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CONICAL PRECOLUMN AS LOADING BUFFER FOR THE MAIN COLUMN

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SUMMARY

A conical high-performance liquid chromatographic precolumn was developed to cope with the problems that arise during the processing of large volumes of biological samples. The shape of the column was designed so as to offer a large loading capacity at the front of the precolumn. The stainless-steel construction, which is pressure resistant up to 40 MPa, can be fully integrated into high-performance systems.

In the present work, the precolumn arrangement was used in the assay of pamoic acid in human plasma and in the isolation of radioactive metabolites from pools of animal urine and of supernatants from liver homogenates.

Apart from extremely polar compounds, which were not retained on the precolumn, recovery of metabolites was practically complete. Almost the same resolution was obtained with the equivalent of 900 ml of urine, purified and enriched on the precolumn, as with a 2-ml sample of the original urine. Likewise, the chromatographic metabolite pattern of 650 ml of supernatant from homogenized liver was similar to that of a deproteinized sample of 2 ml. It is suggested that the precolumn is usable for all chromatographic problems involving enrichment of small amounts of substances in large amounts of complex matrices.

INTRODUCTION

In drug metabolism studies, high-resolution chromatograms of metabolites have become an essential requirement. The matrices in which drug metabolites are available, namely urine, bile and blood plasma, in effect constitute very dilute aqueous solutions of these metabolites, with in addition relatively large amounts of other solutes of various kinds. Thus, an important condition for the optimal resolution of drug metabolites in biological matrices is to get rid of most of the water and also those solutes irrelevant to the problems on hand.

Several studies that were aimed at enrichment of very dilute solutions have been reported. Thus, Huber and co-workers[1,2] and Karger *et al.*[3] reported the development of precolumns for this purpose, and Little and Fallick[4] proposed the use of reversed-phase columns to concentrate aqueous solutions of lipophilic substances.

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Since then a number of studies based on these principles have been reported.

We found that the preconcentration systems described[5–12] did not work properly with biological samples of more than a few millilitres. Clogging of the columns and poor resolution were the most frequent problems. This was not surprising in view of the excess of dissolved material in urine, bile, or plasma, part of which is possibly retained on the first few millimetres of column packing, thus obstructing the flow. Widening of the column entrance would appear to be a promising approach to overcome this problem. The present paper describes the design of, and some experiments with, an arrangement consisting of a conical precolumn with wide opening and a switchable interface to connect it to separation columns. The experiments were chosen so as to test the performance of the precolumn arrangement with different biological matrices.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatographic (HPLC) apparatus (Waters Assoc., U.S.A.) was a combination of two M6000A solvent-delivery systems and an M720 system controller. Samples were added to the columns either by a Model 7125 syringe loading sample injector equipped with a 2-ml sample loop (Rheodyne, U.S.A.), or if larger volumes were involved, with one of the 6000A pumps.

Detection

UV detection was done with a Pye LC-UV monitor. For on-line radioactive measurement we used either the Berthold module BF 22550 (F.R.G.), or an Isomess (F.R.G.) HPLC detector IM 2000.

Precolumns

Construction materials. The precolumns were of stainless steel type AISI 316. All static gaskets were of either polychlorotrifluoroethylene ***(i.e.*** Kel-F) or polytetrafluoroethylene (PTFE) armoured with 25% glass fibre. All gliding gaskets were of PTFE. The whole device was constructed to be operated at pressures up to 40 MPa.

Design. Fig. 1 shows the shape of the precolumn. In essence it is a short wide column, tapering off to attain sufficient width to enable connection to a separation column with a minimum of discontinuities in the flow profile. In order to ensure a smooth flow of fluid from the narrow entrance of the cone-shaped precolumn (precone), and thus its even distribution over the wide surface of the actual packing, the top section of the precolumn is filled with 100-120 mesh glass beads (Chrompack, Middelburg, The Netherlands). The dimensions of the precolumns used by us are specified in Table I.

Fig. 1 also shows the design of the valve that connects the precolumn to the separation columns. The bore of the valve can be made to fit any current type of separation column by means of adaptors, as shown in Fig. 2.

Correction factor. To correct the chromatographic conditions for the use of the different precons, we multiplied all parts of the elution programme (time-related events and, in general, flow-rates) by the empirically established factor U:

$$\frac{(V_{column} + V_{precone})}{(V_{column})} = U \quad (1)$$

where $V_{precone}$ is the volume of the part of the precone filled with reversed-phase material.

TABLE I

DIMENSIONS OF THE PRECOLUMNS USED IN THE PRESENT EXPERIMENTS

Type	*Volume of packed section (ml)*	*Maximum I.D. (mm)*	*Length (mm)*
I	*25.5*	*39*	*45*
II	*5.5*	*24*	27
III	1.5	14	12.5

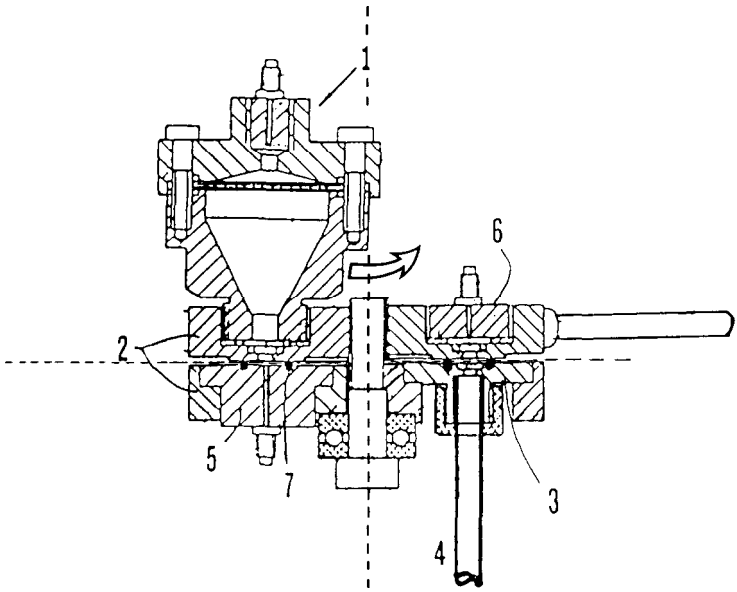

Fig. 1. Pre-column set up: 1 = precone; 2 = valve; 3 = column adaptor (see also Fig. 2); 4 = column; 5 = "column adapter" (for capillary tubing); 6 = insert for capillary tubing connection; 7 = gliding gasket.

Procedures

Assay of pamoic acid in human plasma. Mebeverine is a musculotropic antispasmodic drug[13]. One of its presentation forms is a liquid containing mebeverine pamoate. For the assay of pamoic acid in human plasma we developed an HPLC method with UV absorbance (at 233 and 360 nm) as the detection method. Details of the method are presented in Fig. 5.

Isolation of clovoxamine metabolites from urine. Clovoxamine is a psychotropic compound under investigation in our laboratories for the treatment of depression[14].

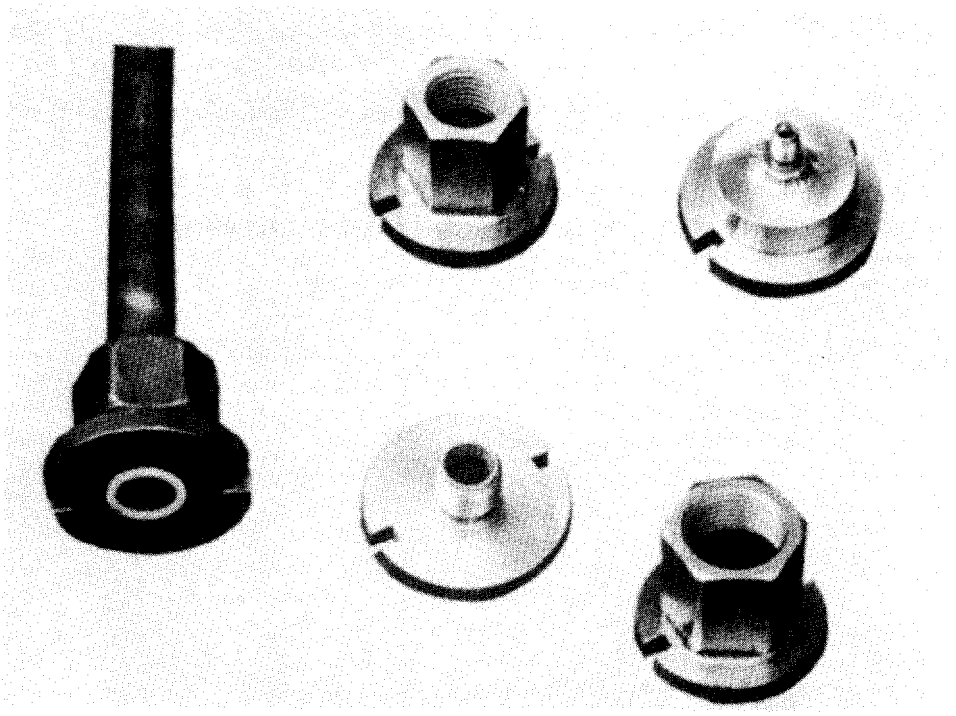

Fig. 2. Adaptors for fitting any current type of separation column.

We investigated the urinary metabolite patterns in animals after administration of the ^{14}C-labelled compound. The site of the radiolabel is indicated in the formula (Fig. 3).

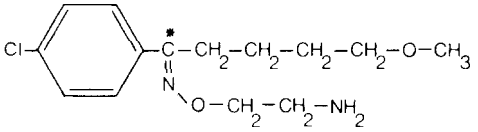

Fig. 3. Structure of clovoxamine. The asterisk indicates the site of the radiolabel.

An HPLC system suitable for the metabolite pattern as well as the isolation of metabolites from urine was developed. Details of the method are contained in Fig. 6. In order to isolate the metabolites from urine they were concentrated on a precone (type I in Table I). After passage of the urine through the cone, the latter was washed with its own dead volume of water. Subsequently it was connected to the separation column and eluted as described.

Isolation of secoverine metabolites from supernatant of liver homogenate. To obtain insight into the primary metabolism of secoverine, currently being tested for the treatment of functional disorders of the gastrointestinal tract[15], *in vitro* experiments were conducted in rat liver homogenates (10,000-*g* supernatant). The compound was labelled with ^{14}C at the site indicated in the formula (Fig. 4).

A chromatographic metabolite pattern was developed using on-line radioactivity measurement. Details of the method are given in Fig. 8. The same separation

system was used for the isolation of metabolites from 10,000-*g* supernatants of liver homogenates. For the development of the chromatographic pattern, we used precone type III (Table I) to remove proteins from the samples. Larger volumes of the homogenates were deproteinized and concentrated by pumping them through precolumn type I. Fig. 9 gives the chromatogram and conditions.

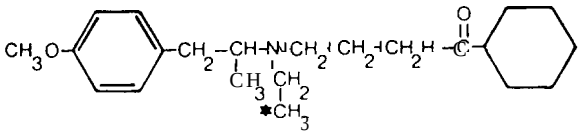

Fig. 4. Structure of secoverine. The asterisk indicates the site of the radiolabel.

Check on the recovery

The recovery in the case of pamoic acid was checked by comparison of peak areas obtained after concentration of a spiked sample on precone type III with those of direct injection of a standard solution in water. With the radiochemical experiments the balance of radioactivity was taken from the pool to be'concentrated and from the waste. When significant amounts of radiolabel were present in the waste, an aliquot of the latter was chromatographed to check for selective losses.

RESULTS

Analysis of pamoic acid in plasma

Fig. 5 shows the results of the analysis of pamoic acid in plasma. This example concerns a recovery experiment of pamoic acid from a spiked plasma sample. The preconcentration step yielded 100% recovery of pamoic acid.

Isolation of metabolites of clovoxamine

Fig. 6 shows the chromatographic pattern of the radioactivity in 1 ml of hamster urine, injected directly into the column. Fig. 7 shows the corresponding result with the equivalent of a pool of 900 ml of urine. It contained 300 μmol (*ca.* 120 mg) of clovoxamine as radioactive metabolites.

Comparison of Figs. 6 and 7 shows that, with regard to major peaks, adequate resolution was obtained after enrichment of the urine. In this instance recovery was *ca.* 90%. The chromatograms reveal that this was largely due to losses of the more hydrophilic metabolites. However, the big difference in ratio (5/8), must be attributed to the heavy quenching of scintillation caused by endogenous material which eluted with metabolite 5.

Isolation of secoverine metabolites from supernatant of liver homogenate

Fig. 8 shows the chromatographic pattern of the secoverine metabolites in 2 ml of supernatant from homogenized liver. Recovery of the radioactivity was over 95%. Fig. 9 shows the corresponding pattern obtained after the processing of 650 ml of supernatant on precone type I. In this instance the recovery was *ca. 70%.* Probably the metabolites represented in Fig. 8 by A and B were too hydrophilic to be retained on the precolumn. Recovery of the radioactivity represented by peaks C-G was prac-

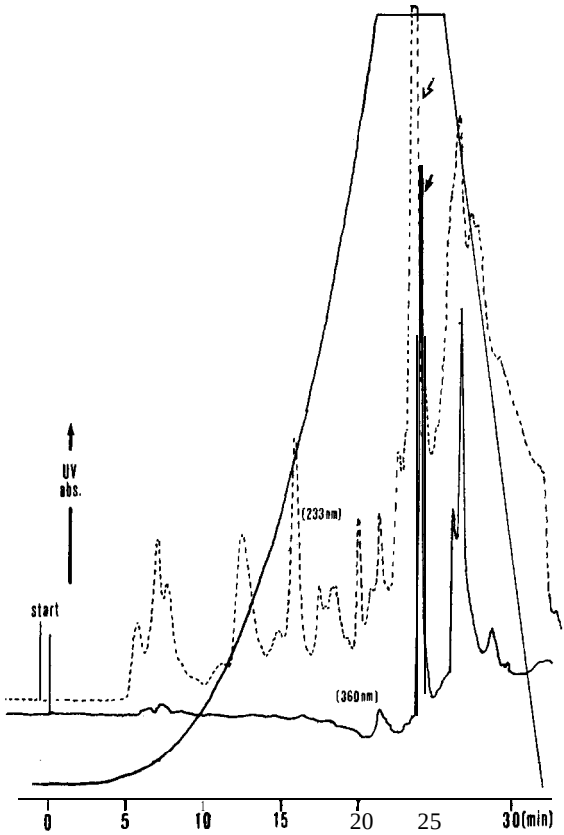

Fig. 5. Pamoic acid (indicated by the arrows) in plasma. Column, 250 × 6.2 mm I.D. packed with Nucleosil7 C_8 combined with precone type III filled with the same material. Gradient elution from water (5 g l^{-1} NH_4HCO_3) to methanol. Injected amount, 1.2 μg pamoic acid in 160 μl plasma. UV detection at 233 and 360 nm.

tically complete. Comparison of the figures shows that almost the same resolution was obtained with the large amount of sample as with less than 0.5% of that amount.

DISCUSSION

We have demonstrated that the use of reversed-phase precolumns of the design presented are suitable for the concentration and purification of drug metabolites contained in complex matrices, such as urine, plasma and tissue homogenates. By connecting a wide-bore precolumn with a small-bore main column we combine a large loading capacity of the precolumn with high resolution and low flow-rate of the main column. This low flow-rate enlarges the concentration factor. Practically the same resolution was obtained with filtered pools as with the very much smaller samples from the corresponding pools.

Recoveries were practically complete except for the hydrophilic metabolites, which were not retained on the stationary phase (C_8) of the precolumns. In none of the experiments did any obstruction of the set-up occur. The lifetime of the precones

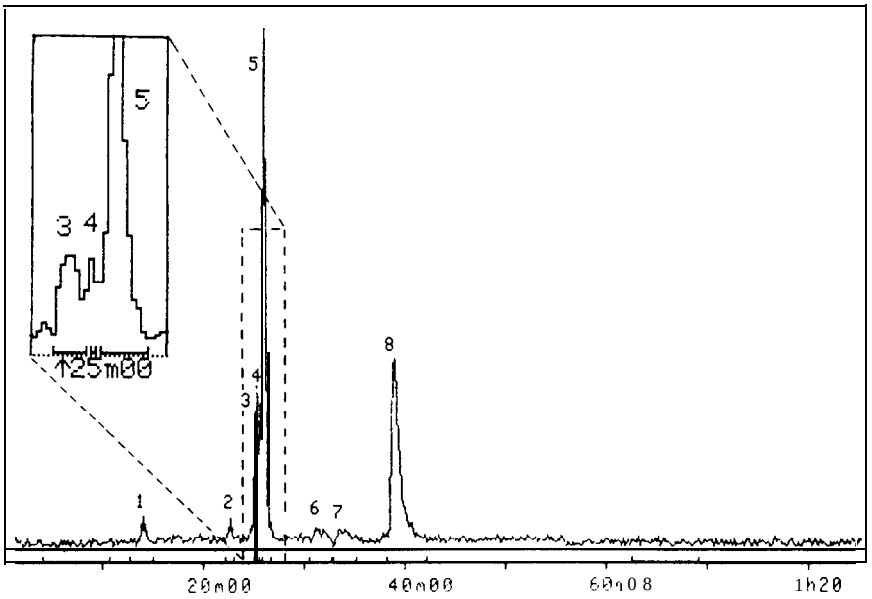

Fig. 6. Metabolite pattern of clovoxamine in hamster urine. The metabolites are indicated l-8. Injected volume, 1 ml. On-line radioactivity detection. Column, 500 × 9 mm I.D. packed with Nucleosil 7 C_8. A combination of gradient and isocratic elution from water to methanol was used.

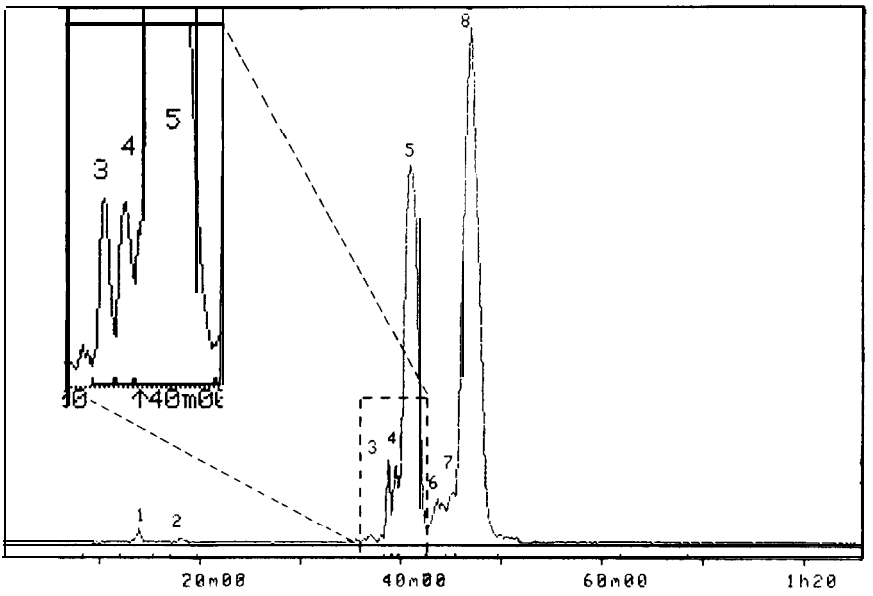

Fig. 7. Isolation of clovoxamine metabolites (peaks l-8) from a pool of hamster urine. Concentrated amount, 900 ml using precone type I. Chromatographic conditions were similar to Fig. 5 but corrected for the use of precone type I.

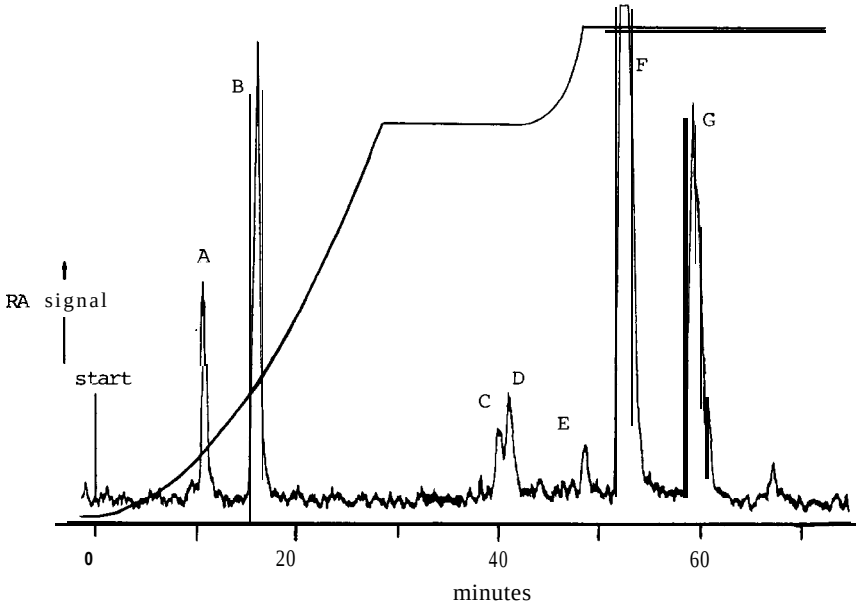

Fig. 8. Radiochemical metabolite pattern of secoverine incubated with liver homogenate. The metabolites are indicated A-G. Column, 500 × 9 mm I.D. packed with Nucleosil 7 C_8. A combination of gradient and isocratic elution from water to methanol was used. Injected volume, 1 ml. For deproteinizing purposes we used precone type III. RA = Radioactivity.

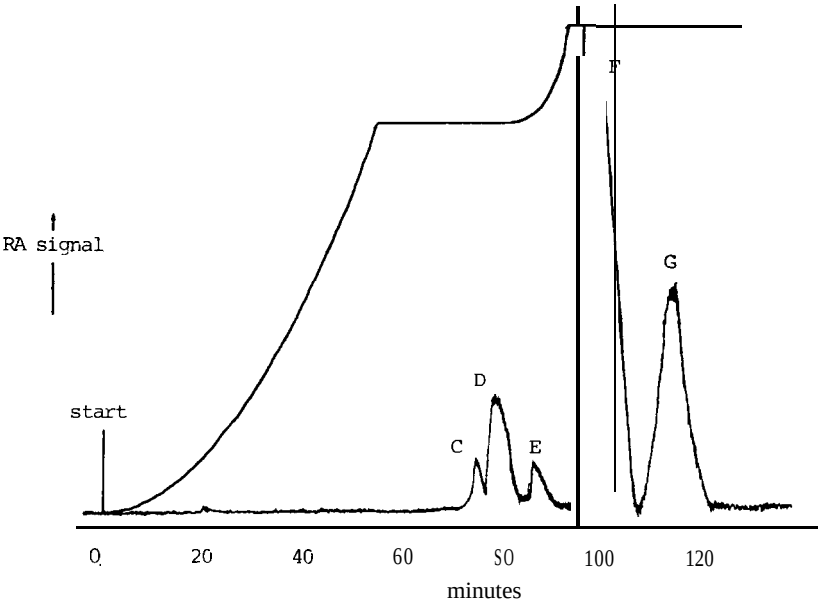

Fig. 9. Isolation chromatogram of 650 ml of liver homogenate containing 10 mg of secoverine metabolites (peaks A-G). Chromatographic conditions were similar to those of the previous pilot pattern.

in these investigations was the same of that of an analytical HPLC column under more delicate circumstances.

The results suggest that our precolumn arrangement is applicable to any problem involving enrichment of small amounts of substances in large amounts of complex matrices. Modes other than the reversed-phase systems presented may also reasonably be applied.

We observed that on a nitrile phase, proteins may be retained from aqueous solutions and accordingly spoil the column by denaturation when an organic solvent is introduced for elution. We could avoid this by rinsing the precolumn with water containing 10% dimethyl sulphoxide.

The principle should be helpful in surmounting loading problems in the current miniaturization of HPLC systems.

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