solvent of methanol, acetonitrile (Rathburn Chemicals) and water (25:25:50)[10] and thus represented a stringent test of resolution.

RESULTS AND DISCUSSION

Performances of the analytical columns used alone

The data in Table 1 provide information as to the chromatographic performance of the system for different diameters of chromatographic columns used alone

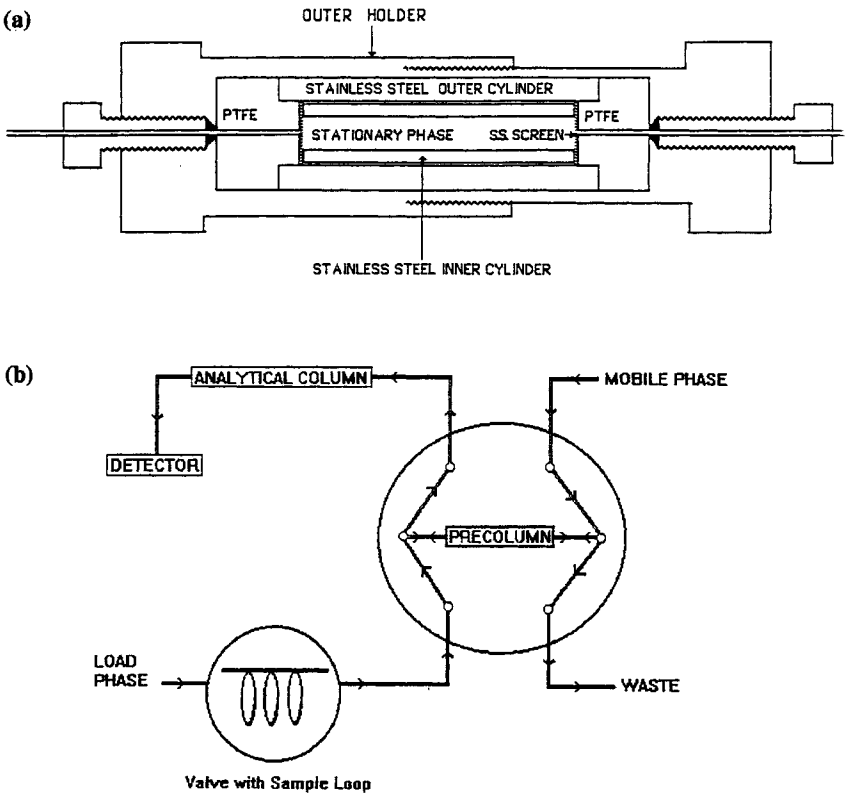

Figure 1. (A) Construction of Precolumn (B) Column switching system.

and, as such, constitute baselines from which the effects of the inclusion of precolumns into the system may be assessed. These results were obtained by injecting an aqueous solution of the six steroids at a concentration of 2 μgcm^{-3} each via a 20 μl loop directly on to the analytical column. To maintain constancy of linear flow rate, volumetric flow rates of 1.0, 0.2 and 0.05 cm^3min^{-1} were used for the 4.6, 2.0 and 1 mm diameter columns respectively. The data show that, as column diameter decreases, the overall system efficiency decreases. At the same time the absorbance data show an overall increase in sensitivity as column diameter decreases. The average sensitivity increase on going from a 4.6 to a 1

TABLE 1 Comparison of Plate Number and Absorbance Maxima for the Analytical Columns used alone

Steroid	Plate Number			Absorbance x 100 (RSD%)		
	Column Diameter			Column Diameter		
	4.6	2	1	4.6	2	1
T	7557	3036	2034	0.671 (1.8)	2.61 (2.9)	3.60 (1.9)
A	8185	3228	2089	0.550 (1.5)	2.28 (1.6)	3.29 (0.83)
17P	7210	3191	2144	0.365 (3.1)	1.54 (3.4)	2.22 (1.0)
20P	7068	3363	1998	0.257 (2.4)	1.17 (2.9)	1.87 (1.4)
P	7023	3677	2331	0.178 (2.0)	0.798 (2.6)	1.28 (1.8)
N	6720	3980	2242	0.132 (3.6)	0.589 (3.4)	0.948(2.9)

mm diameter column being 13 taking into account the decreased path length used with the 1 mm column. It can be seen that the absorbance ratio increases with retention time of the solute being measured which also indicates the existence of extra- column band broadening with the 1 mm diameter column. Such extra column band broadening effects are well established [11,12] The maximum sensitivity increase nevertheless is similar to that reported previously[10].

General characteristics of the column switching system

A 1 x 10 mm precolumn was incorporated with a 100 x 2 mm analytical column and a 1cm^3 loop in a column switching system designed to operate in the backflush mode as represented in Figure 1B. The absorbance of each steroid and the plate number of the corresponding peak were determined as follows. After filling the loop with an aqueous solution containing the six steroids at concentrations of 40 ngcm^{-3}, these were loaded on to the precolumn using different volumes of water, transferred to the analytical column and eluted with the chromatographic solvent.

Two loop volumes were required for maximum loading. It was also found that less than 2% loss was incurred by using 20 loop volumes which can allow extensive

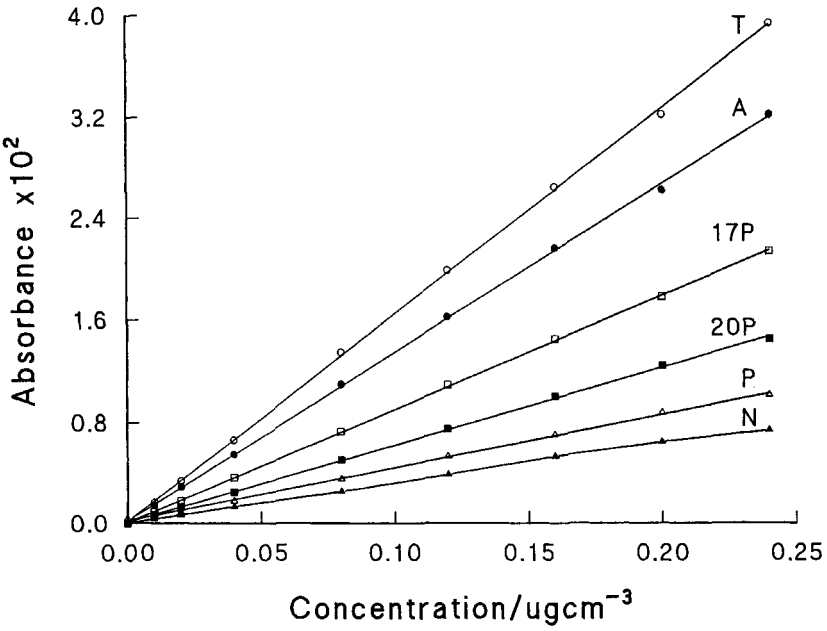

Figure 2. Plots of peak absorbance against concentration for six steroids using a 1x10 mm precolumn and a 4.6x100 mm analytical column. Injection loop volume 1000 μl. Compound identification as in figure.

removal of water soluble endogenous components from biological samples. The linearity of calibration was determined by injecting 1 cm^3 samples of the steroid solution ranging in concentration from 10 - 240 ng cm^3 for each compound. The linearity of plots of absorbance against concentration injected is shown in Figure 2. Regression analysis yielded correlation coefficients greater than 0.998 with a mean relative standard error of the gradients of 1.05%. This confirms the quantitative utility of such preconcentration systems at least for aqueous solutions.

Coupled systems

Each of the three analytical columns were, in turn, coupled with each of the precolumns. Samples of the six steroid test mixture (1 cm^3) at concentrations of 40 $ngcm^{-3}$ were injected and the absorbances and efficiencies of all peaks were

recorded as the mean of 4 replicate injections. The percentage recovery of each steroid was also determined by relating the response to that obtained in the absence of a precolumn by injecting 20 μl of 2 μgcm^{-3} solution.

With the 4.6 and 2 mm diameter columns one way analysis of variance showed no significant difference at the 5% level in the overall system efficiency nor in the maximum peak absorbance for any of the test solutes as a result of incorporating a precolumn in the system and operating in the backflush mode. This was true for all of the precolumn geometries tested. Also, recoveries among all steroids and precolumns were uniformly high. For the 4.6 mm diameter column the mean recovery of all steroids from all precolumns used was 99.3% (sd 1.8%) and for the 2 mm column 99.6% (sd 1.9%). Significant differences in the efficiency and maximum peak absorbance were observed with the 1 mm column. However, when recoveries were based on peak area measurements a mean recovery of 96.5% (sd 2.2%) was obtained.

The effects of precolumn volume on absorbance and theoretical plate number are demonstrated graphically in Figures 3 and 4. In Figure 3 it is evident that coupling any size of precolumn with either a 4.6 or 2 mm diameter analytical column has no effect upon the absorbance values and the same is seen for the effect on theoretical plate number in Figure 4. The plots in these Figures for the 1 mm column show initial drops in both absorbance and efficiency. This appears to be due to extra column band broadening in the column switching system rather than to the precolumn volume since, after this initial drop, both variables appear to be substantially independent of column volume.

The effect of precolumn volume on precolumn load capacity was determined by removing the analytical column and connecting the precolumn directly to the detector. A solution containing 3 μgcm^{-3} of each steroid was pumped through the precolumn at $1 cm^3 min^{-1}$ and the absorbance monitored with time. Breakthrough volumes were determined for each precolumn and the corresponding mass of each steroid calculated. The results are shown in Table 2 where it can be seen that the ratio of mass loading to precolumn volume is approximately constant at

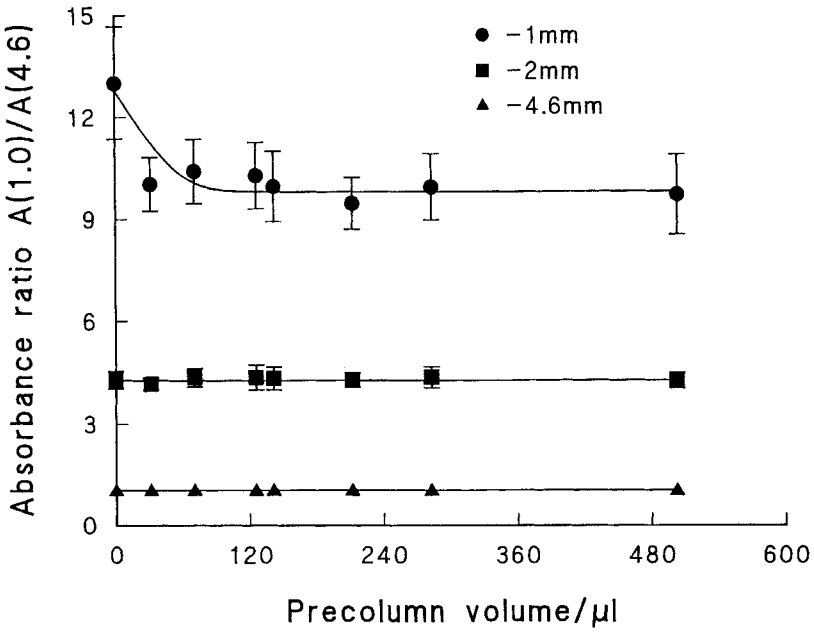

Figure 3. Plots of the mean peak absorbance for all solutes obtained for different analytical column diameters divided by the corresponding value obtained using a 4.6 mm diameter column as a function of coupled precolumn volume.

1.2 - 1.3 for all precolumns other than the two whose diameter to length ratio exceeds 0.5. These precolumns show a significantly lower mass loading. This is attributed to the flow pattern within the column not allowing full saturation of the stationary phase. For all the precolumns investigated, however, the mass loading was in excess of 40 μg of each steroid representing a total analyte loading of 240 μg.

From the above it would appear that analytes can be preconcentrated to an appreciable degree on such coupled column systems using 4.6 and 2 mm diameter columns without appreciable loss of overall system efficiency. The use of 2 mm diameter analytical columns confers a similar increase in sensitivity to that observed with conventional valve and loop injection. When 1 mm diameter

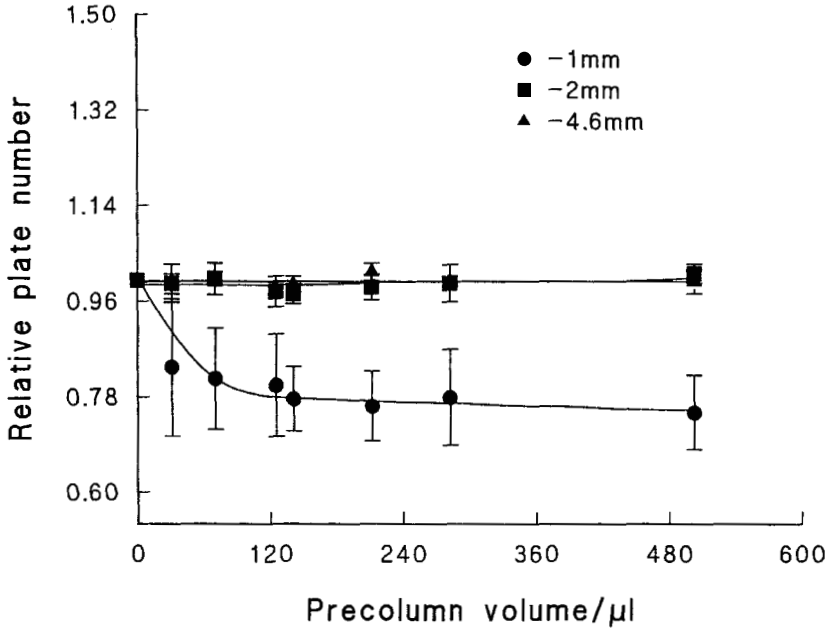

Figure 4. Plots of the relative theoretical plate number for all solutes using different analytical column diameters as a function of coupled precolumn volume.

TABLE 2 Analyte Load Capacity of Different Precolumns

Precolumn Size/ mm	Volume/ mm^3	Mass of each steroid/ μg	Mass/Volume / $\mu g mm^{-3}$	Precolumn Diam./Length
1 x 10	31.4	40	1.27	0.1
2 x 10	125.7	149	1.19	0.2
3 x 10	282,7	369	1.31	0.3
4 x 10	502.7	612	1.22	0.4
3 x 2.5	70.7	64	0.91	1.2
3 x 5	141.4	150	1.06	0.6
3 x 7.5	212.1	248	1.17	0.4

columns are incorporated there is an appreciable drop in the theoretical plate number which is independent of precolumn dimensions but which is associated with the extra column band broadening inherent in the coupled system. This results in lower gains in sensitivity over conventional diameter columns than were realised in the absence of on-line preconcentration.

The above refers only to the preconcentration aspect of column coupling from aqueous solutions which lack matrix interferents. In practice, for such techniques to be useful for the analysis of drugs in biological fluids, the effect of endogenous components present in such complex matrices on recovery and efficiency as well as on the general ruggedness of the system is of considerable importance. For this reason, the performances of the coupled systems were examined for the steroid set in matrices of urine, plasma and saliva. In the present study, the effects of the endogenous species present in such matrices on the recovery, sensitivity and longevity of the system is examined. The equally important aspect of sample clean-up by effective removal of endogenous components which could potentially interfere and thus adversely affect the specificity of the analysis is not addressed. The rationale for this is that any clean-up procedure will be particular for the analyte. That is, more drastic wash procedures may be employed the greater the capacity factor of the analyte in the chromatographic solvent and, conversely, it may not be possible to obtain adequate removal of endogenous materials from weakly retained analytes. The effect of matrix components upon the quantitative aspects of recovery, sensitivity and plate number are, however, likely to be a general characteristic of that matrix and therefore operative for all analytes.

Extraction from urine

The 40 $ngcm^{-3}$ solution used in previous experiments did not allow reliable detection of the first three steroids eluted (T, A and 17P) on the 1 or 2 mm diameter columns due to the increased solvent front following injection of 1 cm^3 of spiked urine and washing with water only. For this reason in the examination of urine matrix, concentrations 0.3 μgcm^{-3} in each of two steroids, T and 17P,

were used. Absorbance, efficiency and recovery of each steroid were determined from water and from urine using 1 x 10 and 4 x 10 mm precolumns coupled in turn with 4.6, 2 and 1 mm diameter analytical columns.

No significant differences were found between matrices of water or urine for absorbance or efficiency and the recovery from urine was identical with that from water alone. The main effect of the urine matrix was an increase in back pressure of approximately 10 bar on loading. The magnitude depended on the concentration of the urine in terms of endogenous components.It was independent of steroid concentration and was reduced following aqueous flushing of the precolumn. Up to 5 cm^3 aliquots of urine could be applied by repeated operation of the 1 cm^3 loading valve and loop. Each precolumn was found to be capable of accommodating at least 20 repeat applications of urine with no deterioration in performance or permanent rise in back pressure. These findings are consistent with the general experience that urine as a matrix is readily pretreated for drug analysis and that direct, on column injection of urine is often possible[13,14].

Extraction from Plasma

Plasma samples spiked with 0.3 μgcm^{-3} of the two steroids were injected via the precolumn and the peak efficiencies and sensitivities were compared with the corresponding values in aqueous solutions for the precolumns and analytical columns specified above. In contrast to the situation in urine, there was an appreciable decrease in overall system efficiency and sensitivity even taking into account band broadening produced with the 1 mm diameter column. Also, the recovery from plasma was significantly lower than from aqueous solution. The recoveries from plasma diluted with 20% water are shown in Table 3. The dilution was found necessary in order to allow loading of plasma samples without excessive increases in system back pressure. It can be seen from Table 3. that the decrease in efficiency and recovery is common to both precolumns but is more marked for the smaller precolumn. The efficiency and thus sensitivity

TABLE 3 Peak Areas of Testosterone and 17α-Hydroxyprogesterone in Water and 80% Plasma and Recoveries from Plasma

		Testosterone			17α-OH-progesterone		
		Peak Area		Percent	Peak area		Percent
Column Size/ mm	Precolumn Size/ mm	Water	Plasma	Recovery	Water	Plasma	Recovery
4.6 x100	1 x 10	501	327	65.3	432	262	60.6
	4 x 10	498	437	87.7	440	350	79.5
2 x 100	1 x 10	625	405	64.8	541	330	61.0
	4 x 10	628	549	87.4	545	415	76.1
1 x 100	1 x 10	1006	684	68.0	899	575	64.0
	1 x 10	1016	842	82.9	890	671	75.4

continue to decrease with successive injections to a value of 40% of the original. Recovery based on peak area measurements remained constant over approximately 10 injections. It was confirmed that the decrease in recovery was due to plasma constituents by recording the peak areas obtained by injection of the above concentration of the two steroids in solutions containing different proportions of plasma to water. The results are shown in Figure 5. where it is seen that for both precolumns tested the peak area decreases monotonically with increasing proportion of plasma in the sample matrix injected. This decrease is more marked for the smaller precolumn. In spite of the decrease in recovery, the linearity of calibration in plasma remains high and plots of peak area against steroid concentration over a range of concentration from 150 - 600 $ngcm^{-3}$ are shown in Figure 6. The lines for plasma show the lower sensitivity consequent on the lower recoveries obtained. It was found also that recovery could not be improved by substituting C-8 for C-18 in the precolumn and that reducing the particle size merely exacerbated back pressure problems.

It is not possible on the basis of the present study unequivocally to specify the reason for the deterioration in recovery when plasma is injected. It is known that

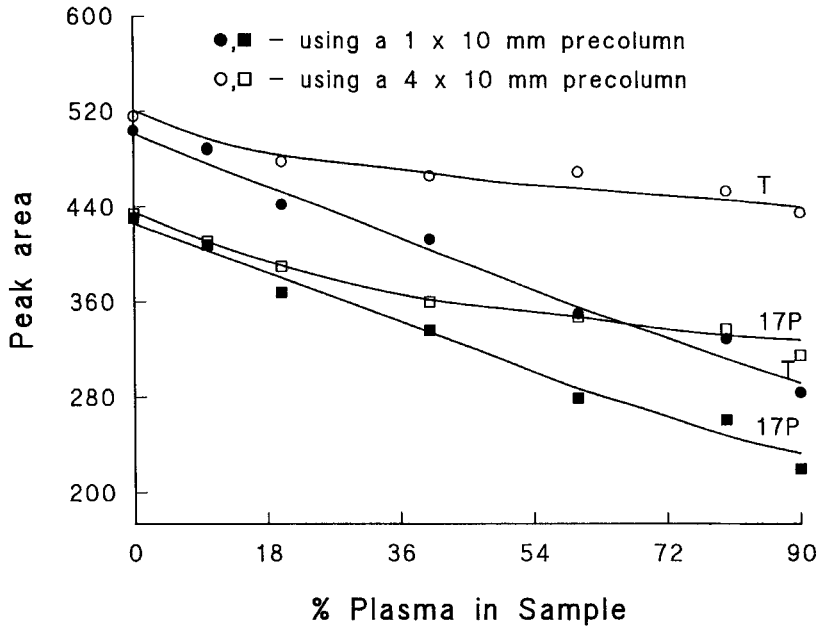

Figure 5. Variation of peak area with proportion of plasma in injected sample for testosterone (T) and 17α-hydroxyprogesterone (17P)

these steroids are appreciably protein bound[15] but it is generally accepted that the interactions of drugs with C-18 stationary phases are sufficiently strong to effect complete recovery. It appears more likely from our experiments that the addition of plasma components alters the C-18 surface irreversibly so that the load capacity is considerably reduced. This is at variance with reports that drug adsorption on C-18 columns is enhanced following several injections of plasma and that such columns should be conditioned by plasma injection before use[16] In the present study the reverse was found and prior injection, even of drug free plasma, was found to produce low efficiencies on subsequent injections of steroids in plasma or in purely aqueous solution. This would appear to indicate some permanent modification of the C-18 surface.

While many reports in the literature suggest that such on-line pretreatment of plasma samples for chromatography is effective[17-19] and that large numbers of

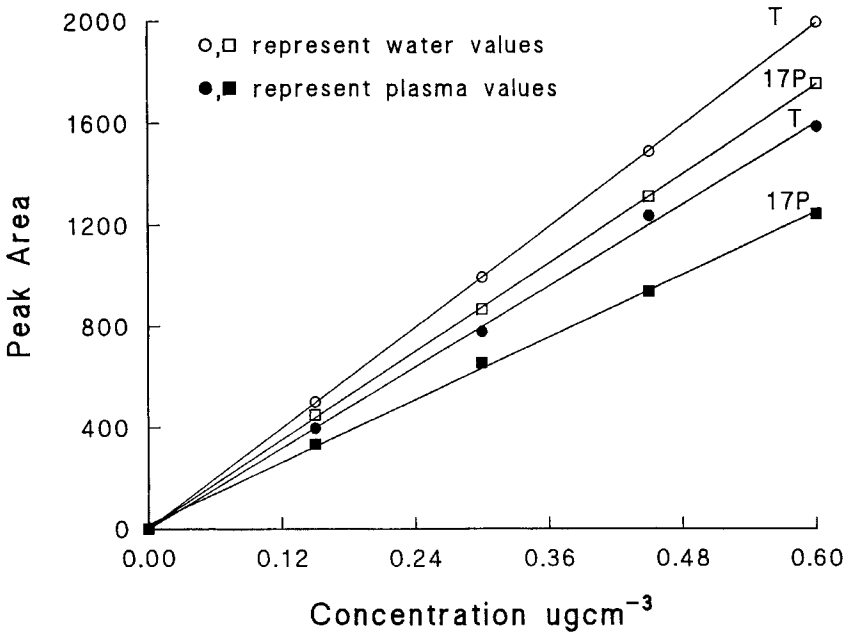

Figure 6. Plots of peak area against concentration for testosterone (T) and 17α-hydroxyprogesterone (17P) injected in water and plasma matrices.

samples may be processed using a single precolumn, there are also many reports in the literature which indicate that additional pretreatment of biological samples is required before a sample is injected into the precolumn[19-21]. A study of the literature of on-line sample pretreatment reveals that about 65% of methods reported use some form of off-line plasma sample pretreatment e.g. protein precipitation before on-line preconcentration by an integral precolumn. In addition, many assays involve the injection of small <100 μl volumes of plasma achieving sample clean-up but no appreciable preconcentration.

Extraction from Saliva

Saliva offers a useful alternative matrix for the assay of drugs for many clinical purposes. It has the advantage of being non invasive and good correlations have

been obtained between saliva and plasma concentrations for some drugs[22,23]. The above experiments were repeated using spiked saliva. Absorbance, efficiency and recovery of each steroid were determined. It was found that injection of samples in the saliva matrix resulted in only slight peak broadening and lowered absorbance while recovery, calculated on the basis of peak area, was identical to that obtained from water. No appreciable pressure rise on saliva injection was observed. As with urine, up to 5 cm^3 aliquots of saliva could be applied by repeated operation of the loading valve and at least 20 repeat samples could be applied to a single precolumn with no deterioration in performance.

GENERAL CONCLUSIONS

It has been shown that, when a column switching sample pretreatment system is operated in the backflush mode, the precolumn dimensions are of little importance with respect to peak broadening or sensitivity when coupled with either 4.6 or 2 mm diameter analytical columns. When coupled with 1 mm diameter analytical columns the increased extra column broadening adversely affects efficiency with consequent loss of sensitivity. From aqueous solutions the recovery is uniformly high. Precolumn concentration coupled with use of narrow bore columns has been shown to improve sensitivity. The analyte loading of the precolumn is proportional to its volume and is in the region of 6 - 7 $\mu g\ \mu l^{-1}$ of stationary phase. On this basis the optimum system among those tested is a 4 x 10 mm precolumn coupled with a 1 mm diameter analytical column although a more rugged system would incorporate a 2 mm diameter analytical column. It has also been shown that such sensitivity advantages can be realised for the biological matrices of urine and saliva and that the endogenous components of these do not appreciably adversely affect the performance of such coupled systems. For the steroid set in plasma, however, it has been shown that such a matrix reduces the efficiency and recovery of the overall system and severely restricts the lifetime of the precolumn. The apparent inconsistency between these findings and more positive reports in the literature concerning on-line plasma pretreatment are explained on the basis that in many reports additional pretreatment is used or that small plasma volumes only are injected.

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