Journal of Chromatography, 255 (1983) 79–90
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,323

AUTOMATED DETERMINATION OF DRUGS IN BLOOD SAMPLES AFTER ENZYMATIC HYDROLYSIS USING PRECOLUMN SWITCHING AND POST-COLUMN REACTION DETECTION

C. E. WERKHOVEN-GOEWIE, C. DE RUITER, U. A. Th. BRINKMAN and R. W. FREI*
Free University, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)

G. J. DE JONG
Duphar B.V., Research Laboratories, P.O. Box 2, 1380 AA Weesp (The Netherlands)
and
C. J. LITTLE and O. STAHEL
Kontron Electrolab Ltd., Ashford, Middlesex (Great Britain)

SUMMARY

Enzymatic hydrolysis of blood samples with subtilisin-A releases protein-bound drugs and permits the repeated (10–50 times) injection of up to 1-ml volumes on short (2–30 mm) precolumns without appreciable build-up of pressure or loss of performance of the precolumn. The principle of fully automated serum and plasma analysis is demonstrated with the drug secoverine as a model compound. After enzymatic hydrolysis of the sample with an equal volume of a 1 mg/ml solution of subtilisin-A for 15 min at 55°C, the model compound is preconcentrated using a microprocessor-controlled column switching unit. Separation occurs in a reversed-phase liquid chromatographic system using a CN-type stationary phase and a buffered aqueous dioxane solution as mobile phase. Detection is done by UV spectrophotometry or fluorometrically after post-column ion-pairing reaction with dimethoxyanthracenesulphonate. The relative standard deviation of the procedure is less than $\pm 6\%$ ($n = 10$).

INTRODUCTION

The principle of on-line preconcentration of drugs from biological samples in high-performance liquid chromatographic (HPLC) systems has already been shown to be advantageous by several workers[1–4]. Preconcentration is usually carried out on relatively short (2–30 mm long) precolumns, packed with a sorbent employed in HPLC, such as chemically bonded silicas[1–3] or polymeric packings[4]. The precolumn serves as a preconcentration and clean-up column and as a guard column to prevent contamination of the analytical column.

In order to prevent clogging of the (pre)column by macromolecules present in serum and plasma samples when injecting millilitre amounts, special precautions have

0021-9673/83/0000–0000/$03.00

to be taken, such as filtration of the sample, flushing and back-flushing the precolumn with buffer, water or a basic solution after loading[3,4], packing the precolumn with relatively large (35–50 μm) particles[3,4], deproteination of the sample by precipitation before sampling or making use of eluents with low or zero organic modifier content to prevent precipitation of proteins on the column[5]. The use of large particles increases band broadening and, in the case of very short precolumns, may lead to decrease in recovery, due to the lower efficiency of the system[6]. A basic wash step can only be carried out with chemically stable packings, such as the styrene–divinylbenzene copolymer Amberlite XAD-2. Filtration and protein precipitation of the sample prior to analysis is unattractive as it causes loss of sample. Moreover, it may cause loss of analyte due to adsorption on the filter or coprecipitation on the proteins and, besides, there is a danger of contamination. Further, such off-line methods are time-consuming and elaborate.

A common problem in the analysis of blood levels of drugs is protein binding. In pharmacokinetic and clinical studies differentiation is made between free, protein-bound and total blood (serum or plasma) levels. Total blood levels can be obtained by (mild) hydrolysis of the sample, in order to release the drug from the proteins. An elegant method for the release of drugs from (tissue) proteins has been developed by Osselton *et al.*[7,8], who employed the proteolytic enzyme subtilisin-A for the release of antibiotics and benzodiazepines. After incubation with subtilisin-A, the sample was filtered over a plug of glass wool and analyzed in the usual manner. This enzyme was found to release the drugs completely, without affecting their chemical structures.

In this study we investigated the applicability of enzymatic hydrolysis as a sample pretreatment step to allow the repeated injection of relatively large (0.25–1 ml) blood samples on short precolumns and, thus, on-line preconcentration in automated HPLC analysis. The determination of the prospective spasmolytic drug secoverine was selected as a model system.

EXPERIMENTAL

Liquid chromatography

Automated preconcentration and HPLC analysis were carried out with a Kontron (Zürich, Switzerland) liquid chromatograph, consisting of two Model 410 HPLC pumps, a MSI 660 autosampler, a Model 200 programmer, a prototype of the MCS 670 column-switching apparatus and a Uvikon 720 LC variable wavelength UV–visible detector. Post-column reaction was carried out in a home-made reactor described earlier[2,9]. The reagent solution and extraction solvent were pumped with a Technicon (Tarrytown, NY, U.S.A.) autoanalyzer pump, Model II. Fluorescence detection was carried out with a Kontron SFM 23 spectrofluorimeter. Signals were recorded on a W + W 900 (Kontron) recorder. Quantitation of the signals was carried out manually. The apparatus used is shown schematically in Fig. 1.

HPLC columns were home-packed at duphar (Weesp, The Netherlands) and at the Free University; the 3- and 10-cm CN cartridges were obtained from Brownlee (Santa Clara, CA, U.S.A.). Packing materials used were 5-μm HPLC-Sorb Sil 60-D 5 CN and 10-μm Polygosil 60-10 CN (both from Macherey, Nagel & Co., Düren, G.F.R.), 10-μm LiChrosorb CN (E. Merck, Darmstadt, G.F.R.) and 7–8 μm Zorbax CN (DuPont, Wilmington, DE, U.S.A.).

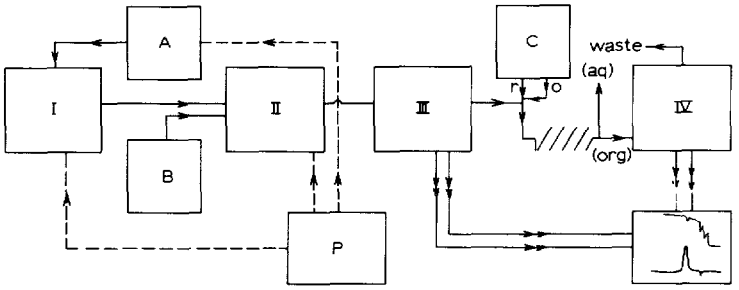

Fig. 1. Apparatus for automated preconcentration and HPLC analysis. A, B = Pumps; P = programmer; C = peristaltic pump; I = autosampler; II = column-switching apparatus; III = UV-detector; IV = fluorescence detector; r = reagent solution; o = organic phase.

Preconcentration took place on two types of precolumns: 30 × 4.6 mm I.D. prepacked cartridges (Brownlee) containing 10-μm LiChrosorb CN, and home-made (1.5–4) × 4.6 mm I.D. precolumns as described earlier[10], which were slurry-packed by hand, with a microspatula. Precolumns were packed with 5- or 10-μm CN bonded phases, or with a coarse (*ca.* 50-μm) C_{18} bonded phase.

Measurement of pressure drop

To study the effect of preconcentration of blood samples on the pressure drop over a precolumn the system shown in Fig. 2 was used, consisting of an Orlita DMP-AE-10.4 (Orlita, Giessen, G.F.R.) dual-head pump, with home-made pulse dampers and manometers (Chrompack, Middelburg, The Netherlands) ranging from 0 to 600 bar (0–60 MPa), a Valco (Houston, TX, U.S.A.) ten-port switching valve and home-made precolumns. The manometers were calibrated and found to have an accuracy of $\pm 10\%$ at pressures of over 50 bar.

Gel electrophoresis

Gel electrophoresis of blood samples was performed using a home-made apparatus, at 300 V and 40 mA, during 3 h and using gel slabs (20 × 20 cm) containing 9–15% polyamide. Linear gradients were formed as described by Egberts *et al.*[11]. Gels were stained in an aqueous solution containing 0.25% Coomassie Brilliant Blue

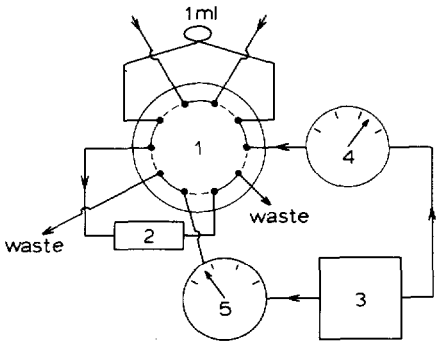

Fig. 2. Apparatus for pressure measurements during preconcentration. 1 = Ten-port switching valve; 2 = precolumn; 3 = dual head pump; 4 and 5 = manometers.

R 250, 45% methanol and 9% glacial acetic acid, during 1 h at 65°C and destained in an aqueous solution containing 5% methanol and 7.5% glacial acetic acid. For identification of the formed bands a marker solution was applied, containing phosphorylase *b* subunit (mol.wt. = 94,000), bovine serum albumin (mol.wt. = 67,000), ovalbumin (mol.wt. = 43,000), carbonic anhydrase (mol.wt. = 30,000), soy bean trypsin inhibitor (mol.wt. = 20,100) and α-lactalbumin (mol.wt. = 14,400).

Chemicals

All chemicals used were of analytical grade quality. Secoverine and 9,10-dimethoxyanthracene-2-sulphonate (DAS) were synthesized at Duphar. 1,4-Dioxane and 1,2-dichloroethane were purchased from Baker (Deventer, The Netherlands). All aqueous solutions were prepared with demineralized water, treated in a Milli-Q (Millipore, Bedford, MA, U.S.A.) ultra filtration system. Eluents were filtered through an all-glass filtration apparatus with 0.2-μm filters (Millipore) and degassed under vacuum before use. Frozen sterilized calf serum was obtained from Flow Laboratories (Irvine, Great Britain). Frozen human plasma was a gift from the blood transfusion service of the Onze Lieve Vrouwe Gasthuis (Amsterdam, The Netherlands). Subtilisin-A was purchased from Novo (Bagsvaerd, Denmark), proteinase K from E. Merck, pepsin and chymotrypsin from Sigma (St. Louis, MO, U.S.A.) and pronase from Boehringer (Mannheim, G.F.R.).

RESULTS AND DISCUSSION

As a model system for the investigation of the applicability of enzymatic hydrolysis in the preconcentration of drug-containing blood samples in an on-line HPLC system, the analysis of secoverine[2] was chosen. Secoverine is preconcentrated and chromatographed on CN-bonded silica and can be detected either directly with UV absorption (detection limit 1 μg) or fluorimetrically, after post-column ion-pairing with DAS as counter ion (detection limit 20 pg–1 ng, depending on the conditions used)[2].

We have studied the effects of enzymatic hydrolysis of blood serum and plasma on the pressure drop and performance of the precolumns of various (1.5–30 mm) lengths and packed with 5–50 μm particles. In order to test the general applicability of the method, we have investigated possible interferences of the matrix in UV absorption and fluorescence detection with and without a post-column reactor.

Chromatography of secoverine

In a previous paper[2] the optimization of the HPLC and detection system for secoverine was described. Columns packed with HPLC-Sorb Sil 60-D 5 CN were invariably used, and best results were obtained with dioxane–water mixtures as the eluent. In an attempt at further optimization, we investigated the behaviour of secoverine on three other types of CN material (*cf.*, Experimental). The retention of secoverine was much greater than on HPLC-Sorb CN. Peak shapes were about the same (asymmetry factor, $A_{0.1}$ = 2–3). Consequently, if the analysis time has to be maintained constant, a distinctly higher modifier content of the mobile phase is required. This is a drawback in the post-column extraction system, as higher percentages of polarity modifier promote the extraction of DAS into the organic phase, thereby causing an increased background signal.

With HPLC-Sorb CN as packing material we also investigated the use of acetonitrile or tetrahydrofuran. The latter, which is both structurally and physicochemically very similar to, but less toxic than dioxane, could not be used as, upon mixing with a 0.01 *M* phosphate buffer of pH 3, clouding occurred. With acetonitrile, peaks were extremely asymmetric ($A_{0.1} = 7$).

In this study, HPLC of secoverine was therefore performed in CN/dioxane–0.1 *M* sodium phosphate buffer (pH 3.2) systems. In the experiments on the effect of enzymatic hydrolysis on precolumn performance, use was made of a HPLC-Sorb CN (250 × 4.6 mm I.D.) column with dioxane–buffer (15:85) as the eluent. In the experiments testing the functioning of the complete automated system including post-column reaction, a Polygosil (250 × 4.6 mm I.D.) column was used with dioxane–buffer (40:60) as the eluent.

Breakdown products of serum and plasma upon enzymatic hydrolysis

Table I shows literature data on the properties of some enzymes that were tested for the hydrolysis of polypeptides in blood samples. The enzymes hydrolyze polypeptides at specific amino acid sites, which are indicated in Table I. The enzymes (50 μg) were dissolved in 1 ml of buffer of the appropriate pH, mixed with an equal volume of plasma and incubated at the optimal temperature (according to the literature) for 10, 30 or 60 min in a stirred water-bath. Aliquots of the treated and untreated plasma samples were subjected to electrophoresis, together with a marker solution, as decribed in the Experimental. The proteins present in blood plasma are known to be 30–60% globulin (mol.wt. = 90,000–160,000), 52–66% albumin (mol.wt. = 69,000) and about 6–7% fibrinogen (mol.wt. = 400,000)[12]. The latter is absent from serum. Their respective dimensions are 18 × 3, 16 × 4 and 66 × 3 nm[13]. Gel electrophoresis of plasma and serum samples showed a strong broad band corresponding to mol.wt. = 40,000–55,000. This band remained present with about the same intensity in the samples treated with pronase, chymotrypsin and pepsin, while it partly disappeared and shifted to lower-molecular-weight regions upon treatment with subtilisin-A and proteinase K. With both these enzymes, intense bands were found at mol.wt. < 15,000 (small peptides and amino acids) and around mol.wt. = 30,000. A small band of mol.wt. = *ca.* 100,000 present in plasma completely disappeared upon treatment with subtilisin-A and proteinase K. From the electrophoretogram it was

TABLE I

SURVEY OF ENZYMES INVESTIGATED

Enzyme	*Activity sites*[12]		*Optimal conditions*	
	Primary	*Secondary*	*pH*	*T(°C)*
Chymotrypsin	Trp, Phe, Tyr	Leu, Met, His, Asp, NH_2	7.8	25
Pepsin	Trp, Phe, Tyr Met, Leu	Various	2	37
Proteinase K	Random	Random	7.8	25
Pronase	Random	Random	7.8	25
Subtilisin-A	Aromatic and aliphatic	Various	8.2	55

concluded that proteinase K is slightly more effective in hydrolyzing blood plasma proteins than is subtilisin-A and that the other three enzymes were not noticeably active under the present conditions. As the mild action towards labile drugs of subtilisin-A in protein-bound drug analysis is well documented[7,8], contrary to the situation with proteinase K, which is known to be an extremely strong hydrolyzing agent[12], we continued our studies with the former enzyme. The use of proteinase K may however be favourable in the analysis of thermolabile drugs, as its optimal incubation temperature is 25°C compared to 55°C with subtilisin-A, and will therefore be studied in the future.

In another gel electrophoresis experiment the optimal conditions for subtilisin-A treatment were investigated using serum samples. The incubation time was varied between 15 and 120 min, temperature between 20 and 55°C and subtilisin-A concentration between 0.05 and 1 mg/ml in borate buffer, pH 8.5. Maximal hydrolysis was found to have occurred after 15 min at 55°C or after 60 min at 20°C. A concentration of 1 mg/ml, as recommended in the manufacturer's brochure, was found to be more effective than one of 50 μg/ml, and was therefore used in all further experiments presented below.

Effect of enzymatic hydrolysis on pressure drop during and after preconcentration

The apparatus used is shown in Fig. 2. A ten-port switching valve was mounted with a 1-ml injection loop and a precolumn (2.2 × 4.6 mm I.D.). The valve was connected to two HPLC pumps, each delivering water. Between each pump and the valve was placed a manometer, which was read at regular intervals. With this set-up the effect of precolumn loading with (hydrolyzed) serum and a back-flush wash step with water on the pressure drop over the precolumn was studied. For convenience, the pressure drop is expressed as the relative pressure drop, P_{rel}

$$P_{rel} = \frac{P - P_0}{P_{max} - P_0} \times 100\% \quad (1)$$

where P is the pressure measured, P_0 the initial pressure drop over the precolumn and P_{max} the pressure which is reached upon complete clogging of the system ($P_{max} = 50$ bar). The initial pressure drop over the precolumn depends on the density and particle size of the packing, and the porosity of the frits at both ends of the precolumn. It is generally 0–10 bar at a water flow-rate of 1 ml/min and when using 5–10 μm particles.

Untreated serum samples. After sampling 1 ml of newly received, up to 2-weeks old thawed calf serum, the precolumns were completely clogged (P_{rel} rising sharply to 100%). With three other bottles of calf serum, which had been thawed and divided into 10-ml aliquots upon their arrival at different dates in the laboratory and then stored for several days up to several months at −20°C, the results were different. With 2–6 months old samples, complete clogging of the precolumns was observed only after sampling some 7–10 ml. With several 9-months old serum samples, complete clogging never occurred. Obviously, next to refreezing —the effects of which are well known— ageing also affects the breakdown of proteins. It is therefore extremely important to use freshly thawed blood samples, preferably stored at −60°C, as model solutions (for clinical studies).

A precolumn which was almost completely clogged ($P_{rel} = 90\%$) after injec-

tion of 5 ml of 2-months old serum was back-flushed with 10 ml water and the relative pressure was found to decrease to 10%. This indicates that clogging is caused by particles which cannot completely enter the pores of the packing material and become trapped at the top of the column.

Hydrolyzed serum samples. The effect of enzymatic hydrolysis with subtilisin-A upon the pressure drop during preconcentration of 1-ml aliquots of a serum sample, containing 1 mg subtilisin-A, is illustrated in Fig. 3. Four representative examples out of 20 series of experiments are shown: 7–9 ml of 2-months old serum, diluted with 10% water or subtilisin solution, were sampled at regular intervals; each 10 min for 1-ml and each 2.5 min for 250-μl aliquots. It is evident that sample size in the range studied has no noticeable influence on the pressure drop profile. The relative pressure increased from 10 to 40% for hydrolyzed and from 30 to about 100% for untreated samples. In other studies, consistently lower relative pressures were observed with hydrolyzed samples compared to untreated samples of equal age. Typically, with fresh, hydrolyzed serum samples, P_{rel} increased further to 75%, with 2-weeks old serum to 50% and with 1-month old serum to 10% after eight subsequent 1-ml injections. Upon introduction of back-flush wash steps, the pressure could be kept consistently low (P_{rel} = 0–10%) with all samples. It was also observed that dilution of the blood samples in an equal volume of enzyme solution had a favourable effect on the pressure profile, due to the decreased viscosity of the samples. This aspect was not thoroughly studied, but 1:1 dilution in enzyme solution was invariably used in further studies presented below.

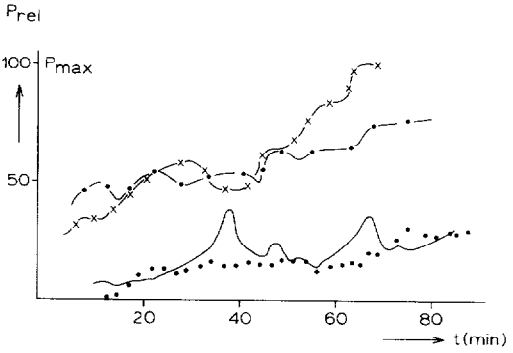

Fig. 3. Relative pressure over precolumn during preconcentration. Untreated serum: × — ×, 1-ml samples taken every 10 min; ●—●, 250-μl samples every 2.5 min. Hydrolyzed serum: ·····, 1-ml samples every 10 min; ———, 250-μl samples every 2.5 min.

Precolumn packing material. The influence of particle size on P_{rel} was studied by injecting untreated —but diluted— and hydrolyzed serum samples; 5-, 10- and 50-μm particles were used. With untreated serum samples slightly more favourable results were obtained for the 50-μm particles: maximal pressure was reached after three 0.5-ml injections with 50-μm particles, and after one injection with 10-μm particles. Since, however, large particles are known[6] to contribute significantly to band broadening, 5–10 μm particles were selected for further study.

Effect of enzymatic hydrolysis on precolumn performance

The configuration of valves for on-line preconcentration is shown in Fig. 4. Blood serum samples were hydrolyzed by incubation with an equal volume of subtilisin-A (1 mg/ml) solution in 0.1 *M* borate buffer of pH 8.5 and shaking for 15 min at 55°C.

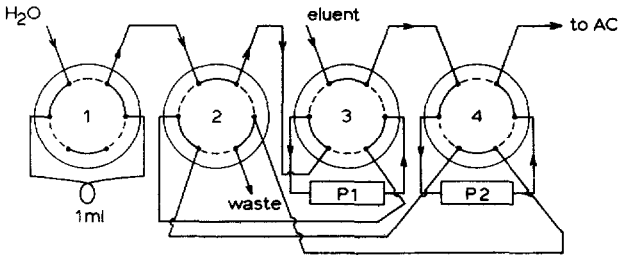

Fig. 4. Switching-valve configuration for alternate preconcentration + back-flush wash step on two precolumns (P1 and P2) and desorption onto an analytical column (AC).

Aliquots (1 ml) of untreated or hydrolyzed blood serum samples were spiked with 15 μg secoverine and preconcentrated on a 2 × 4.6 mm I.D. precolumn. After washing the loaded precolumn with 2 ml water in the back-flush mode, the sample was transferred by the eluent used as mobile phase to the analytical column. Detection took place by means of UV absorption at 274.5 nm.

With untreated serum, two analyses could be carried out without problems. After the third one the pressure increased sharply, which was found to be mainly due to clogging of the 0.5-μm top frit of the analytical column. The recovery of the analyte now was only some 10%. The fast decrease in performance after preconcentration of untreated blood serum or plasma samples has repeatedly been observed by us, both with short and long precolumns. It must be due to precipitation of proteins on the precolumn packing material when changing the solvent from water to the organic-modifier-containing eluent. This is also indicated by the occurrence of the frit clogging, since dissolved protein molecules are at least one order of magnitude smaller than the pores of the frit.

With subtilisin-A treated serum samples, neither pressure problems nor loss of performance was observed after twenty injections, equalling a total injected amount of 10 ml serum. After twenty injections the analyte recovery was still 100%. The repeatability was good (relative S.D. = 3%, n = 6). With subtilisin-A treated plasma samples the same trend was observed. After fourteen injections (7 ml plasma) the pressure drop over the precolumn plus analytical column still had its initial value and the recovery remained 95–100%. With these experiments, repeatability was less good (relative S.D. = 6%, n = 6) than in the previous series. This is probably due to the presence of compounds which interfere in the UV detection. These compounds are more abundant in hydrolyzed plasma than in hydrolyzed serum, and make quantitation of the analyte signal less precise.

Interference of subtilisin-A treatment with detection

Subtilisin-A is a relatively small protein with molecular weight 27,287, composed of a single polypeptide chain of 274 amino acid residues. It is a white crystalline

powder, readily soluble in water to give a colourless solution, which is stable under normal working conditions. The UV spectrum of subtilisin-A shows two broad bands (Fig. 5a) in the 200–240 and 260–290 nm regions. Upon excitation with light of any wavelength between 230 and 280 nm, a fluorescence emission spectrum with maximum at 300 nm is obtained (Fig. 5b). Batch measurements of the fluorescence intensity of 0.01–1 mg/ml subtilisin-A solutions in water in a 3-ml cuvette with 1-cm pathlength showed loss of signal at higher concentrations. A plasma sample treated with 1 mg/ml subtilisin-A showed strong fluorescence between 260 and 500 nm upon excitation with light of 230–380 nm.

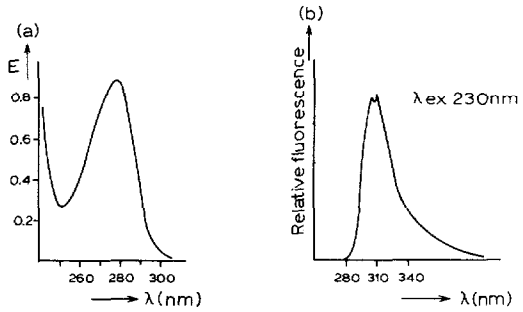

Fig. 5. UV (a) and fluorescence (b) spectrum of subtilisin-A.

In a HPLC test system with a CN column and methanol–0.1 *M* phosphate buffer (25:75) as eluent, 20 μl and 1 ml of a subtilisin-A solution of 75 mg/ml in water were injected and the concomitant signals on a UV detector set at 225 and 254 nm, and at 0.01 a.u.f.s., were measured. Subtilisin-A was not retained on the column and produced a large t_0 peak. For the 20-μl injection this peak would interfere with solutes having a capacity factor (k') < *ca.* 1.4 at 254 nm (*i.e.*, solutes with higher k' values would be completely resolved from the t_0 peak). For the 1-ml injection the t_0 peak would interfere with solutes having $k' < 2.3$ at 254 and $k' < 4.0$ at 225 nm. In order to minimize interference with UV or fluorescence detection it is therefore recommended that the precolumn be flushed with water to remove subtilisin-A, before transfer to the analytical column.

As subtilisin-A hydrolyzes large protein molecules to smaller polypeptides and amino acids, subtilisin-A treated blood samples may be expected to contain many compounds which will strongly interfere with UV, fluorescence or electrochemical detection. The interference with UV detection at 225 and 254 nm was tested in the same system as described above. A 20-μl injection of a serum sample containing 75 mg subtilisin-A per litre showed a t_0 peak at 254 nm which would interfere with solutes having $k' < 4.6$. With untreated serum this figure is 4. Upon direct injection of 1 ml of subtilisin-treated serum on the analytical column, both at 225 and 254 nm the disturbance lasted for over 40 min ($k' > 30$)! When preconcentrating the same sample followed by a 1-ml wash step with water, the resulting t_0 peak would, at 225 nm, have interfered with solutes having $k' <$ *ca.* 7. Consequently, after preconcentration of (subtilisin-A treated) blood samples a wash step is absolutely necessary to remove a major part of the interfering compounds. Such a wash step will also serve to protect the analytical column. Our experiments indicate that, without additional clean-up, it

is not possible to analyse compounds which are only slightly retained on the analytical column, unless a highly selective detection mode is used.

Extremely specific detection modes are offered by post-column reaction detectors which use reagents that are (almost) inactive towards the bulk of peptides and amino acids present in the matrix and/or make use of a specific extraction step. An example of such a detection system is the ion-pairing reaction with DAS, followed by continuous extraction into 1,2-dichloroethane and fluorescence detection (λ_{ex} 383 nm, λ_{em} 452 nm)[2].

Automated analysis of secoverine. The applicability of the combined method of preconcentration and post-column reaction extraction to the automated analysis of secoverine in enzymatically hydrolyzed blood plasma samples was tested in the system shown in Fig. 1, using the valve configuration given in Fig. 4 for precolumn switching.

500-μl aliquots of 175 ng secoverine spiked and subtilisin-A treated (1:1 diluted) blood plasma were sampled by the autosampler and preconcentrated on a CN precolumn. The performances of two types of precolumns (length 30 and 1.5 mm) were compared in this experiment. In order to test the performance of the system under normal HPLC conditions, a Polygosil CN/dioxane–buffer (40:60) system was chosen for HPLC.

With the 30-mm precolumn, 47 subsequent analyses were performed over a period of 2 days. The performance of the precolumn and analytical column was excellent throughout the whole experiment, although the pressure in the system increased by 50%. This was found to be due to clogging of the top frit of the analytical column. Also, disturbances of the post-column reaction detector occasionally (about once every hour) occurred which led to a less precise quantitation. These disturbances are probably due to the large dead volume of the precolumn (0.5 ml), which causes the transfer of rather large amounts of solutes to the HPLC system.

With the short (1.5-mm) precolumn, 20 analyses were performed without pressure problems or disturbance of the post-column system. The repeatability was good (relative S.D. = 4%, n = 20) (see Fig. 6). In both cases the precolumns showed no loss of performance after completion of the run. Memory effects studied by injecting "blank" water samples were found not to occur.

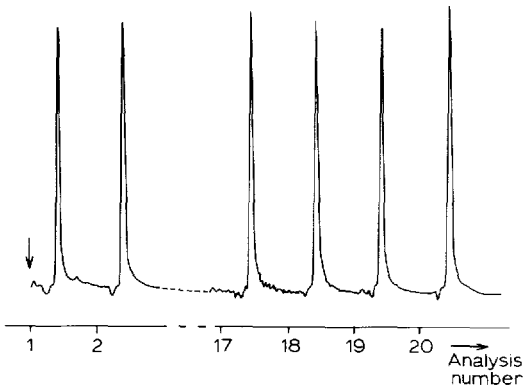

Fig. 6. Repetitive injection of 500-μl preconcentrated secoverine-spiked (170 ng) and subtilisin-A treated blood plasma. Cycle time: 12 min. Detection: post-column ion-pairing + fluorescence detection (see text).

CONCLUSIONS

Enzymatic hydrolysis of blood serum and plasma samples offers a rapid and easy sample pretreatment which, combined with a back-flush wash step of the short precolumn used as concentration column, permits the continuous, automated analysis of total drug levels in up to 1-ml aliquots in an on-line HPLC system.

No pressure problems are observed and precolumns show no loss of performance after injection of a total amount of *ca.* 10 ml of hydrolyzed plasma or serum sample. With untreated samples, precolumns were irreversibly affected upon injection of 1 ml of serum. As enzymatic hydrolysis produces a large number of small peptides and amino acids, the method should be used in combination with selective separation and/or detection systems.

APPENDIX

Overall procedure for the automated preconcentration of secoverine

Time	*Event*	*Action*
0	File 13 start	
0	File 12	
0	File 11 start	
0	Start autosampler (S_1)	Filling sampler-loop with S_1 (1 ml)
1.8	Start file 6	Flushing of PC I with water. PC II with eluent
	(flow 2 ml/min, % B 100, Aux 3, dur 3.1)	Preconcentration on PC I
3.8	Aux 1, dur 1	Backflushing of PC I with water
4.8	flow 0	
4.9	Aux 2, dur 1	Elution of sample from PC I
6	End file 6, return to file 10	Analysis of S_1 (6 min)
6.1	Autosampler move 1 step forward	
8.2	End file 10, return to file 12	
8.3	Start file 11	
8.3	Start autosampler (S_2)	Filling of sampler-loop with S_2 (1 ml)
10.1	Start file 5	Flushing PC II with water, PC I with eluent
	(flow 2 ml/min, % B 100, Aux 2, dur 3.1)	Preconcentration on PC II
12.1	Aux 1, dur 1	Backflushing of PC II with water
13.1	flow 0	
13.2	Aux 3, dur 1	Elution of sample from PC II
14.3	End file 5, return to file 11	Analysis of S_2 (6 min)
14.4	Autosampler move 1 step forward	
16.5	End file 11, return to file 12	
16.6	End file 12, return to file 13	
16.7	End file 13	

Time	*Function*	*Value*	*Duration*	*Comments*
File number 5				
0	Flow	2		
0	% B	100		Pump
0	Aux	2	3.1	PC I conditioning and loading
0.9	Aux	1	1	Backflush water over PC I
3	Flow	0		
3.1	Aux	3	1	PC I flush with eluent
4	end			

(Continued on p. 90)

File number 6				
0	Flow	2		
0	% B	100		Pump water
0	Aux	3	3.1	Conditioning + loading of PC II
0.9	Aux	1	1	Backflush PC II with water
3	Flow	0		
3.1	Aux	2	1	Elute sample of PC II
4.2	END			
File number 10				
0	Aux	7	0.1	Autosampler start; fill loop
1.8	Call File	6	1	Start flushing water over PC II
2.9	Aux	8	0.1	Move autosampler one step
5	END			
File number 11				
0	Aux	7	0.1	Autosampler start; fill loop
28	Call File	5	1	Start flushing water over PC I
2.9	Aux	8	0.1	Autosampler move one step
5	END			
File number 12				
0	Call File	10	1	Preconcentration + elution of PC II
0.1	Call File	11	1	Preconcentration + elution of PC I
0.2	END			
File number 13				
0	Call File	12	30	
0.1	END			

ACKNOWLEDGEMENTS

We thank D. Kroese and G. Koomen for carrying out some of the experiments, and W. H. Mager (Department of Biochemistry of the Free University, Amsterdam) for helpful assistance with the gel electrophoresis experiments.

REFERENCES

1 J. Lankelma and H. Poppe, *J. Chromatogr.*, 149 (1978) 587.
2 R. J. Reddingius, G. J. de Jong, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.*, 205 (1981) 77.
3 W. Roth, K. Beschke, R. Jauch, A. Zimmer and F. W. Koss, *J. Chromatogr.*, 222 (1981) 13.
4 R. A. Hux, H. Y. Mohammed and F. F. Cantwell, *Anal. Chem.*, 54 (1982) 113.
5 K.-G. Wahlund, *J. Chromatogr.*, 218 (1981) 671.
6 C. E. Werkhoven-Goewie, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, 51 (1982) 2072.
7 M. D. Osselton, M. D. Hammond and P. J. Twitchett, *J. Pharm. Pharmacol.*, 29 (1977) 460.
8 M. D. Osselton, I. C. Shaw and H. M. Stevens, *Analyst (London)*, 103 (1978) 1160.
9 R. W. Frei and J. F. Lawrence, *Chemical Derivatization in Analytical Chemistry*, Plenum, New York, 1981, pp. 306–309.
10 H. P. M. van Vliet, Th. C. Bootsman, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 185 (1979) 483.
11 E. Egberts, P. B. Hachett and P. Traub, *Hoppe-Seyler's Z. Physiol. Chem.*, 357 (1976) 1779.
12 H. R. Mahler and E. H. Cordes, *Biological Chemistry*, Harper and Row, New York, 2nd ed., 1966, p. 117.
13 J. S. Fruton and S. S. Simmonds, *General Biochemistry*, Wiley, New York, 1958, p. 150.