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FULLY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A NEW CHROMATOGRAPH FOR PHARMACOKINETIC DRUG MONITORING BY DIRECT INJECTION OF BODY FLUIDS*

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SUMMARY

A new fully automated high-performance liquid chromatograph is described which detects drugs from directly injected plasma (urine, saliva) without sample pretreatment. The apparatus consists of a programmable automatic sampling unit, which is connected via two alternating working pre-columns to an analytical column ("alternating pre-column sample enrichment"). The new device is able to operate with directly injected body fluids like an auto-analyzer and is especially useful for pharmacokinetic and clinical studies, where drug concentrations have to be determined from plasma, urine or saliva.

INTRODUCTION

In pharmacokinetics, which deal with the description and interpretation of time-dependent levels of drugs in the body, non-radioactive analytical methods are gaining in prominence because of their selectivity in the specific detection of drugs or metabolites and their sensitivity in the nanogram range [1—5]. In this field, high-performance liquid chromatography (HPLC) is one of the most important techniques for the separation and the determination of drugs and their metabolites in body fluids [5, 6].

The use of an automatic sampler and the application of auto-analyzer reactor systems in connection with HPLC was a great step forward in the partial automatization of liquid chromatography [7—16]. But a still unsolved problem, especially for pharmacokinetic investigations, was the question how to avoid the classical sample pretreatment steps for biological samples prior to HPLC analysis.

*Dedicated to Prof. Dr. Leopold Horner on the occasion of his 70th birthday.

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The application of a pre-column in its property as a protecting unit to extend the lifetime of the analytical column is a very common technique in liquid chromatography [2, 14]. Moreover, the employment of small columns with hydrophobic packing materials yields good results in the preconcentration of xenobiotics [4, 7, 17, 18].

Concerning the development of HPLC methods for biological samples (e.g. plasma, serum, saliva, urine), one of the most time-consuming steps, involving considerable sources of error, is sample pretreatment and enrichment prior to injection into the chromatograph. An approach towards the on-column sample-enrichment technique using a gradient elution system and microbore columns has been described [19].

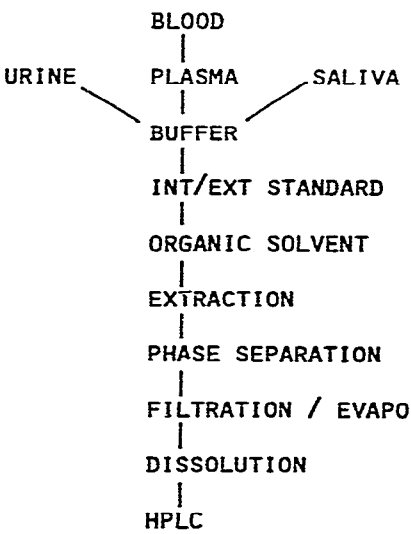
So far it has not been possible to determine drugs by repeated injection of native body fluids into a liquid chromatograph. Thus, we adapted the idea of the pre-column as a protecting device for the analytical column and, at the same time, the possibility to adsorb and preconcentrate drugs on small reversed-phase columns, for the development of a new technique.

The following report describes a novel automatic high-performance liquid chromatograph, with alternating pre-column sample enrichment, for direct plasma (urine, saliva) injection without classical sample pretreatment steps. Our automated system uses the "on-column" enrichment technique on small pre-columns and the pre-column backflushing technique in addition with pre-column re-equilibration.

EXPERIMENTAL

Sample pretreatment

Steps in "classical" sample pretreatment of biological fluids for subsequent HPLC runs are shown in Fig. 1. Nearly all steps are susceptible to errors and



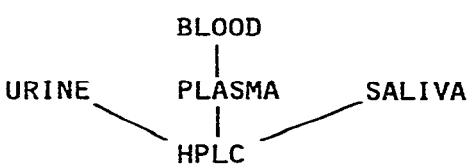


Fig. 1. "Classical" steps in the pretreatment of body fluids prior to high-performance liquid chromatography.

Fig. 2. Handling of body fluids prior to automated HPLC with alternating pre-column sample enrichment.

waste time and laboratory capacity. The application of the recently developed automatic HPLC device, with alternating pre-columns, reduces these steps significantly (Fig. 2).

Fully automatic HPLC

The apparatus, illustrated schematically in Figs. 3 and 4, consists of an injection system (autosampler) and two pneumatically driven valves, which alternatingly connect two pre-columns with the injection system and pump A. The columns can also be connected in the "backflush mode" with the analytical column and pump B. The programmable autosampler, affiliated to a time relay, controls the whole analytical procedure.

Columns

The pre-columns (typical dimensions 25 mm × 4.6 mm I.D.) are made by dividing commercially available Knauer columns (Dr. Knauer, West Berlin, G.F.R.) into smaller columns 25 mm long. They are dry packed. In the examples described in this paper we used C_{18}-Corasil (Waters Assoc., Königstein/Ts, G.F.R.) with a particle size of 37—50 μm (see legends to Figs. 6 and 9).

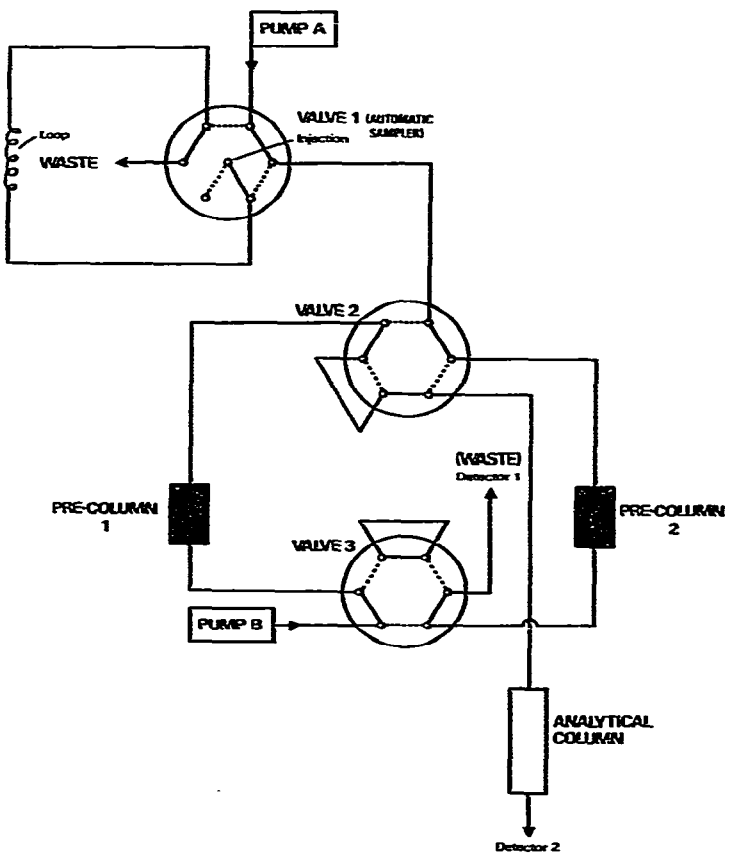


Fig. 3. Alternating pre-column switching technique for sample enrichment, demonstrated with three 6-way valves.

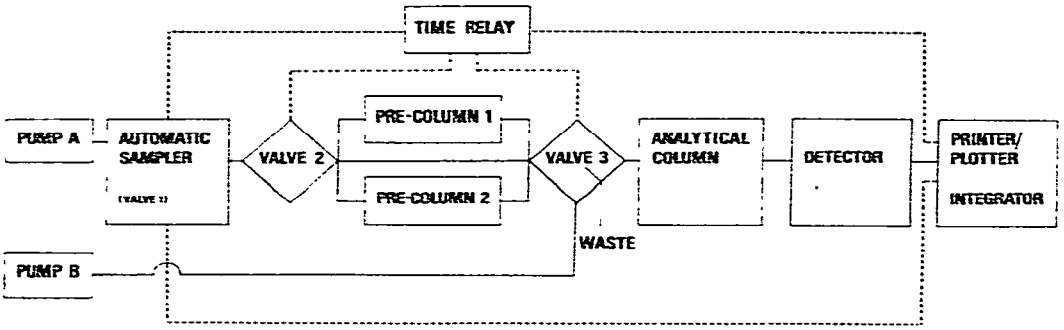


Fig. 4. Flow-chart of the automated HPLC system with alternating pre-column sample enrichment. (— — —), electronic connections.

The analytical columns (typical dimensions 120 mm × 4.6 mm I.D.) are filled by the usual slurry technique with LiChrosorb RP-18 (5 μm) from Merck (Darmstadt, G.F.R.) for analysis of R-A 233 (Dr. K. Thomae GmbH, Biberach/Riss, G.F.R.) (Fig. 6), and with ODS-Hypersil (5 μm) from Shandon Southern Products (Cheshire, Great Britain) in the analysis of AR-L 115 BS (Dr. K. Thomae GmbH).

Detailed description of technique

Samples of body fluids (maximum 48 samples) are pipetted into the sample holder of the automatic sampling device (e.g. WISP, Waters). Volumes of between 10 and 2000 μl of plasma, urine or saliva, preferentially between 10 and 150 μl can be automatically injected. It should be checked that the samples contain no solid particles which might block the injection needle. Pre-column 1 (PC1) onto which the first sample is injected, has been conditioned with the purge phase (water or buffer solution) delivered by pump A (Fig. 3). After the injection to PC1 (typical dimensions 25 mm × 4.6 mm I.D.) the pre-column is washed for a further 5 min with water (buffer).

The adsorption material for the pre-columns is reversed-phase or ion-exchange material with a particle size of about 20—50 μm. Here the substances to be detected are selectively adsorbed on C_{18}-Corasil (37—50 μm) and thus enriched. At the same time all accompanying water-soluble co-products are eliminated with the purge phase (pump A). A second pre-column (PC2) has been added to save time. While PC1 is reconditioned, PC2 is eluted in the backflush mode onto the analytical column and vice versa (= alternating pre-column sample enrichment). Simultaneously to the application—injection step, the autosampler (WISP) activates an electronic controller (time relay) which controls the purge-phase period, after which it switches the pneumatic valves and starts the printer/plotter integrator. This switching process causes two subsequent steps: pre-column 1 (PC1) where the injected drug has been absorbed is switched to the solvent stream of pump B. Pump B delivers the eluent cocktail, necessary for separation and chromatography, in the backflush mode from PC1 to the analytical column (typical dimensions 120 mm × 4.6 mm I.D.), filled, for example, with reversed-phase material (particle size 5—10 μm). Parallel to this process, PC2 is switched to the eluent stream of pump A (purge phase) which removes the rest of the organic solvent from

the pre-column. The whole working cycle thus consists of an equilibration phase, an adsorption phase and a purge phase on the pre-columns and a chromatographic phase on the analytical column. The automatic sample device (WISP) has been computed for one working cycle and the printer/plotter is stopped by an internal equilibration delay device. During the print-out of the results (Hewlett-Packard, Model 3370) the second pre-column (PC2) is switched to the purge stream of pump A for re-equilibration (equilibration phase). After the next injection step, the electronic timer restarts. It then switches again to the pneumatically working valves and activates the printer/plotter, and so on (Fig. 5).

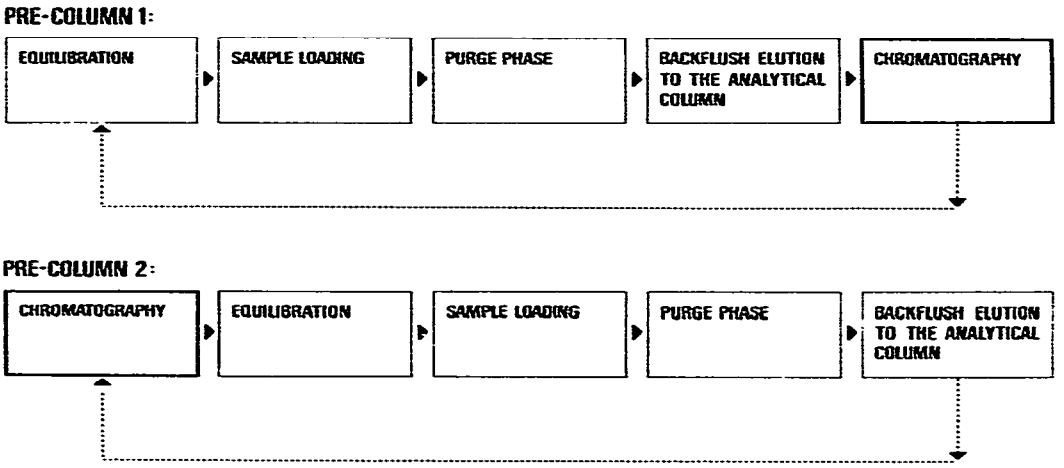


Fig. 5. Parallel working steps of pre-column 1 and pre-column 2, in the automated high-performance liquid chromatograph with alterning pre-column switching.

RESULTS

Chromatography

Drugs which can be separated and detected by reversed-phase or ion-exchange chromatography should also be detectable using automated HPLC with direct injection of body fluids. So far the new method has been used for the determination of AR-L 115 (a new cardiotonic agent) from urine, saliva and plasma, and for Rapenton^{R} (R-A 233 BS), an anti-platelet drug, and dipyridamole (Persantine^{R}), from both plasma and urine.

Typical chromatograms for automatic HPLC (direct sample injection) with alternating pre-column sample enrichment from an overnight run are shown in Figs. 6—8. It can be seen that the example demonstrated in Fig. 6 shows an HPLC determination with an extremely high precision [coefficient of variation (C.V.) = 1.4%].

As the printer is immediately started by the time relay (Fig. 4) with the backflush elution from the pre-column to the analytical column, we get only the main cut-out of the chromatogram with the relevant peaks and the calculated areas. Because of the peculiarity of the novel pre-column switching technique, the peaks from the first, third, fifth, etc., runs derive from pre-

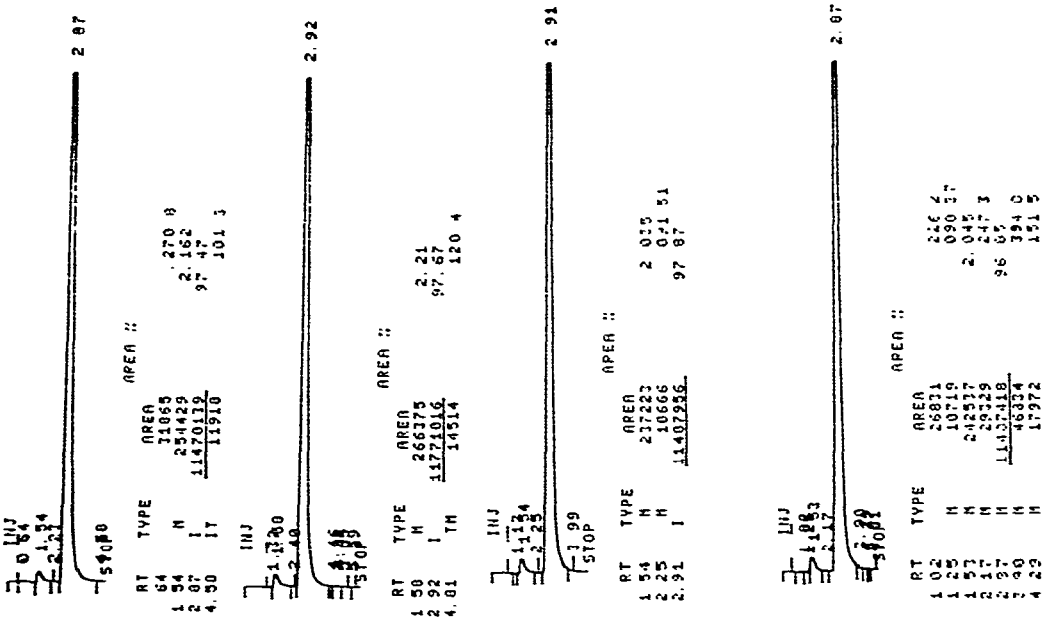


Fig. 6. Typical chromatograms of directly injected human plasma (injection volume = 150 μl), spiked with 100 ng/ml R-A 233, a new anti-platelet drug. C.V. = 1.4%. HPLC conditions: reversed-phase material (Corasil^R, 37—50 μm) in the pre-columns and RP-18 (LiChrosorb^R, 5 μm) in the analytical column. Column dimensions: pre-column 25 mm × 4.6 mm I.D.; analytical column 120 mm × 4.6 mm I.D. Pump A, water; pump B, methanol—0.2 *M* Tris buffer (pH 8.6) (80:20). Flow-rate, 1 ml/min. Fluorimetric detection: 465/510 nm.

column 1 and those from the second, fourth, sixth, etc., runs come from pre-column 2.

Backflush—elution

Immediately after the injection of a drug—plasma solution to the pre-column, the drug is adsorbed and enriched in the very first area on top of the pre-column. This effect could be demonstrated by the application of drugs which show a strong fluorescence in visible light. It is therefore important that the adsorbed material is eluted from the pre-column in the backflush mode to prevent zone-spreading effects. The effect of the 25-mm pre-column to peak width and tailing was negligible in all cases tested.

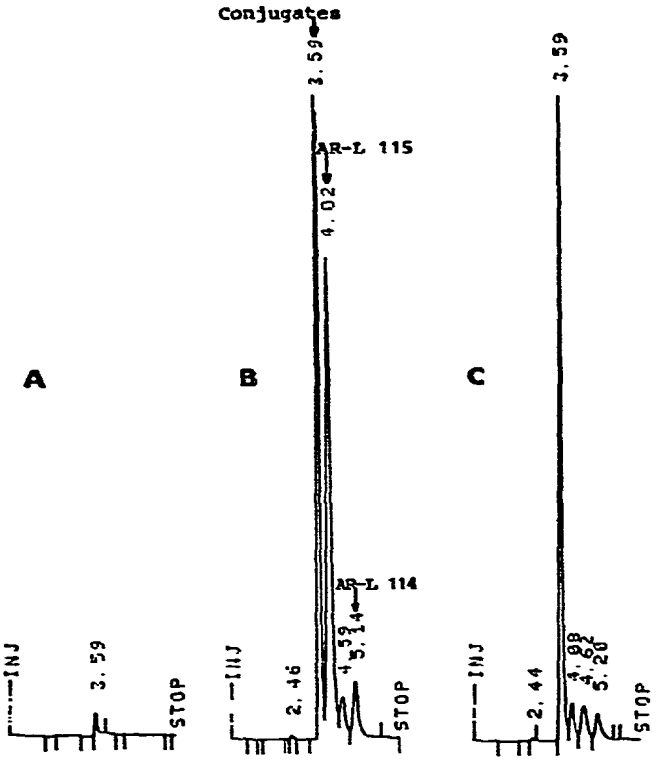


Fig. 7. HPLC chromatogram from native urine (injected volume = 100 μl) after oral administration of 100 mg of AR-L 115. (A) Blank, (B) sampling period 0—4 h, (C) sampling period 4—8 h. For HPLC conditions see Fig. 9; analytical column is 120 mm long.

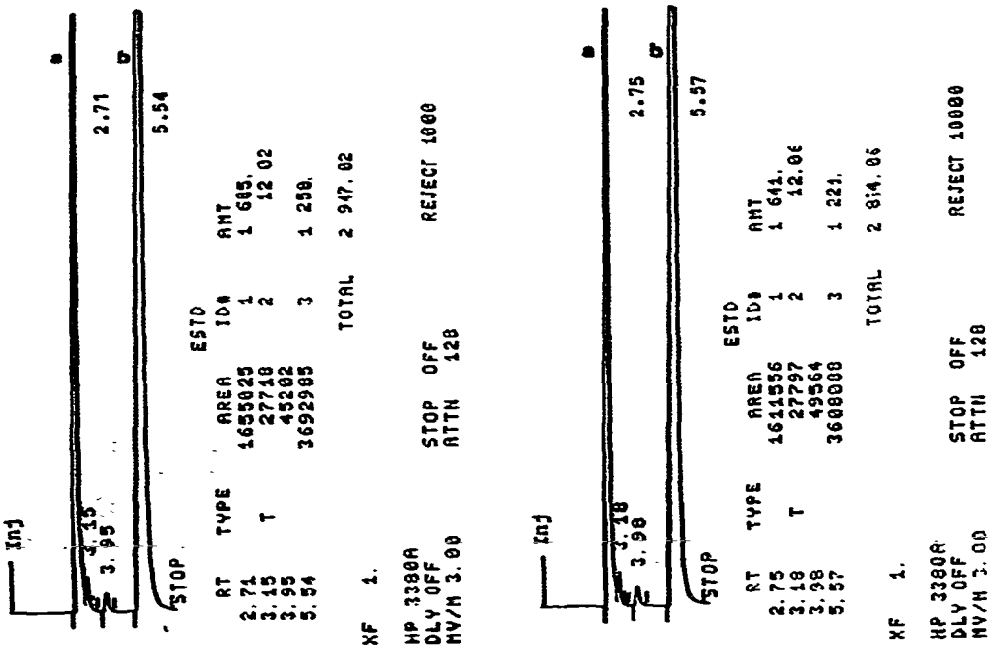


Fig. 8. HPLC chromatogram from native saliva (injected volume = 50 μl) 0.5 h after oral administration of 75 mg of AR-L 115 BS (for HPLC conditions see Fig. 9). (A) AR-L 115; (B) metabolite AR-L 113. Fluorimetric detection: 330/370 nm.

Stability of the automated system

The stability of the system in the time range of 24 h and also between days is extremely high. Fig. 9 shows a chromatogram injected at zero time and 24 h later. The C.V. between day-to-day analyses was in the range of 3—5%.

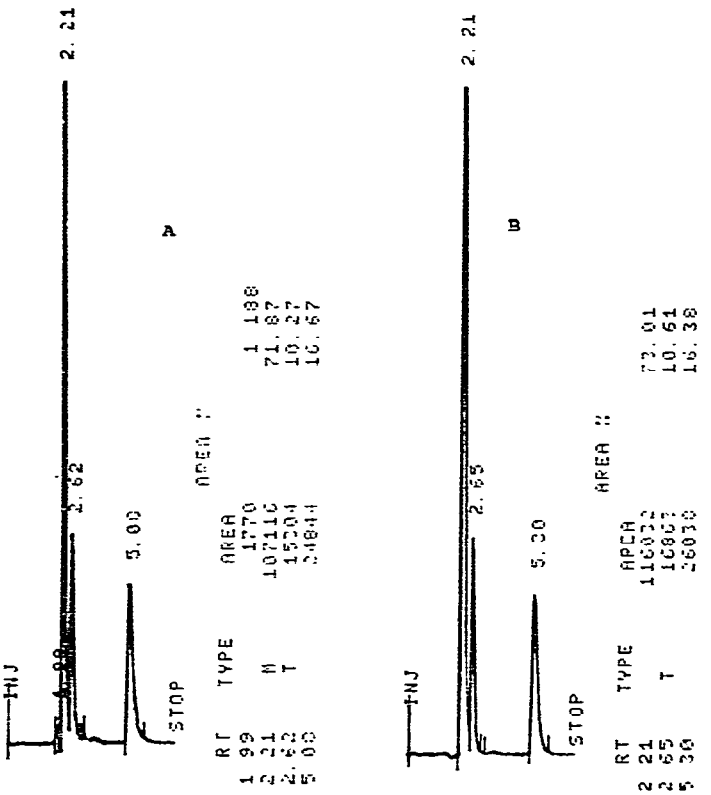


Fig. 9. HPLC chromatograms of AR-L 115, a new cardiotonic drug (t_R = 2.21 min) and two metabolites from a pharmacokinetic run at 0 time (A) and the same injection 24 h later (B). HPLC conditions: reversed-phase material (Corasil^R,37—50 μm)in the pre-columns and ODS-Hypersil^R (5 μm) in the analytical column. Column dimensions: pre-column 25 mm × 4.6 mm I.D.; and analytical columns, 120 mm × 4.6 mm I.D. Pump A: water pump B: acetonitrile—0.03 *M* phosphate buffer (pH 6.8) (1:2). Flow-rate, 1 ml/min. Fluorimetric detection: 330/370 nm.

Lifetime of the columns

In a series of about 1000 analyses of AR-L 115 BS, with an injection volume of about 10—150 μl of plasma, where normal tap water was used for washing the pre-columns and with acetonitrile—phosphate buffer (pH 6.8), there was no need for a column exchange. The results showed constant quality as regards selectivity and resolution.

Protein binding

We tested the influence of protein binding by comparing the recovery

after injection of spiked water and spiked plasma solutions with different concentrations of Rapenton^R. No effect of protein binding on recovery could be found in this case. The affinity of the protein-bound drug to the reversed-phase packing material in the pre-column seems to be stronger than the interaction with the protein. It seems to be possible that differences in recovery may result with strongly protein-bound drugs and "weak" reversed-phase packings in the enrichment column.

Memory effects

Memory effects from the pre-column ("substance bleeding") may be easily identified by injecting blank solutions after a run with definite amounts of drug. No memory effect could be observed.

Precision and accuracy

The fully automated system reaches a high level of precision (Table I). For the calibration curve of Rapenton^R (R-A 233 BS) (HPLC conditions as in Fig. 6), and anti-platelet drug, we obtained coefficients of variation of 1.3% and 1.2% (within day) and of 2.6% (day-to-day) for concentrations of 50 ng/ml and 1000 ng (n = 5). In all cases tested we obtained identical results with the automated technique compared to the normal HPLC using organic extracts of plasma samples. In the case of ^{14}C-labelled R-A 233 BS, we obtained nearly identical results for injected and eluted radioactivity.

TABLE I

PRECISION AFTER AUTOMATICALLY REPEATED INJECTIONS IN A CALIBRATION RUN FOR RAPENTON^R, AN ANTI-PLATELET DRUG

See Fig. 6.

Injected volume (μl plasma)	Concentration (ng/ml)	Coefficient of variation (CV %); n = 5
150	50	1.3
150	100	1.5
150	200	3.5
100	400	2.5
50	600	2.3
50	800	1.9
10	2000	0.8

Internal/external standard

The commonly used internal standard method with an additional compound, which needs to show chromatographic and spectroscopic properties similar to the compound to be detected, is of great importance in HPLC with classical sample pretreatment (Fig. 1). As in the case of fluorimetric determinations, it is sometimes difficult to find a suitable standard compound. With the recently developed HPLC technique, the drug which is to be detected may be used simultaneously as an external standard. There is no need for an additional standard compound. Vials containing plasma samples spiked

with the pure compound give us information about the stability of the whole automated system and about the range of linearity of the method.

CONCLUSION

The fully automated high-performance liquid chromatograph with an "alternating pre-column sample enrichment" device is able to sample body fluids such as plasma, urine or saliva directly on pre-columns, which act simultaneously as enrichment columns. From a theoretical point of view the new technique should be applicable to all classes of compounds that are detectable by reversed-phase and ion-exchange liquid chromatography. The system carries out overnight runs and thus increases laboratory capacity for routine analysis. The HPLC method reported is a useful and precise analytical tool, particularly in pharmacokinetic investigations, where drug concentrations have to be determined from body fluids.

Characteristic features of the device are: no sample pretreatment, direct injection of body fluids (plasma, urine, saliva), only one pipetting step per sample, no further standard compound required, peak width independent of the injected volume, fully automated chromatography, high precision, overnight runs, and low costs.

The new fully automated HPLC system may find application in the fields of drug development, drug monitoring (clinical and outdoor), clinical chemistry, control of chemical syntheses, stability testing, and forensic chemistry.

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