Journal of Chromatography, 204 (1981) 65–76
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,224

APPLICATION OF COLUMN SWITCHING IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO ON-LINE SAMPLE PREPARATION FOR COMPLEX SEPARATIONS

F. ERNI*, H. P. KELLER, C. MORIN and M. SCHMITT

Analytical Research and Development and Biopharmaceutical Department, Sandoz Ltd., Basle (Switzerland)

SUMMARY

High-performance liquid chromatography column switching offers interesting possibilities for a powerful combination of simple sample clean-up and efficient separation. A very flexible column-switching set-up using two six-port valves as switching devices is presented. The approach is suitable for automation and can easily be put together from commercially available components. The arrangement can be used for different kinds of "cuts" (front-cut, heart-cut, end-cut), for back-flushing and for on-line concentration. Varying the separation parameters with gradient elution and/or different stationary phases in the sub-separation systems offers many possibilities, including two-dimensional high-performance liquid chromatography. The set-up presented proved to be valuable both during optimisation and for routine work.

Applications of this technique to the analysis of biological samples (animal feed, urine, plasma, etc.) are discussed. They demonstrate that a chromatographic clean-up is very efficient and may be the method of choice when the compounds to be analysed are chemically labile and when there is a high risk of artefact formation with classical clean-up techniques.

INTRODUCTION

High-performance liquid chromatography (HPLC) plays a key role in the determination of pharmaceutically active compounds in biological materials[1]. However, with the new possibilities of automation and data processing[2,3], sample preparation has become a new bottleneck. It often determines the total analysis throughput as well as the analysis costs for assays in complex matrices. The analysis of drugs in biological materials is part of the general problem of determining a single compound or a few specific compounds in *trace* amounts in a very complex mixture. Reversed-phase materials have introduced new possibilities for simple sample preparation and for sample concentration[4–6], but in many cases time-consuming off-line sample clean-up steps, such as liquid–liquid extraction and liquid–solid adsorption, are still necessary and, unlike in capillary gas chromatography, an increase in separa-

0021-9673/81/0000–0000/$02.50

tion performance is still an important goal of modern HPLC. The never-ending fight with interferences in determinations of biological materials can be approached by increasing the performance of the separation (which means more theoretical plates and an exponential increase in the cost in terms of hardware and time[7]) or by achieving greater selectivity in the detection[8–10] and/or the separation, *e.g.* with multidimensional HPLC[11,12].

In the literature, different approaches to column switching have demonstrated its potential as an alternative method for gradient elution[13] and for increased selectivity using mixed columns[14–18]. Recently, column-switching techniques have been used for biological materials[19–21].

In this paper the possibilities of column switching as an on-line method for sample preparation are discussed. On-line sample concentration techniques[4–6], cut-techniques as used in gas chromatography[22,23], and the selectivity increase with mixed columns of different stationary phases, as in multidimensional HPLC[11,12], are presented.

DESCRIPTION OF THE SYSTEM

Goals

In routine determinations in biological materials with HPLC the sample clean-up is critical for the following reasons:

(1) Complicated sample clean-up is time-consuming and expensive;

(2) There is a risk of loss of the interesting compound (recovery);

(3) Sensitive compounds may decompose during the sample treatment.

The goal of all sample preparation systems therefore must be to cope with these problems. The following points have to be considered if the method is to be used for routine determinations:

(1) The total analysis time (sample preparation and chromatography) should be shortened;

(2) To facilitate the operation, the sample preparation should be on-line to the HPLC separation system;

(3) The sample preparation should be automated;

(4) The reproducibility should be good enough to use external standardisation, which eliminates complicated searches for adequate internal standards and the problem of interferences with the internal standard;

(5) The sample preparation should be mild so that sensitive compounds can be handled;

(6) For trace analysis, a concentration step is necessary;

(7) To minimise the problem of interferences no loss of the separation power of the chromatographic system is acceptable: on the contrary, an improvement of the selectivity of the separation is desirable;

(8) To reduce the time for the development and optimisation in a given analytical problem the sample preparation system should be generally applicable, so that the experimental parameters can be changed on a rational basis and with high flexibility.

Basic principles

Some years ago we demonstrated[4–6] that it is possible to inject on to reversed-

phase HPLC columns very large volumes of aqueous sample without loss of separation efficiency. Up to millilitres of urine or other aqueous biological liquid samples can be concentrated on the column and afterwards analysed. This sample concentration is on-line and has been used with success for years. Unfortunately, not only the compounds of interest but also many interferences are concentrated on the column. They elute together with the substance or stick to the top of the column. The use of a precolumn to concentrate the sample makes possible the use of back-flushing[21], which can significantly increase both the life of a separation system and the reproducibility of retention times. As shown in Figs. 1 and 2, if the precolumn and the second column are put in the loops of six-port valves, cutting techniques can be used. With a *front-cut*, only the first part of the chromatogram, including the interesting peak(s), is analysed by columns 1 and 2. After the elution of the last peak of interest from column 1, the precolumn is bypassed by switching valve 1 and can be cleaned by the flow of good eluting solvent using *back-flush* or forward-flush mode. With

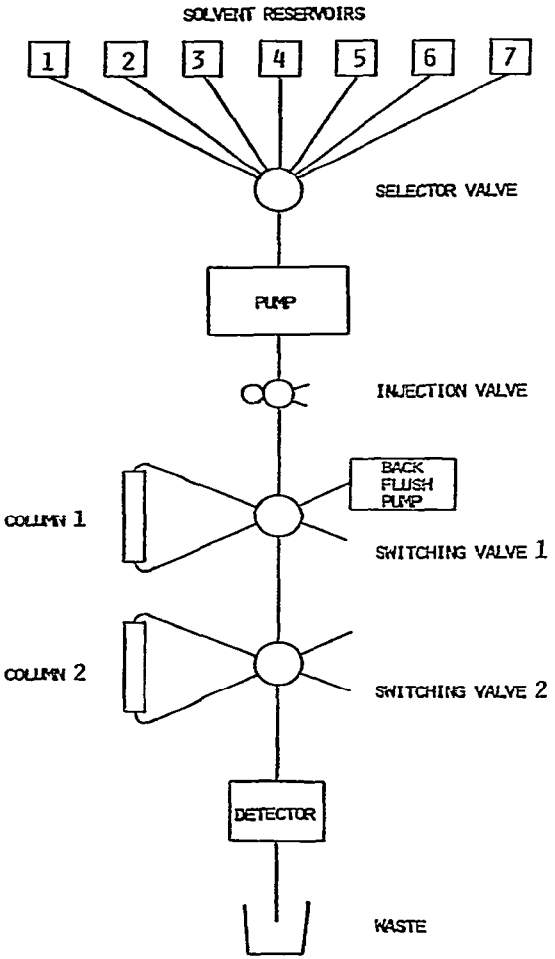

Fig. 1. Diagram of the column-switching system with a step-gradient elution solvent delivery system.

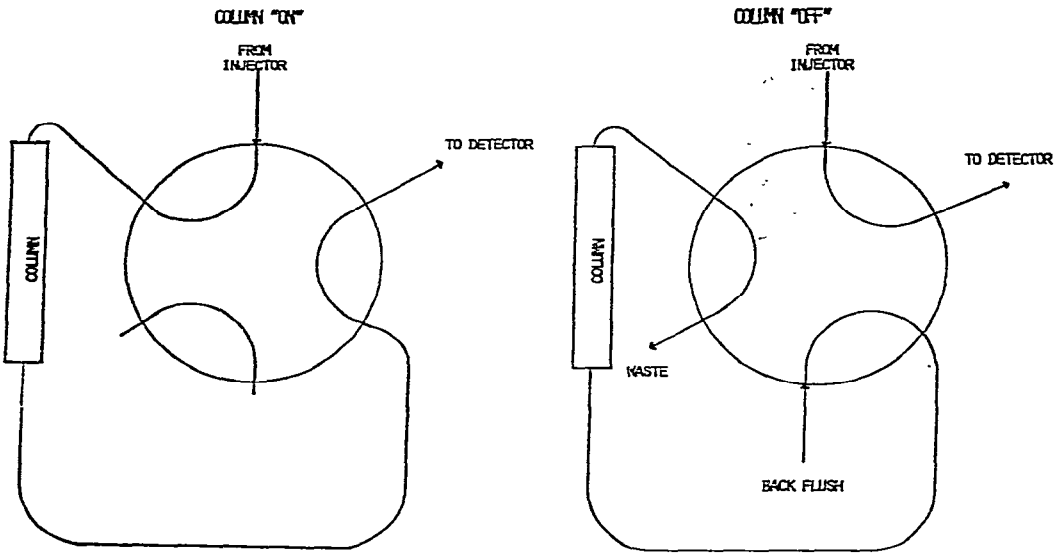

Fig. 2. Flow scheme of the six-port column-switching valves (Rheodyne, Berkeley, CA, U.S.A., type 7010 A, or Valco Instruments, Houston, TX, U.S.A., type ACL-6-UHPa-N60).

this front-cut the analysis time can significantly be shortened because late-eluting background peaks are not analysed on the second column.

Very often the first part of the chromatogram contains a large amount of polar substances. The tailing of these peaks interferes with the compound(s) of interest. It is therefore useful to eliminate these polar interferences using an *end-cut* technique, thus bypassing the separation column 2 during the elution of the first part of the chromatogram. A practical application of this end-cut technique was recently demonstrated by Gfeller[21]. By combining the front-cut and the end-cut, only the relevant part of the chromatogram (*heart-cut*) is analysed by the second column. This minimises the interference of fast-eluting and late-eluting background peaks and also minimises the ballast load on the main separation column that may change the separation characteristics (selectivity and performance) of the column 2 from run to run.

The approach described here (Fig. 1) uses two six-port valves as switching valves (Fig. 2) and therefore has the following advantages:

(1) Front-cut, end-cut and heart-cut techniques are possible;

(2) Cut techniques and back-flushing can be combined;

(3) The combination of hardware shown in Fig. 1 has proved to be optimal for method development and daily checks because each column can be run separately to optimise chromatographic conditions and switching times;

(4) The switching valves can easily be automated.

This system has proved to be an excellent sample-preparation system for different biological samples because only simple clean-up steps, if any, are necessary. It is also very simple and especially suitable for sensitive compounds.

The selectivity of the system can be varied by using different columns in the switching system. If the first column contains a stationary phase with generally

smaller capacity factors (k') than that in the second column (*e.g.* RP-2 in column 1 and RP-18 in column 2), a second concentration on top of column 2 is achieved. This leads to narrower peaks (meaning higher efficiency) of the total separation system. The two different columns also have different separation selectivities, which is especially important for trace analysis. Only a small part of the first column is separated on the second column, with the result that fewer peaks overlap. Information theory[11] shows that with this two-dimensional selectivity system, the information produced by column switching increases with increasing difference between the selectivities of the two columns. Our experience has shown that a combination of Diol phase (column 1) and RP-18 (column 2) gives excellent preconcentration on the second column, together with the advantage of a large selectivity difference. In addition, the combination of column switching with step-gradient elution gives more flexibility: not only can different stationary phases be used in the two columns but it is also possible to use different mobile phases for sample clean-up on the first column and final separation on the second column.

EXPERIMENTAL

Apparatus

The chromatographic system used in this work consists of either a two-pump gradient solvent delivery system or a step-gradient elution system described in an earlier paper[4], an automatic sample injection system[24], six-port switching valves and a variable-wavelength UV detector.

Reagents

All solvents used were HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain) or LiChrosolv grade (E. Merck, Darmstadt, G.F.R.). The water was double-distilled. For the buffer solutions analytical grade reagents were used (E. Merck, and Fluka, Buchs, Switzerland).

The animal feed contains a mixture of cereals, plant and animal proteins, mineral substances, amino acids and vitamins (Type 850 G4, NAFAG, Gossau, Switzerland). The drugs Fluorproquazone and Endralazine were from Sandoz, Basle, Switzerland.

APPLICATIONS

Analysis of medicated feed

During the development of a drug, long-term toxicological studies have to be carried out. For this purpose laboratory animals get a feed spiked with the drug. The homogeneity and the stability of the medicated feed have to be checked to ensure "Good Laboratory and Development Practice" (GLDP). The analysis of the drug in the complex matrix of the feed mixture is often very complicated and time-consuming. A good selectivity of the separation system and an excellent sensitivity for the detection is necessary. Fig. 3 shows the determination of Fluorproquazone[21] in animal feed using the column-switching system described in Fig. 1. The reproducibility of the system is better than 2% relative standard deviation (R.S.D.) at 25 ppm, the calibration curve is linear from 1 to 100 ppm with a correlation coefficient of $r = 0.99998$,

and no systematic error from interferences was detected. Because the sample preparation is simple and the reproducibility good, external standardisation can be used. The total analysis time is only 14 min and the total analysis system operates fully automatically.

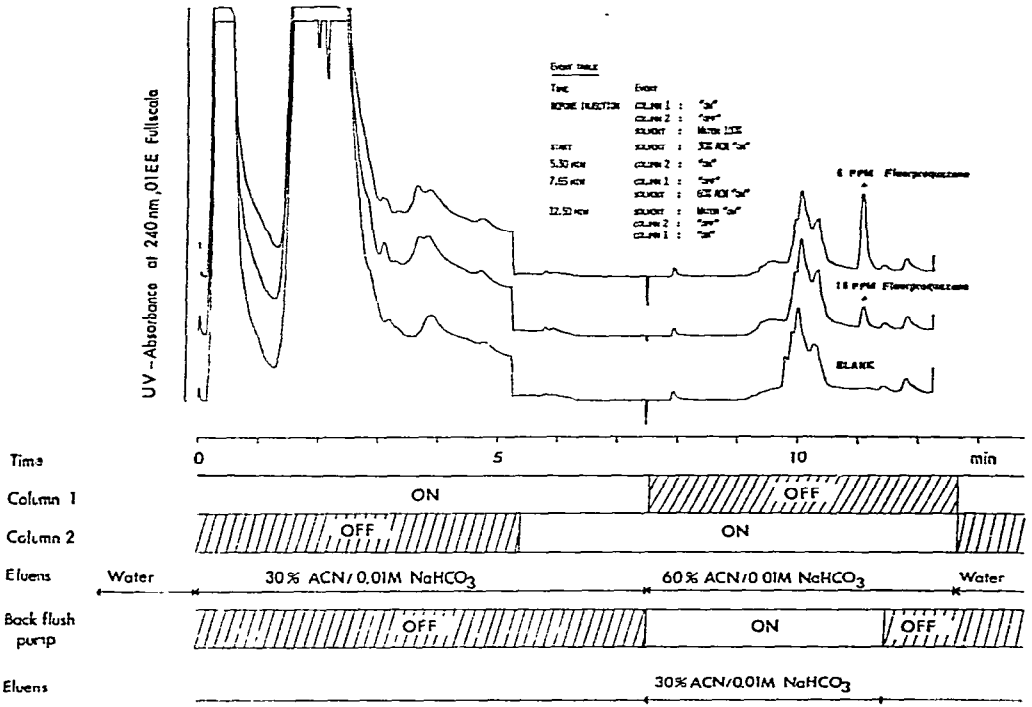

Fig. 3. Analysis of Fluorproquazone in medicated feed with the step-gradient system[4] and column switching (see Fig. 1). Pump: Model 100, Altex Scientific, Berkeley, CA, U.S.A.; automatic sample injector[24]; switching valves: Rheodyne 7010 A; UV detector: Model LC 55 (Perkin-Elmer, Norwalk, CT, U.S.A.); Lab. Data System HP 3354B (Hewlett-Packard, Avondale, PA, U.S.A.). Column 1: RP-2, 5 μm, 3 cm × 4.7 mm I.D. (Knauer, Oberursel, G.F.R.). Column 2: RP-18, 5 μm, 12 cm × 4.7 mm I.D. (Knauer). Sample preparation: stir 100 g of medicated feed with 200 ml of methanol for 10 min, centrifuge, inject 50 μl of the clear solution. ACN = acetonitrile.

The determination of a drug metabolite in plasma and urine samples

The pyruvate of the new antihypertensive drug Endralazine (EP)[25] is a main metabolite in blood and urine of humans and most animals investigated. For pharmacokinetic studies and for the development of dosage forms a bioanalytical method for EP had to be developed with sufficient sensitivity to detect this compound in the lower ng/ml range (ppb) in body fluids. Because of the relatively high solubility in aqueous solution and the sensitivity to hydrolysis in acidic media, classical liquid–liquid clean-up methods could not be used to extract this acidic compound.

From 1 ml of plasma the compound is extracted at neutral pH into chloroform–propan-2-ol as an ion-pair using tetrabutylammonium counter-ions (see Fig. 4 for details). Injection of the extracted residue on to a paired-ion reversed-phase system with UV detection at 280 nm yields a chromatogram where the compound EP elutes

with a k' value of *ca.* 3. The extract contains a large number of coextracted endogenous compounds partly strongly retained. An elution gradient cannot be applied to this HPLC system at the required high detector sensitivity. Therefore the column-switching technique is an excellent alternative way of reducing the analysis time of *ca.* 45 min to *ca.* 15 min (see Fig. 4). The extract is injected on to a short reversed-phase column from which only the front part is transferred on to the second column (Fig. 5), where the separation and detection of the compound of interest occur. The stationary phase and the isocratic mobile phase are the same for both columns. The remaining part of the extract is back-flushed from the first column with the same mobile phase. This simple two-column method was used for several hundred plasma samples from dogs and humans administered with Endralazine. The linearity (r = 0.9995 in the range from 0 to 500 ng/ml) and reproducibility (R.S.D. = 10% for 100 ng/ml standards) are satisfactory with external standardisation.

All liquid–liquid and liquid–solid extraction clean-up techniques tried on urine failed, giving very high blanks. Therefore a column-switching technique with on-line sample clean-up and external standardisation was developed. Urine is buffered

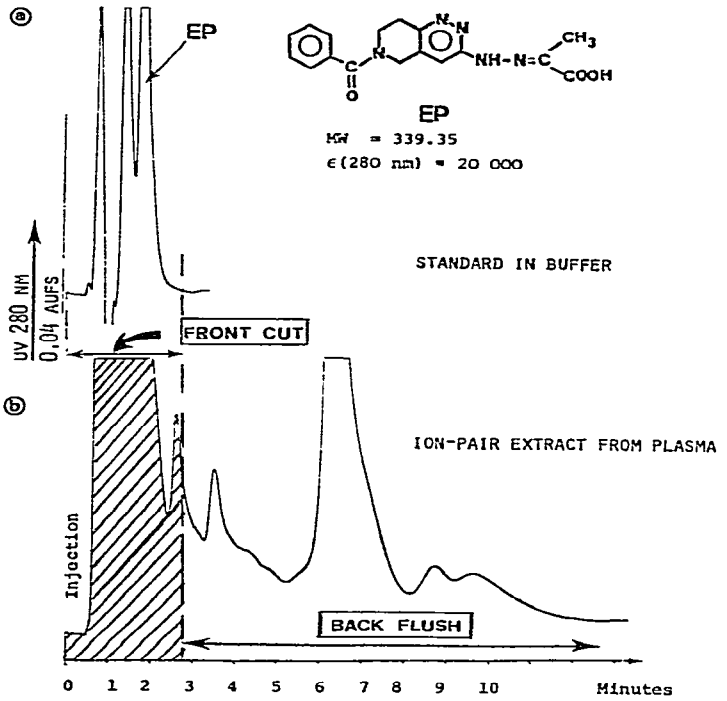

Fig. 4. Analysis of plasma samples: front cut on the first column C_1. (HPLC equipment and chromatographic conditions as in Fig. 5.) Sample (a): 200 ng of pyruvate of Endralazine (EP) dissolved in 150 μl of 0.01 M KH_2PO_4 pH 7.4. Sample (b): extract of 200 ng of EP from 1 ml of heparinised human plasma. Extraction procedure: add 1 ml of plasma to 0.1 M tetrabutylammonium chloride, pH 7.0; extract EP as ion-pair into 10 ml of chloroform–propan-2-ol and evaporate the organic layer to dryness at 40°C under reduced pressure. The residue is reconstituted in 0.01 M KH_2PO_4 pH 7.4 (150 μl). These chromatograms on column C_1 are obtained with the switching valve 1 ON and switching valve 2 OFF (see Figs. 1 and 2).

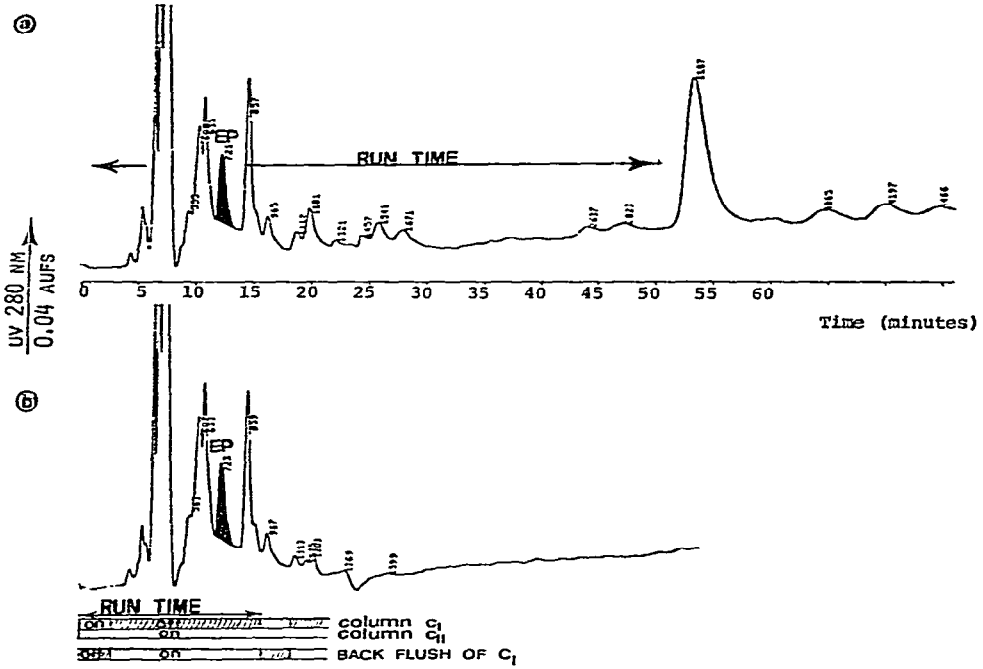

Fig. 5. Analysis of plasma samples: (a) without column switching (switching valves 1 and 2 ON), (b) with column switching (front-cut on column C_I of 165 sec, see Fig. 5). HPLC equipment: pumps (2), Model SP-740 (Spectra-Physics, Santa Clara, CA, U.S.A.); automated sample injector valve, type 7010 (Rheodyne) filled with a 130-μl sample loop; UV detector, Model LC3 (Pye-Unicam, Cambridge, Great Britain); chromatography data system, Model SP-4000 (Spectra-Physics); Valco air-actuated switching-valves type ACV-6-UHPa-N60 (Valco Instruments, Houston, TX, U.S.A.). Column C_I: HPLC-Guard RP-8, 10 μm, 30 × 4.6 mm I.D. (Brownlee Labs., Santa Clara, CA, U.S.A.). Column C_{II}: LiChrosorb RP-8, 5 μm, 250 × 4.6 mm I.D. Mobile phase: 0.01 *M* tetrabutylammonium chloride (pH 7.0)–acetonitrile (75:25), flow-rate 0.75 ml/min. (See Fig. 4 for details of sample preparation.)

at pH 8 (see Fig. 6 for details) and injected on to a first column containing LiChrosorb RP-2 and eluted with an almost aqueous phase containing 0–1.5% acetonitrile (Fig. 6). By means of a heart-cut, the fraction containing the compound of interest is transferred on to the second column, filled with LiChrosorb RP-8, and conditioned with the same mobile phase. The strongly retained compound EP on the top of the second column is then eluted with a stronger mobile phase after a very steep gradient step (Fig. 7 and Table I). During the elution of the second column the first column is flushed with a strong mobile phase using an auxiliary pump. At the end of the run both columns are reconditioned with the initial mobile phase. The system proved to be very stable for several weeks, giving a constant retention time of 1682 sec $\pm$1.06 R.S.D. (n = 20 days). The method has a reproducibility for peak areas of better than 5% R.S.D. (n = 6) for a sample concentration of 250 ng/ml. The calibration curve sample concentration range 0–2500 ng/ml gave a correlation coefficient of r = 0.999. With 500-μl injection volumes the limit of detection is *ca.* 20 ng/ml for 1:3 diluted urine, corresponding to *ca.* 150 ng/ml urine.

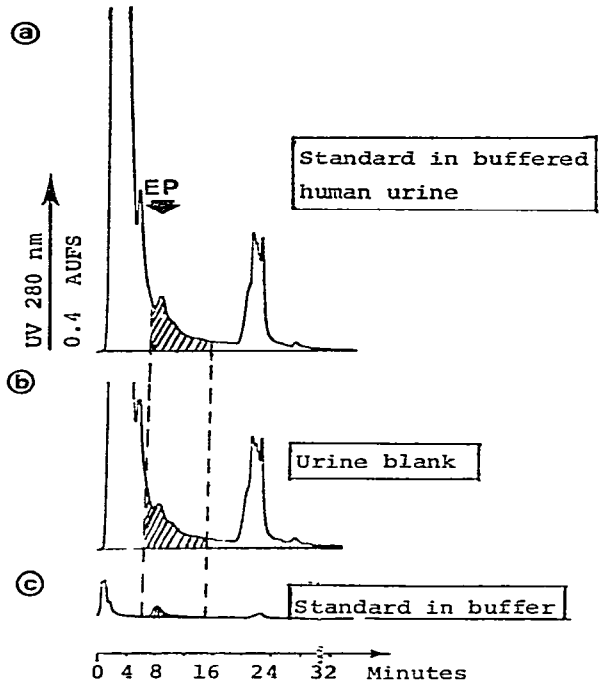

Fig. 6. Analysis of urine samples: heart-cut on the first column C_1. Mobile phase A with a flow-rate of 1.5 ml was used (see Fig. 7). Sample preparation: urine (3 ml) is buffered with 0.2 *M* Na_2HPO_4 pH 8.0 (5 ml) and stabilised with 0.5 *M* Na_2EDTA (0.5 ml) to prevent metal-catalysed oxidation. The pH of the mixture is adjusted to 8.0 by dropwise addition of 1 *N* NaOH, and the volume made up to 10.0 ml with water. Aliquots of 500 μl of this mixture are injected.

DISCUSSION

The three examples show that column switching is an excellent technique for the analysis of complex mixtures. The sample concentration on the first column and the heart-cut to a second independent separation system has proved to be a very efficient sample preparation procedure for medicated feed and urine samples. Preliminary experiments also show that blood plasma samples can be analysed in a similar way to urine samples. The total analysis times are very short and the reproducibilities and recoveries are excellent. The analytical procedure is very mild and therefore well suited to the analysis of sensitive compounds. Because the sample handling is simple and operation automated, accurate determinations at very low concentrations are possible. The two-dimensional HPLC approach minimises interferences and enhances significantly the selectivity. The clean-up effect depends on the quality of separation and efficiency of the first column. The optimisation of the chromatographic conditions, especially for the first column, is very important and demands a careful adjustment of the mobile-phase composition in those cases where large volumes of sample are injected. The on-line concentration between the first and the second column ensures good starting conditions for the separation in the second column.

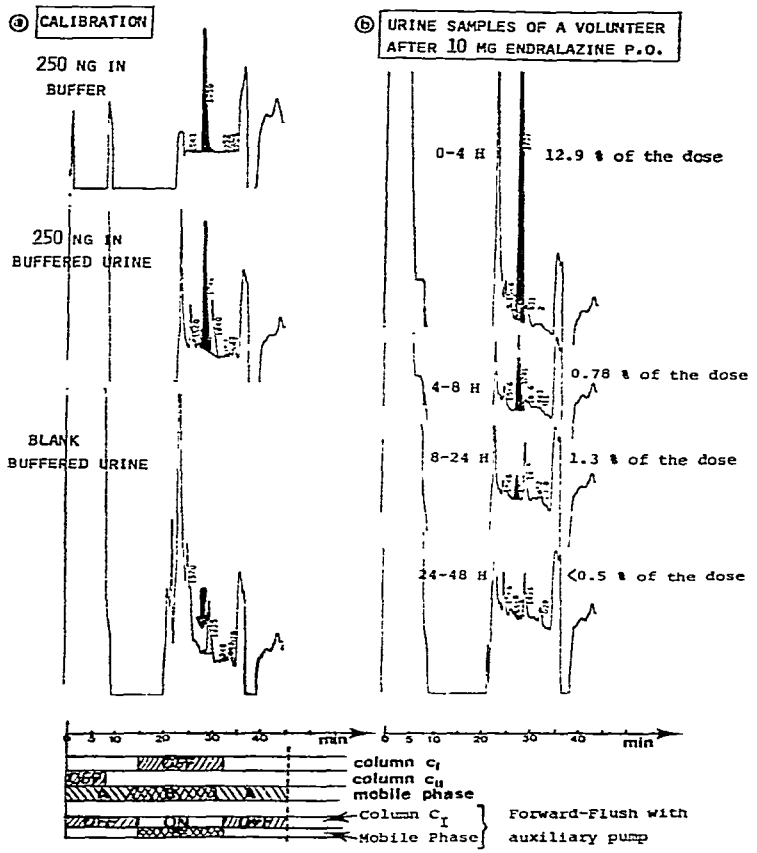

Fig. 7. Analysis of urine samples using column switching and a gradient system. (a) Calibration chromatograms; (b) urine samples of a volunteer who received orally 10 mg of Endralazine. (See Fig. 6 for sample preparation.) HPLC equipment: pumps (2), Model AX-110 (Altex Scientific); gradient controller, Model AX-420 (Altex); sample injector, Model WISP 710A (Waters Assoc., Milford, MA, U.S.A.); switching valves (2), type ACV-6-UHPa-N60 (Valco Instruments); chromatography data system, Model SP-4000 (Spectra-Physics); UV detector, Model LC-55 (Perkin-Elmer). Column C_I: LiChrosorb RP-2, 10 μm, 60 × 4.6 mm I.D. Column C_{II}: LiChrosorb RP-8, 5 μm, 250 × 4.6 mm I.D. Mobile phase A: 0.005 *M* NaH_2PO_4 (pH 5.4)–0.005 *M* Na_2EDTA–acetonitrile (985:3:15). Mobile phase B: water–0.005 *M* Na_2EDTA–H_3PO_4 conc.–acetonitrile (800:2:0.200:200). Injection volume: 500 μl. See Table I for the experimental timing.

The instrumental set-up with two switching valves has proved very efficient for the method development. Routine application of these column-switching techniques showed that the column can be used for several hundred biological samples, indicating that the first column is effectively flushed and regenerated, and that the second column is well protected from column plugging and decomposition when complex samples are injected.

TABLE I

TIMING OF THE AUTOMATED ANALYSIS OF URINE SAMPLES

(1) Programme of the autosampler WISP; (2) programme of the time file of the chromatographic data-system SP-4000; (3) programme of the gradient system.

Time (min)	*Programmation*	*Event*
0–0.08	(1) → (2) → (3)	injection, run start
	(3) flow-rate 1.5 ml/min	
	(3) 0% B	sample clean-up on C_I with mobile phase A
	(2) column C_{II} off	
7.50	(3) flow-rate 0 ml/min	
8.20	(2) column C_{II} on	
8.50–9.50	(3) flow-rate 0–1.2 ml/min linear	transfer of cut from C_I to C_{II} with
14.50	(3) flow-rate 0 ml/min	mobile phase A
14.70	(2) column C_I off	
15.00–16.00	(3) flow-rate 0–1.2 ml/min linear	separation and quantitation
25.00	(2) start integration	on C_{II}
31.67	(2) stop integration	flush of C_I with mobile phase B with
32.00	(3) flow-rate 0 ml/min	auxiliary pump
	(3) 0% B	
32.20	(2) column C_I on	
32.50	(3) flow-rate 1.2 ml/min	reconditioning of C_I and C_{II}
40.00	(1) start injection	
45.20	(2) column C_{II} off	
45.33	(2) print report	
46.00		start new cycle

REFERENCES

1 *Symposium on Drug Analysis in Biological Material — Principle Aspects, Stockholm, November 1–3, 1978.*
2 *Symposium on Automation in HPLC, Basle (Switzerland), January 16–17, 1979*, papers published in *Chromatographia*, 12(June 1979).
3 F. Erni, K. Krummen and A. Pellet, *Chromatographia*, 12 (1979) 399.
4 F. Erni and R. W. Frei, *J. Chromatogr.*, 130 (1977) 169.
5 P. Schauwecker, R. W. Frei and F. Erni, *J. Chromatogr.*, 136 (1977) 63.
6 K. Krummen and R. W. Frei, *J. Chromatogr.*, 132 (1977) 429.
7 M. Martin, G. Blu, C. Eon and G. Guichon, *J. Chromatogr. Sci.*, 12 (1974) 438.
8 C. N. Kenyon, A. Melera and F. Erni, *J. Chromatogr. Sci.*, 18 (1980) 103.
9 F. Erni and A. Melera, submitted for publication.
10 R. W. Frei and A. H. M. T. Scholten, *J. Chromatogr. Sci.*, 17 (1979) 152.
11 F. Erni and R. W. Frei, *J. Chromatogr.*, 149 (1978) 561.
12 E. L. Johnson, R. Gloor and R. E. Majors, *J. Chromatogr.*, 149 (1978) 571.
13 A. Wehrli, *Chimia*, 28 (1974) 690.
14 J. F. K. Huber and F. Eisenbeiss, *J. Chromatogr.*, 149 (1978) 127.
15 J. F. K. Huber and R. Vodenik, *J. Chromatogr.*, 122 (1976) 331.
16 H. Hulpke and U. Werthmann, *Chromatographia*, 12 (1979) 390.
17 F. W. Willmott, I. Mackenzie and R. J. Dolphin, *J. Chromatogr.*, 167 (1978) 31.
18 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979, p. 694.
19 G. C. Davis and P. T. Kissinger, *Anal. Chem.*, 51 (1979) 1960.
20 D. D. Koch and P. T. Kissinger, *Anal. Chem.*, 52 (1980) 27.
21 J. C. Gfeller and M. Stockmeyer, *J. Chromatogr.*, 198 (1980) 162.

22 W. Bertsch, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 1 (1978) 85.
23 R. J. Miller, S. D. Stearus and R. R. Freemann, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 55.
24 F. Erni and H. Bosshard, *Chromatographia*, 12 (1979) 365.
25 R. Salzmann, H. Bürki, O. Chu, B. Clark, P. Marbach, R. Markstein and H. Reinert, *Arzneim.-Forsch.*, 29 (1979) 1843.