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PROCEDURES FOR DIRECT INJECTIONS OF UNTREATED BLOOD PLASMA INTO LIQUID CHROMATOGRAPHIC COLUMNS WITH EMPHASIS ON A PRE-COLUMN VENTING TECHNIQUE

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SUMMARY

The stability of reversed-phase liquid chromatographic systems when untreated blood plasma samples are applied was studied with respect to retention, peak efficiency and column back-pressure. The influence of the content of organic solvents in the eluent, the flow-rate and the particle diameter of the support was investigated. Many of the column-deteriorating effects seem to be due to denaturation of the plasma proteins and the kinetics of this process. The stability is increased by decreasing the content of organic modifier in the eluent. The flow-rate should be moderate, as both high and low flow-rates give decreased stability. The stability increases with increasing particle size of the solid phase.

A small pre-column is used (1) as a guard column and (2) to effect a pre-separation of the solute from plasma matrix components by a pre-column venting technique. The pre-column venting technique considerably increases the lifetime of the system and especially the separation column. Under optimal conditions hundreds of injections (10 μl of plasma) can be performed without loss of stability.

INTRODUCTION

During the last decade liquid chromatography has become one of the most widely used techniques for the determination of drugs and related compounds in biological fluids[1,2]. The determination often involves a number of time-consuming steps, such as extraction, precipitation etc., prior to injection into the chromatographic system. Such sample handling procedures can also induce errors in the quantitative determination. There is a need for simplified sample handling, *i.e.*, a direct injection procedure in order to increase the sample throughput and accuracy.

The aim of this study was to investigate the performance and stability of simple isocratic liquid chromatographic systems for the direct injection of untreated blood plasma samples. Such systems may be suitable when only a small volume of plasma is sufficient, which is the case for many types of compounds that are present in high concentrations in the plasma or give a good detection sensitivity. A few applications of these kinds of systems have been presented, but the procedures do not seem to

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have found wide acceptance, possibly owing to experimental difficulties and a lack of knowledge about the compatibility of the chromatographic system with the untreated biological sample. In previous work we examined some of the experimental conditions that influenced the column performance in the determination of acidic drugs, *i.e.*, solvent composition[3] and protein binding[4]. Other workers have determined various drugs in untreated plasma[5–9], but discussions on column performance are scarce.

A related technique for direct injections was developed by Roth *et al.*[10] using a two-stage procedure, including sample enrichment on a pre-column, followed by a backflush elution into the separation column. This technique has been automated and can be used to inject plasma samples in the millilitre range. Several applications have been published[11–15].

This work is focused on the different experimental factors that affect the chromatographic system when untreated blood plasma is injected. The composition of the eluent, the flow-rate, the particle size of the support and the sample size are discussed. A small pre-column is used not only as a guard column but also as a pre-separation column in order to use a pre-column venting technique[16], which increases the lifetime of the separation column.

EXPERIMENTAL

Chemicals

Naproxen, quinidine and theophylline were of pharmacopoeial grade and N,N-dimethyloctylamine (DMOA) (ICN–K & K Laboratories, Plainview, NY, U.S.A.) was distilled. Serum albumin was human fraction V, essentially fatty acid-free, obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade. LiChrosorb RP-2, LiChrosorb RP-8 and LiChroprep RP-8 were obtained from E. Merck (Darmstadt, F.R.G.) and had particle diameters of 6 μm (RP-2), 5 and 10 μm (RP-8) and 25–40 μm (LiChroprep RP-8). Partisil-5-CCS/C8 was obtained from Whatman (Clifton, NJ, U.S.A.) and had a mean particle diameter of 5 μm.

Equipment

The pump was an LDC 711-47 solvent delivery system (Milton-Roy minipumps with a pulse damper) (LDC, Riviera Beach, FL, U.S.A.). The injector was a Rheodyne 7120 syringe loading injector (Rheodyne, Berkeley, CA, U.S.A.), equipped with 10-, 20- or 100-μl loops. A Valco CV-3-HPax three-port valve (Valco Instruments, Houston, TX, U.S.A.) was used as a venting valve. The detector was an LDC 1203 UV monitor operating at 254 nm. The separation columns were LiChroma tubes (316 stainless steel) (Handy and Harman Tube Co., Norristown, PA, U.S.A.), 100 × 4.6 mm I.D., equipped with modified Swagelock connectors and Altex (Altex, Berkeley, CA, U.S.A.) 2-μm stainless-steel frits. The pre-column, 3.2 mm I.D. was constructed similarly to the separation column after modifications of the fittings[17]. It can be prepared in lengths from 0 to 10 mm. Altex stainless-steel frits were placed in both ends.

A laboratory-built pressure regulator was constructed according to ref. 18, and was used to regulate the back-pressure when using the pre-column venting technique.

Procedures

Blood plasma samples were obtained by adding 100 μl of a stock solution of a drug compound to 900–1000 μl of carefully homogenized pooled blank plasma. Prior to the injection, the plasma sample was carefully centrifuged at about 5000 *g* and in some instances diluted with buffer. All experiments were performed at 25°C. The buffers had an ionic strength of 0.1.

For strongly protein-bound drugs the plasma was diluted (1:1) with solutions of phosphoric acid or sodium hydroxide[4].

The separation columns were slurry-packed with a high-pressure pump. The pre-columns were also slurry-packed by using an ordinary LC pump. The slurry was prepared in methanol–dichloromethane (1:1). Methanol was used as pumping liquid. The void volume was determined by injection of sodium nitrate.

In determinations of peak efficiency, the plate number (N) was calculated from the retention time and the base width. The peak efficiency is expressed either by the plate number, the theoretical plate height ($H = L/N$) or the reduced plate height ($h = H/d_p$), even if the chromatographic system consisted of a pre-column combined with a separation column and the two columns contained particles of different size. Further, in these instances, the column length (L) and particle diameter (d_p) used in the calculation were those of the separation column.

The column resistance factor (ϕ') is expressed by

$$\phi' = 100 \cdot \frac{P t_m d_p^2}{\eta L^2}$$

were P (bar) is the column back-pressure, t_m (sec) is the retention time of an unretained solute, d_p (μm) is the particle diameter of the support, η (cp) is the viscosity and L (mm) is the column length.

RESULTS AND DISCUSSION

Effect of plasma injections on the chromatographic system

A common problem with the direct injection of untreated blood plasma samples into isocratic chromatographic systems has been the decrease in performance after a number of injections. Changes in the retention[5,6], column back-pressure[3–6] and peak efficiency[4–6,8] have been observed.

The chromatographic system can be influenced both chemically and physically after the application of such a complicated matrix as blood plasma. The physical influences are, *e.g.*, those which influence the transport of liquid through the column. If large amounts of material are deposited in the column, its porosity will decrease, resulting in an increased back-pressure (if a constant-flow pump is used) and an increased linear flow-rate. This can lead to a decreased retention time. Chemical influences are those which influence the distribution of a solute between the stationary phase and the mobile phase, *i.e.*, which causes a change in its capacity ratio.

With a complicated sample matrix these influences will sooner or later show up, but if their reason is detected, the chromatographic system can be designed in such a way that the disturbances are eliminated or minimized.

In this study, we used a small pre-column as a guard column but also as a

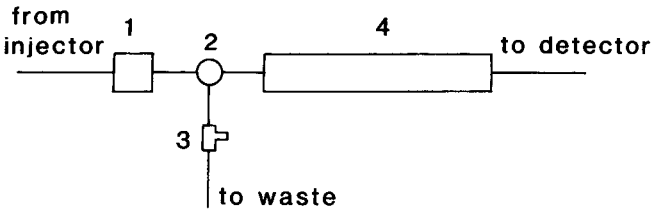

Fig. 1. Pre-column venting system. 1 = Pre-column, 10 × 3.2 mm I.D.; 2 = three-port valve; 3 = laboratory-built pressure regulator[18]; 4 = separation column, 100 × 4.6 mm I.D.

pre-separation column to perform a pre-column venting[16]. With the pre-column venting technique, illustrated in Fig. 1, a valve is placed between the pre-column and the separation column and unretained matrix components are vented to waste.

The pre-column adsorbs highly retained components of the sample and has to be changed at certain intervals. The time to make the change is when the performance of the whole chromatographic system is such that the resolution and quantitation are no longer satisfactory.

An alternative to changing the pre-column may be to regenerate it, *e.g.*, by eluting the retained compounds by using a suitable eluent. This possibility has not been studied in detail, but rinsing the column with methanol was not successful. In practice, a change of the pre-column may be more appropriate, as pre-columns are easily repacked (5–10 min) and use only about 50 mg of support material.

Below is discussed how the retention, column back-pressure and peak efficiency are influenced by different experimental conditions and how this information can be used to optimize the chromatographic system.

Sample preparation and pre-column venting: effect on column back-pressure

Careful centrifugation of the blood plasma sample prior to injection is of great importance, as a thawed sample often contains clots and solid particles, which on injection will give immediate increases in the column back-pressure owing to restrictions of the inlet frit or of the column packing. After centrifugation, a clear solution is obtained, which gives only a very slight increase in the column back-pressure.

In previous work[4] and in a few experiments in this work the plasma samples were filtered, but we found that to be an unnecessary pre-treatment step if they were carefully centrifuged at about 5000 *g*.

The plasma sample is viscous because of its high content of macromolecules. Owing to their size, they are often excluded from the pores of the stationary phase. When the excluded front passes the column, there is always a slight increase in the back-pressure, but it returns to the original value after the front has passed.

In the non-venting procedure, where all of the sample passes through the pre-column and the separation column, occasional increases in the back-pressure were observed, but this effect was much reduced by dilution of the plasma.

In the pre-column venting procedure, dilution of the plasma sample has been found to be unnecessary, because even with the non-diluted plasma the occasional pressure increases were absent. A continuous pressure increase, however, much smaller than in the non-venting procedure is always present. This indicates that the weakly retained plasma matrix components cause a greater deterioration of the chro-

matographic system when, in the non-venting procedure, they are allowed to pass through the separation column also. This phenomenon may be connected with a kinetic effect on the deterioration process, which will be discussed below.

Test substances

The test substances used were predominantly drugs suitable for direct injections, *i.e.*, compounds with a good detectability or which occur at high plasma concentration levels. Theophylline and naproxen have high therapeutic concentration levels, which can be assayed in small volumes or even diluted plasma. Quinidine has a lower concentration level, 2–4 μg/ml, but owing to its good detectability this concentration can be determined in 10 μl of plasma.

In previous work[4] we found that the strongly protein-bound drug naproxen (binding degree over 99% at therapeutic concentrations) gave skewed chromatographic peaks from directly injected plasma samples. Symmetrical peaks of naproxen could be obtained if the protein-binding equilibrium was shifted to yield a lower degree of binding[4]. In this work we added phosphoric acid or sodium hydroxide to the plasma sample for this purpose. Quinidine and theophylline have lower binding degrees, 74–88 and 53–65%, respectively, and no special precautions had to be taken.

In some experiments with the pre-column venting technique, some other substances that gave a suitable retention were chosen, *e.g.*, 1-naphthylacetic acid and 2-methoxybenzoic acid.

Chromatographic phase systems

For the construction of phase systems for direct injection of untreated blood plasma samples it is essential to have the possibility of regulating the retention of the solute and to separate it from plasma matrix compounds. Secondly, the mobile phase must be compatible with the plasma sample, *i.e.*, plasma proteins must be kept in solution. The phase systems used in this study were liquid–solid systems. Various parameters were used to regulate the retention, *e.g.*, the hydrophobicity of the stationary phase, the pH of the eluent and organic modifiers or hydrophobic ions in the eluent.

The use of aqueous buffers with alkylsulphates in the eluent[3] gave less possibility of regulating the retention and low peak efficiencies ($h \approx 20$). If methanol was chosen to regulate the retention[4], the capacity factors could be varied within a wider range and a sufficient efficiency was achieved ($h = 5$–7). The addition of organic modifiers to the eluent may be expected to give problems, as they may precipitate proteins that can block the column. We have found that it was possible to use up to 50–60% of methanol in the eluent without incurring this problem, but above 70% the proteins precipitated and a high back-pressure was obtained after a few injections.

Stability of the chromatographic system

The first experiments were performed with the non-venting procedure. Fig. 2 shows that the peak efficiency deteriorated when plasma was injected, and it is clear that the stability decreased with increasing methanol content in the eluent. In these experiments the pre-column was exchanged after 10–15 injections to prevent highly retained plasma compounds from entering the separation column, as we thought that they might cause the deterioration. The retention of the solute was stable in these systems.

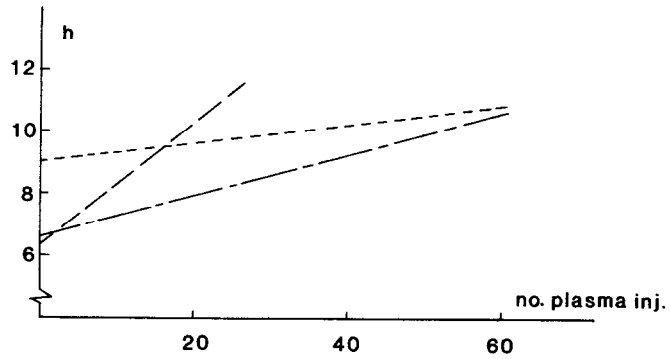

Fig. 2. Effects of blood plasma injections on peak efficiency at different methanol concentrations. ----: Column, LiChrosorb RP-8 (5 μm); eluent, 5 m*M* DMOA in phosphate buffer (pH 3.0)–methanol (84:16); sample, quinidine in blood plasma diluted (1:1) with buffer (20 μl); $k' = 8.0$. — · — · —: Column, LiChrosorb RP-2 (6 μm); eluent, phosphate buffer (pH 7.0)–methanol (7:3); sample, naproxen in blood plasma diluted (1:1) with 0.5 *M* phosphoric acid (10 μl); $k' = 5.8$. ———: Column, Partisil-5-CCS/C8 (5 μm); eluent, phosphate buffer (pH 5.9)–methanol (1:1); sample, naproxen in blood plasma diluted (1:1) with 0.5 *M* phosphoric acid (10 μl); $k' = 3.0$. Pre-columns: 5 × 3.2 mm I.D., containing the same packing as the separation column. Data points scattered ±5% around the curves.

Small continuous increases in the column back-pressure were observed, less then 0.5 bar per injection, but they were small compared with the pressure capacity of the pump. In some instances, higher pressure increases were observed, but the restrictions were then often located in the frit of the bed support.

From Fig. 2 it can be seen that a slight deterioration was obtained when a low concentration of methanol in the eluent (16%) was used. The efficiency decreased by only 15% within 60 injections of 10 μl of blood plasma.

The phase system containing 50% of methanol in the eluent, with which there was a rapid deterioration of the peak efficiency, was chosen for a closer study of the causes of the instability. The retention properties of plasma matrix compounds must then be considered.

The main solutes in blood plasma are proteins (6.5–8.0%) and lipids (0.4–0.6%)[19]. Serum albumin is excluded from the pores of the stationary phase[4] and the same can be expected for many other proteins. A used pre-column has a yellowish colour and a cement-like consistency, which is probably caused by strongly retained lipid material.

In order to determine whether the highly retained compounds cause the instability, experiments were performed in which the pre-column was exchanged after each plasma injection. This prevents compounds still present in the pre-column from entering the separation column during elution. Immediately after the peak of naproxen had been eluted the pump was stopped, the pre-column was disconnected and the efficiency of the separation column was measured. Calculations have shown that compounds having capacity ratios above approximately 200 will remain in the pre-column when this procedure is used. The separation column still deteriorated, as shown in Fig. 3. Obviously the deterioration was caused instead by weakly retained compounds (*e.g.*, excluded proteins), which had entered the separation column.

The influence of the most weakly retained matrix compounds was studied by

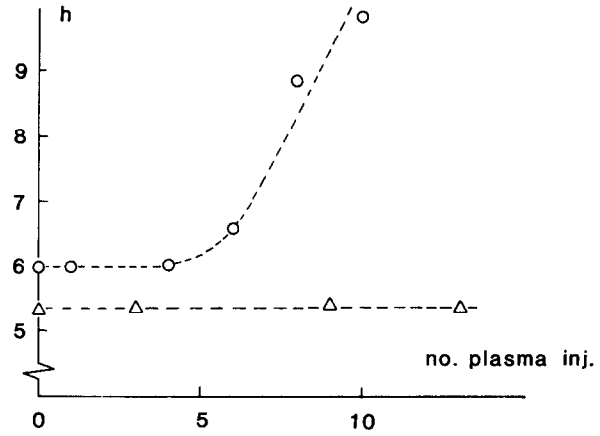

Fig. 3. Efficiency of the separation column after plasma injections by pre-column non-venting and venting procedures. ○, Non-venting; pre-column (5 × 3.2 mm I.D.) exchanged after each plasma injection. △, Venting; pre-column (10 × 3.2 mm I.D.) not changed. 5 μl of plasma per injection. Column: Partisil-5-CCS/C8 (5 μm). Eluent: phosphate buffer (pH 5.9)–methanol (1:1). Sample: naproxen in buffer (10 μl).

a pre-column venting procedure. Weakly retained compounds are vented and cannot enter the separation column, resulting in a completely stable separation column (Fig. 3). The deterioration was obviously caused by compounds having a lower retention than that of naproxen.

In order to obtain some indications of the nature of the column deterioration phenomenon, the plate height of the separation column was measured at different flow-rates before and after feeding plasma into the column. When the plasma was injected, a pre-column was added and 50 injections, corresponding to a total volume of 250 μl, were made. The results presented in Fig. 4 indicate a change in the mass-transfer term of the Van Deemter equation after plasma samples have been injected. This points to a decreased diffusion rate in the stationary zone and might be caused

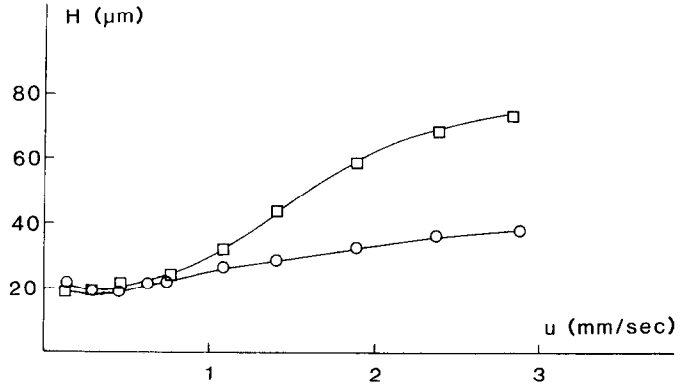

Fig. 4. Dependence of plate height on flow-rate after plasma injections. ○, Before plasma injections; □, after 50 injections of 5 μl of blood plasma. During plasma injections, a 5 × 3.2 mm I.D. pre-column and 84 ml of eluent at a flow-rate of 1.0 ml/min were used. Sample: naproxen in buffer. No changes in k' or peak symmetry were observed. Column: Partisil-5-CCS/C8 (5 μm). Eluent: phosphate buffer (pH 5.9)–methanol (1:1).

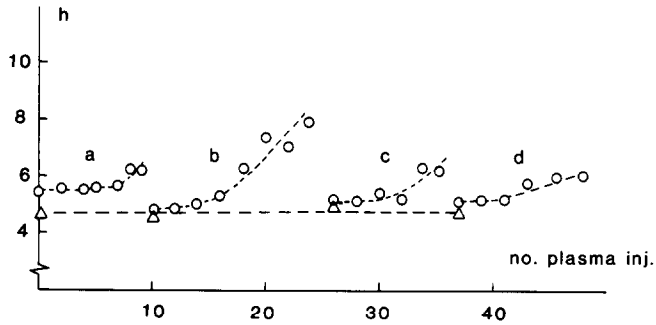

Fig. 5. Efficiency during pre-column venting and change of pre-columns. △, Separation column; ○, separation column + pre-column (10 × 3.2 mm I.D.). Pre-column venting, 0.19 ml. Series a, b, c and d are for different pre-columns. Column: LiChrosorb RP-8 (5 μm). Eluent: phosphate buffer (pH 7)–methanol (6:4). Sample: △, naproxen in buffer; ○, naproxen in plasma diluted (1:1) with 0.5 *M* phosphoric acid (10 μl).

by some compound from the plasma matrix being transformed from a soluble form into a form that adhered to or was adsorbed on the column particles. Such compounds might well be proteins.

Pre-column venting

The results demonstrated that the lifetime of the separation column can be increased by the pre-column venting procedure. The efficiency (*h*) of the combined pre-column–separation column deteriorated after a certain number of injections. This

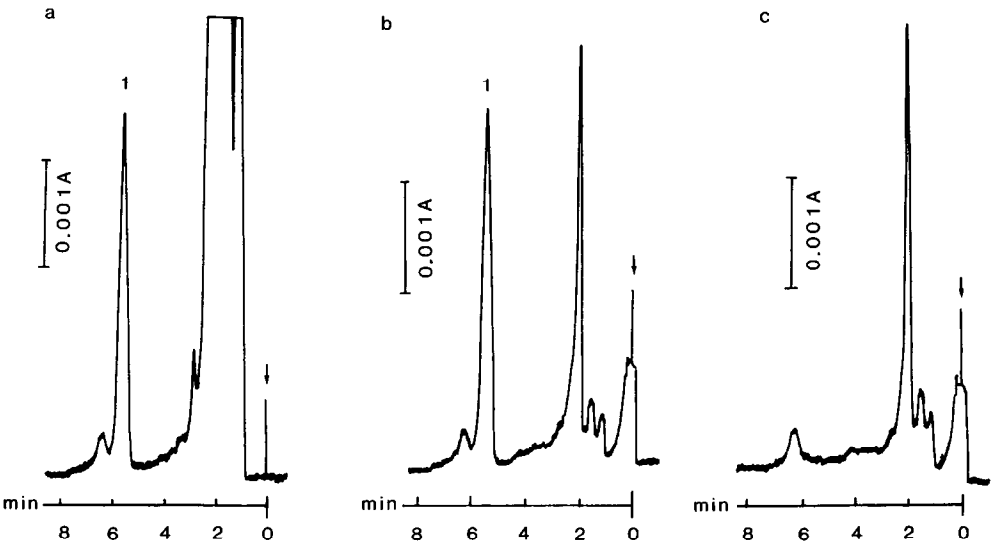

Fig. 6. Chromatograms of blood plasma injections. (a) No venting, naproxen (1) in plasma; (b) pre-column venting 0.18 ml, naproxen (1) in plasma; (c) pre-column venting 0.18 ml, blank plasma. Pre-column: 10 × 3.2 mm I.D. Injection volume: 10 μl. Naproxen sample: 8 μl/ml in blood plasma containing 0.05 *M* NaOH. Phase system as in Fig. 5 but 10-μm particles in pre-column. Flow-rate: 1.0 ml/min. Detection: 254 nm.

is illustrated in Fig. 5, where a very unstable phase system, with 40% of methanol in the eluent, was used. When the pre-column was exchanged, the original peak efficiency was obtained.

A further advantage of the pre-column venting technique is that the resolution of the solute is increased, as the highly UV-absorbing front is vented to waste, as illustrated in Fig. 6.

The pre-column venting technique requires a pre-separation of the solute from the weakly retained plasma components on the pre-column. The venting must obviously last only until the solute starts to emerge from the pre-column, which requires that the capacity ratio and the band broadening of the solute in the pre-column are known. The venting volume is given by $V_R - 3\sigma_v$, where V_R is the retention volume and σ_v is the standard deviation of the solute zone emerging from the pre-column.

Assume a capacity ratio of 5 and that the pre-column (10 × 3.2 mm) is as well packed as the separation column, *i.e.*, $h = 5$, and that it has a porosity of 0.7. The void volume of the precolumn is then 56 μl and the venting volume can be estimated to 0.28 ml, *i.e.*, a venting time of 17 sec if the flow-rate is 1 ml/min.

Such calculations will only give a rough estimate, however, as the band broadening in the injector and the connections and the fact that the solute can have a different capacity ratio when the plasma sample passes through the pre-column also have to be considered. The capacity ratio can be affected by different equilibria in the plasma, *e.g.*, protein binding[4]. If it is assumed that the influence of protein binding is negligible, then it is clear that the plasma itself, in many instances, causes a step gradient, *i.e.*, the solute is more retained in the plasma plug than in the eluent owing, for example, to the presence of methanol in the eluent.

A better estimate of the venting volume may be obtained, however, by injecting the solute dissolved in buffer, as a similar step gradient as for the plasma sample will be created. The step gradient will improve the pre-separation of the solute from the plasma matrix, as the proteins will migrate excluded in the plasma plug.

Some experimental variables were studied in order to observe their effect on the stability of the chromatographic system. The stability was measured for three parameters: (1) the reduced plate height, (2) the column resistance factor and (3) the retention time. The stability is defined as a change in the measured parameters of 10% from the original values. The experimental variables were the flow-rate, sample volume, type and concentration of organic modifier and the particle diameter of the solid phase.

Most of the stability studies were performed in an accelerated mode, *i.e.*, a series of blank plasma samples were injected at short time intervals, using the pre-column venting technique, and the three parameters were measured after each series. A few experiments indicated that the same stability values were obtained in routine use of the procedure, where plasma injections were made at longer time intervals.

Moderate flow-rates gave good stability of the peak efficiency, in contrast to low flow-rates, when it was very bad, as illustrated in Table I. With a low flow-rate, the residence time of the plasma components in the pre-column becomes high and the poor stability obtained obviously indicates that the process, which leads to the deterioration, is so slow that it was not completed during the shorter residence times at the higher flow-rates. It is assumed that this effect is associated with the denaturation of plasma proteins, which occurs when they come into contact with the eluent

TABLE I

EFFECT OF FLOW-RATE ON STABILITY

Pre-column venting. Solid phase: LiChrosorb RP-8 (5 μm). Pre-column: 10 × 3.2 mm I.D. Eluent: phosphate buffer (pH 6)–methanol (4:1). Solute: theophylline, $k' = 2.7$. Sample: 10 μl of blood plasma–buffer (1:1).

Flow-rate (ml/min)	*h*			*ø'*		
	Starting value	*Stability**		*Starting value* $\times 10^{-3}$	*Stability**	
		No. of injections	*Total plasma volume (μl)*		*No. of injections*	*Total plasma volume (μl)*
0.42	9.1	8	40	2.5	45	225
0.68	7.1	30	150	2.1	25	125
0.87	9.2	40	200	2.3	80	400
1.09	10.3	35	175	2.2	45	225
1.40	7.5	20	100	2.0	70	350

* 10% change of the measured parameter from the starting value.

and the column material. This conclusion is supported by published data on the kinetics of protein denaturation, caused by various alcohols[20] and by contact with the column material[21]. The reason for the decreased stability at high flow-rates may be physical damage of the pre-column top. The stability of the column resistance factor is independent of the flow-rate.

The deterioration effect of proteins is clearly demonstrated in Table II, where it is shown that injection of albumin solutions limits the stability, whereas injected buffer solutions do not.

Increasing concentrations of organic modifier decrease the stability, even if the concentration is not high enough to cause substantial precipitation of plasma proteins[3]. This is evident from Table III.

Table IV illustrates the effect of the type of organic modifier in the eluent. The

TABLE II

EFFECT OF PROTEINS ON STABILITY

Pre-column venting. Conditions as in Table I; flow-rate, 0.68 ml/min.

Sample (10 μl)	*h*		*ø'*	
	Starting value	*Stability*: No. of injections*	*Starting value* $\times 10^{-3}$	*Stability*: No. of injections*
Buffer	7.2	>400	1.4	>400
Albumin (0.50 m*M*)	6.6	50	1.7	100
Blood plasma	8.4	16	2.3	18

* 10% change of the measured parameter from the starting value.

TABLE III

EFFECT OF METHANOL CONCENTRATION ON STABILITY

Pre-column venting. Solid phase: LiChrosorb RP-8 (5 μm). Pre-column: 10 × 3.2 mm I.D. Eluent: methanol–buffer (pH 6). Solutes: theophylline (T) and naproxen (N). Sample: 10 μl of blood plasma–buffer (1:1).

Methanol in eluent (%)	*k'*	*h*			*ø'*		
		Starting value	*Stability**		*Starting value*	*Stability**	
			No. of injections	*Total plasma volume (μl)*	*× 10⁻³*	*No. of injections*	*Total plasma volume (μl)*
5	15.3 (T)	6.0	130	650	2.0	160	800
10	7.7 (T)	7.6	80	400	2.1	30	150
20	2.7 (T)	7.1	30	150	2.1	25	125
40**	5.2 (N)	4.5	8	40	2.4	12	60

* 10% change of the measured parameter from the starting value.
** pH 7.

results indicate an increasing stability in the order methanol < 1-propanol < ethylene glycol for the same concentration. Ethylene glycol also gave an increased stability of the back-pressure when it was used as an additive to eluents containing a monohydric alcohol (*cf.*, Table III). This indicates that the monohydric alcohol can be used to regulate the retention and ethylene glycol to increase the stability. Although ethylene glycol also influences the retention, it is not as effective as methanol and 1-propanol, as it gives a lower slope of a plot of log *k' versus* percentage of water in the eluent. Ethylene glycol is well known to prevent the denaturation of proteins[22]. The monohydric alcohols are known to denature proteins, as opposed to polyhydric alcohols[20]. Other types of polyhydric alcohols[20] can be used as modifiers in order to increase the stability of plasma proteins. Ion-pair reagents (*e.g.*, alkyl sulphates and

TABLE IV

EFFECT OF TYPE OF ORGANIC MODIFIER ON STABILITY

Pre-column venting. Solid phase: LiChrosorb RP-8 (5 μm). Pre-column: 10 × 3.2 mm I.D. Eluent: modifier–buffer [A, phosphate buffer (pH 6); B, posphate buffer (pH 3) with 10 m*M* acetic acid; C, phosphate buffer (pH 7)]. Solutes: theophylline (T), 2-methoxybenzoic acid (M) and naproxen (N). Sample: 10 μl of blood plasma–buffer (1:1).

Modifier	*Buffer*	*k'*	*h*			*ø'*		
			Starting value	*Stability**		*Starting value*	*Stability**	
				No. of injections	*Total plasma volume (μl)*	*× 10⁻³*	*No. of injections*	*Total plasma volume (μl)*
10% methanol	A	7.7 (T)	7.6	80	400	2.1	30	150
10% 1-propanol	B	3.8 (M)	7.7	140	750	1.7	130	650
10% ethylene glycol	A	2.7 (M)	9.6	250	1250	2.0	160	800
40% methanol + 10% ethylene glycol	C	3.1 (N)	8.6	18	90	2.3	30	150

* 10% change of the measured parameter from the starting value.

TABLE V

EFFECT OF PRE-COLUMN PARTICLE SIZE ON STABILITY

Conditions as in Table I; flow-rate, 0.68 ml/min. N_{sep} = plate number of separation column; N_{tot} = plate number of combined pre-column–separation column.

Pre-column particle diameter (μm)	*Starting values*		*Stability* of h*		*ø'*		
	N_{sep}	N_{tot}	*No. of injections*	*Total plasma volume (μl)*	*Starting value* $\times 10^{-3}$	*Stability**	
						No. of injections	*Total plasma volume (μl)*
5	3100	3000	30	150	2.1	25	125
10	1800	1600	90	450	1.7	90	450
25–40	2450	1450	>150	>750	1.6	110	550

* 10% change of the measured parameter from the starting value.

alkylamines), commonly used to regulate the retention of ionic solutes, were found not to influence the stability significantly, which was to be expected, as similar compounds have been found not to denature albumin[22].

Table V shows the results when different particle sizes of the support were used in the pre-column. Large particles (25–40 μm) increase the stability of the efficiency enormously, as expected because the system has a low peak efficiency from the start owing to the large contribution to band broadening from the pre-column. The magnitude of the contribution to h for a 10% increase from its starting value will, of course, be different for different starting values. Calculations show that if the contributions to h from each plasma injection are equal the stability, as defined here and expressed in terms of number of injections will be proportional to the starting value of h.

Taking this into consideration, the stability is still greater for pre-columns with larger particle diameters. The reason for this might be that the interparticle channels are wider so that denatured proteins tend to adhere to the particle surfaces to a lesser extent.

When choosing a pre-column for the pre-column venting technique, two points have to be considered: (1) the required peak efficiency of the combined pre-column–separation column needed to resolve the solutes of interest for final quantitation and (2) the required peak efficiency of the pre-column needed to resolve the early emerging plasma components from the solutes of interest on the pre-column.

The beneficial effect on the stability of large particles in the pre-column can only be utilized for less demanding separations. If a high peak efficiency is needed, smaller particle diameters have to be used despite their lower stability.

The minimum peak efficiency of the pre-column required to resolve quantitatively the solute from early eluted plasma matrix components will depend on the capacity ratio of the solute in the pre-column. In order to estimate the required efficiency of the pre-column, some calculations were made. For simplicity, it is assumed that the deteriorating early eluted plasma components (a) have a capacity ratio of zero. To vent 99.9% of these, the venting should occur until passage through the pre-column of a volume of eluent corresponding to $V_{R(a)} + 3\sigma_a$. To avoid the

loss of a solute of interest (b), the venting should cease before $V_{R(b)} - 3\sigma_b$. $V_{R(a)}$, $V_{R(b)}$, σ_a and σ_b are the retention volumes and the standard deviations of the peak bands in the pre-column.

For example, calculations have shown that the required plate number must be above 18 if the capacity ratio is 5, but above 36 if the capacity ratio is only 2. A comparison with experimental data showed that a pre-column (including the connections) with a particle diameter of 5 μm gives a contribution to band broadening, expressed as the reduced plate height, of about 10–25. If it is assumed that pre-columns with other particle diameters give corresponding efficiencies, the plate numbers of the pre-columns will be 80–200, 40–100 and 13–33 for 5-, 10- and 30-μm particles, respectively.

In practice, as mentioned earlier, the plasma sample often causes a step gradient when it passes through the pre-column. The venting may then be carried out with lower plate numbers than have been calculated on the basis of the retention in presence of the eluent.

It is obvious that the pre-column size and packing material have to be optimized with respect to the retention of the solute in the pre-column.

Table VI illustrates the effect of the amount of plasma injected. It is not the number of plasma injections but the total volume applied that affects the stability, and there seems to be a correlation between the stability of the column resistance and the peak efficiency.

The retention times were very stable in all these experiments, changes of less than 2–5% being observed.

CONCLUSIONS

The advantages of the pre-column venting technique are as follows: the separation column is stable, because it is not reached by the deteriorating plasma components; the possibility of deterioration due to the slow denaturation process is decreased, because the residence time of the plasma matrix in the column system de-

TABLE VI

EFFECTS OF AMOUNT OF PLASMA INJECTED ON THE STABILITY OF THE EFFICIENCY AND THE COLUMN RESISTANCE

Pre-column venting. Conditions as in Table I; flow-rate, 0.68 ml/min.

Injection volume (µl)	*Plasma dilution*	*h*			*ø'*		
		Starting value	*Stability**		*Starting value*	*Stability**	
			No. of injections	*Total plasma volume (µl)*	*× 10⁻³*	*No. of injections*	*Total plasma volume (µl)*
10	1:4	9.5	55	110	2.3	55	110
10	1:1	7.1	30	150	2.1	25	125
10	–	8.4	16	160	2.3	18	180
20	1:1	9.5	14	140	2.3	30	150
100	1:1	9.4	4	200	2.3	4	200

* 10% change of the measured parameter from the starting value.

creases; and the resolution of the solute is increased in comparison with non-venting procedure.

The choice between the pre-column venting and the pre-column non-venting procedure is mainly governed by the demands on the lifetime of the separation column. Our results indicate that the separation column can be given extremely high stability by pre-column venting, in contrast to the non-venting procedure.

The system described can easily be adapted to the determination of different types of compounds in untreated blood plasma, and the results presented give the basis for optimization of the stability with respect to the phase composition and the flow-rate.

It can be estimated that quantitation can be performed with an acceptable accuracy using external standardization, even if the peak efficiency changes by 10%. This means that under favourable conditions at least 50–200 plasma injections can be made. Even under unfavourable conditions, such as with the phase system containing 50% of methanol in Fig. 2, quantitation is possible and recoveries close to 100% and relative standard deviations below 5% have been obtained when external standardization was performed at close intervals.

Work is in progress to apply the pre-column venting technique to the determination of drugs in small-volume plasma samples[23].

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