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Fully-automated assay by liquid chromatography for routine drug monitoring in body fluids — method development with biological samples*

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Abstract: A fully-automated liquid chromatograph based on alternated pre-column enrichment technique has been developed for routine drug monitoring of body fluids using a column switching technique. Advantages of the method include the facility of direct injection of the body fluid onto the chromatograph and the simultaneous detection of both polar and non-polar metabolites under isocratic or gradient elution conditions.

Keywords: *Fully-automated LC; direct injection of body fluids; pre-column clean-up and enrichment; biological samples.*

Introduction

The aim of industrial biopharmaceutical research is to develop active drugs. One of the main objectives during drug development is to establish the drug- or metabolite-related pharmacological and toxicological profile as an aid to understanding the preclinical animal data; these findings are then transformed and compared with those in man.

Recent developments in capillary gas chromatography (GC), and especially in liquid chromatography (LC) with its wide variety of detection systems, column packing materials and solvent systems, have provided potential improvements in techniques for bioanalytical assays [1, 2]. In particular, HPLC may be automated to various degrees [3–7]. The present paper describes the development and potential value of fully-automated LC for routine drug monitoring in body fluids using a column-switching technique [8, 9].

General Considerations

Sample clean-up for HPLC using column switching

The development of a bioanalytical assay to detect compounds in body fluids usually comprises several stages such as: sample pretreatment, concentration of the analyte,

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application of the derivatization reaction, injection into and separation on an appropriate column, detection, quantitation and data handling.

The natural constituents of the plasma matrix, including fatty acids, lipids, proteins, salts, sugars and potentially the whole endogenous pool of compounds and metabolites, may cause several problems during method development and therefore demand selective steps in pretreatment of the sample.

The conventional methods for sample pretreatment comprise various steps that include: withdrawal of blood, preparing the plasma, adding buffer, adding an internal or external standard, adding an organic solvent, extracting into the organic phase, phase separation, concentration of the sample, reconstitution into an appropriate solvent, and injection into the liquid chromatograph.

In general, the selective separation and concentration of compounds from a biological matrix can be achieved by using procedures such as liquid/liquid extraction, liquid/solid extraction, centrifugation, precipitation and derivatization.

In the current work, interest was focused on liquid/solid extraction because this procedure seemed to be the most convenient to implement in an automated LC system.

Nature of analytes in biochemical samples

Pharmaceutical research and clinical pharmacology generally involves the analysis of non-polar, highly lipophilic xenobiotics and their polar, hydrophilic metabolites. A basic requirement for an automated LC assay is therefore its capability to detect mixtures of polar and non-polar lipophilic xenobiotics simultaneously; in particular conjugates should be quantified in the presence of their aglycones.

Column-switching Techniques

Alternating pre-column switching with simultaneous clean-up and sample enrichment techniques using valve-switching methods have been employed for several experimental purposes such as sample clean-up, trace enrichment, sample fractionation, multicolumn chromatography and derivatization procedures [10]. The application of valve switching also provides a good approach for sample enrichment as part of a fully automated LC-system [8]. The simplest approach is shown in Fig. 1.

Two valves and two columns are mounted to enable custom-made on-column pretreatment to be performed. A sample of injected body fluid is flushed through the injector loop of valve 1 (V1) at the top of the pre-column. The lipophilic compound of interest is adsorbed onto the lipophilic matrix of the reversed-phase material, whereas the polar components of the plasma, such as salts and proteins, are flushed through. When valve 2 (V2) is switched in, solvent system B is then connected to the pre-column; the eluent then flushes the enrichment material from the top of the pre-column to the analytical column. This analytical column should be protected by means of a guard column in order to increase its life. In order to clear the enrichment column from the organic solvent of solvent system B, valve 2 is switched back to the initial position; this operation allows the equilibrium of solvent system A to be re-established on the pre-column.

The technique described can be optimized with respect to time if two pre-enrichment columns are used so that they operate in an alternating mode, as described elsewhere [8, 11].

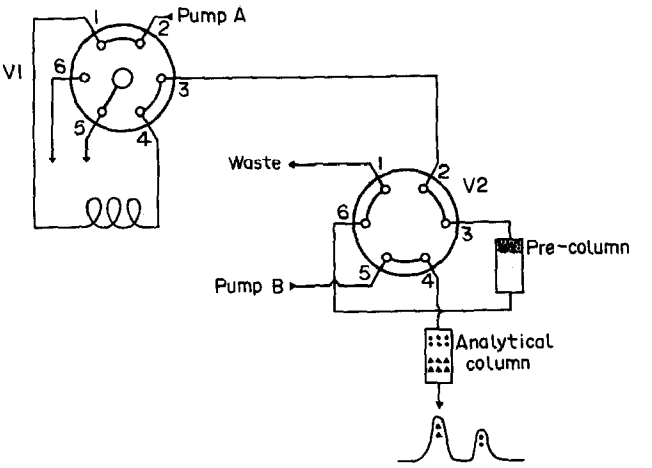

Figure 1
Pre-column sample enrichment using one pre-column for clean-up and enrichment, together with one analytical column.

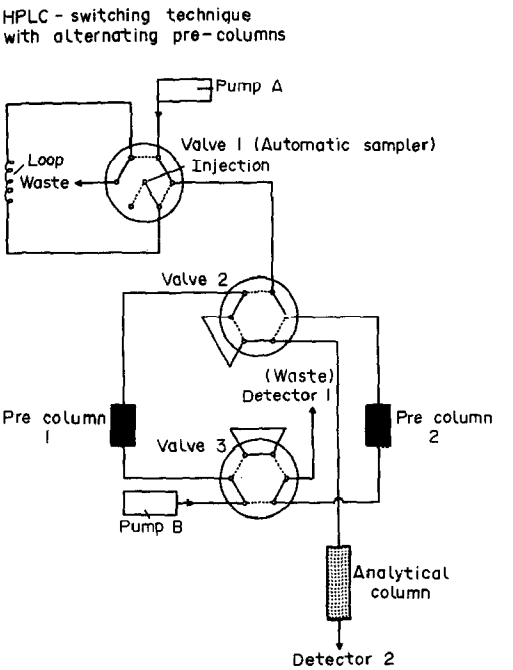

Figure 2
Alternating pre-column sample enrichment using automated alternating pre-column switching technique [8].

This arrangement, in which alternating pre-column sample enrichment is carried out with one analytical column, has to be modified when gradient elution is required.

However, a small modification in the system can be made by adding a second analytical column, connected appropriately to the existing pre-column switching system; this modification permits a simple and convenient approach to the use of fully-automated gradient LC with alternating pre-column enrichment (Fig. 3).

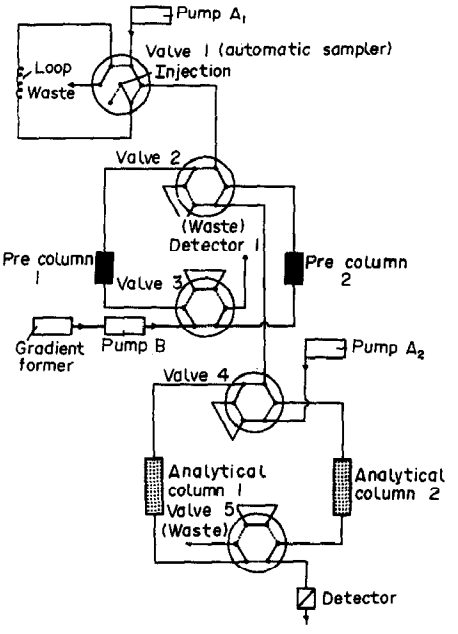

Figure 3
Column-switching systems for fully-automated gradient LC using two pre-columns and two analytical columns.

Commercially available reversed-phase materials with particle sizes in the range of 30–50 μm are best suited for packing pre-enrichment columns. Some of these materials have been examined to determine their performance in pre-concentrating a typical mixture of metabolites with different polarities. Native rat bile, consisting of a mixture of ^{14}C-labelled positive inotropic agent Pimobendane® (UD-CG 115 BS) [4,5-dihydro-6-[2-(4-methoxyphenyl)-1H-benzimidazo-5-yl]-5-methyl-3(2H)pyridazonine], its O-desmethylated metabolite UD-CG 212 BS, and polar conjugates including UD-CG 212 O-sulphate were directly injected onto the pre-enrichment column filled with various reversed-phase materials (Fig. 4), and separated on a Hypersil ODS 5-μm column (12.5 cm × 0.46 cm i.d.), with a flow rate of 1.8 ml/min under gradient elution conditions. The eluents were solvent A (methanol–water (35:65, v/v), containing 2 g ammonium acetate/l) and solvent B (methanol–water (90:10, v/v) containing 2 g ammonium acetate/l). The conditions were: initially, 100% A, 0% B; t = 3 min, 70% A, 30% B; t = 6 min, 40% A, 60% B; and t = g min, 100% A for 3 min.

In all cases the pre-column was flushed with water and with a 1% m/v ammonium acetate solution in water. Water was purified with an ion-exchange system from Waters (Milli-Q-Purification System). The washing phase from the pre-column was collected immediately after passage through the enrichment column; any radioactivity not bound to the reversed-phase matrix was determined by liquid scintillation counting (Fig. 4, Table 1).

The results revealed that under the present experimental conditions, different reversed-phase materials possess different affinities for the injected compounds. Ammonium acetate significantly increased the enrichment capacity of the pre-column. Amberlite XAD-2, which has been reported to permit the concentration of drugs [12] as efficiently as do the more porous reversed-phase materials, showed the highest affinity in the present investigation although the influence of XAD-2 due to band broadening is limiting its practical application as filling material for pre-columns.

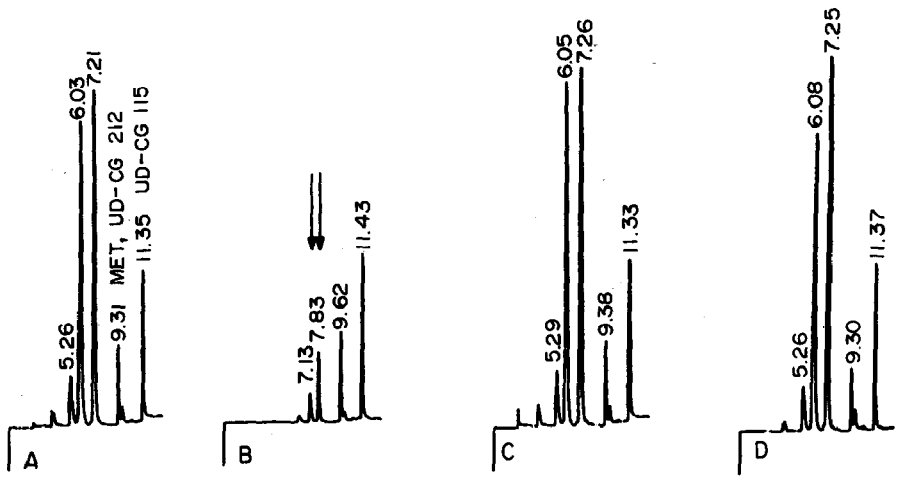

Figure 4
Liquid chromatograms (gradient elution) of native rat bile after intraduodenal application of ^{14}C-UD-CG 115 BS, samples (10 µl) were injected directly onto an analytical column (A), and identical samples separated after pre-column enrichment using different washing eluents and washing times: B, water, 3 min; C, 1% m/v ammonium acetate, 3 min; D, 1% m/v ammonium acetate, 25 min; flow rate 1.5 ml/min. Fluorescence detection: λ_{ex} = 332 nm, λ_{em} = 405 nm.

Table 1
Reversed-phase materials as pre-column enrichment packings, tested with native ^{14}C-labelled metabolites of the drug UD-CG 115 BS

Reversed phase material	Manufacturer	Particle size (µm)	Shape	% Radioactivity* eluent: (water)	% Radioactivity eluent: (water + 1% ammonium acetate
1 Bondapac Corasil (C_{18})	Waters	37–50	Pellicular	67.0	0
2 Phenyl Corasil	Waters	37–50	Pellicular	77.0	0
3 Amberlite XAD2	Serva	50–100	Porous resin	3.7	0
4 VYDAC 201 RP	Macherey & Nagel	30–50	Pellicular	70.0	0
5 Perisorb. RP	Merck	30–40	Pellicular	90.0	3
6 LiChrosorb. Diol	Merck	10	Porous	91.0	73
7 LiChroprep. RP 8	Merck	5–20	Porous	22.0	0
8 LiChroprep. RP 18	Merck	25–40	Porous	1.0	0
9 Sep-Pak C_{18}	Waters	55–105	Porous	0.0	0
10 Permacoat (C_{12})	Permacoat Products	25–40	Porous	1.0	0

* Injected radioactivity: 4500 dpm (10 µl).
The table records the percentage radioactivity not bound to the reversed-phase material after flushing for 3 min (flow rate 1.5 ml/min) with water, in comparison with 1% m/v ammonium acetate solution (injected samples: native rat bile, cf. Fig. 4A).

Selection of eluent for the pre-column washing procedure

One of the main problems with a biological sample that contains metabolites of differing polarity and lipophilicity is the choice of an appropriate solvent system so that the various drugs are retained on the pre-enrichment columns, while the bulk of unwanted plasma constituents are flushed through.

After oral administration of oxazepam, dog urine was directly injected onto an analytical column; the chromatogram was compared with those obtained after use of the column-switching method. For these experiments, oxazepam and metabolites were separated on Hypersil ODS 5 μm (12.5 cm × 0.46 cm i.d.) with a flow rate of 2.0 ml/min under gradient elution conditions. The gradient was started with 100% A (A: acetonitrile–water (10:90, v/v) containing 2 g ammonium acetate/l) and 0% B (B: acetonitrile–water (70:30, v/v) containing ammonium acetate/l) for 5 min; at t = 5 min, 70% A and 30%; t = 10 min, 25% A and 75% B; t = 14 min, 100% B and t = 16 min, 100% A for 2 min. Detection was carried out at 254 nm.

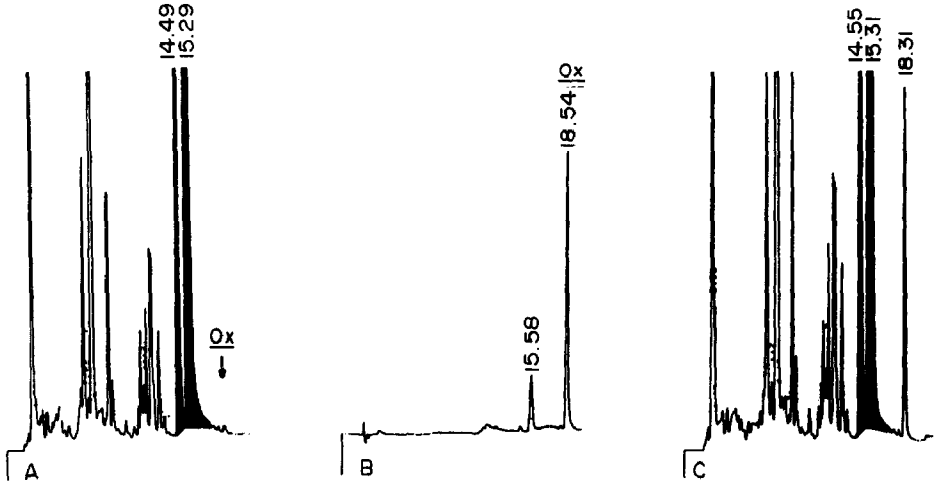

Figure 5
Liquid chromatograms (with gradient elution) after injection of 10 μl native dog urine following oral administration of 25 mg/kg of oxazepam (ox). A. Direct injection onto the analytical column (ODS-Hypersil, 5 μm, 12 cm × 0.46 cm i.d.) and after concentration on pre-enrichment columns (2 cm × 0.46 cm i.d. dry packed with SEP PAK C_{18}), washed with different mobile phases: B, water; C, 1% ammonium acetate; flow-rate, 1.5 ml/min. B + C was spiked with 1 μg of oxazepam.

Chromatogram A (Fig. 5) shows a variety of UV-absorbing peaks and oxazepam metabolites but no parent compound. After applying the pre-column washing procedures, nearly all polar compounds were eluted from the pre-column except oxazepam itself, which was added to the sample as a marker compound. However, after replacing water as the washing eluent by a 1% m/v ammonium acetate solution, all UV-absorbing compounds were retained on the pre-columns under identical pH-conditions (pH 6.5).

Column life

It is advisable to protect the analytical column by an additional guard column in order to increase the life of the main column. The guard column may be changed after 200–400 injections if 10–50 μl of plasma is injected. The pre-enrichment columns can be used for up to 1000–2000 injections if 10–50 μl of plasma is injected. Problems arising from

increased back pressure may occur if plasma samples show very viscous properties. In that case, dilution with water or any physiological buffer and additional centrifugation at 3000 rpm will change the consistency of the biological sample.

Internal standard

With the fully-automated HPLC system with pre-column sample enrichment, no additional standard compound is necessary. Plasma samples, spiked with the analyte, may be used simultaneously as an external or instrumental standard. This is especially advantageous in fluorescence spectroscopy, where it is often difficult to find an appropriate standard compound.

Applications

The current literature encompasses many applications in column switching and column enrichment technology. In the field of drug monitoring, fully-automated LC has been applied to diuretics [13], analgesics [14, 15], a tranquillizer (W. Roth, Dr., K. Thomae GmbH, Internal report 1982), vasodilators [8], positive inotropic agents [11], antiepileptics [16], immunosuppressants [17, 18] and nucleosides [19]. These examples confirm the quality, precision and accuracy of the automated LC system.

Conclusions

The automated LC equipment with alternating pre-column sample enrichment, which is extensively described elsewhere [8, 11], can be built up by the use of commercially available LC modules such as pumps, detectors, integrators and switching valves; it is therefore easily accessible to every LC user. Other advantages include improved accuracy and simultaneous detection of polar and non-polar metabolites. Body fluids can be injected directly onto the chromatograph for drug monitoring. The need for classical pretreatment of the sample, for manual handling steps and for an internal standard is obviated. The method is simple and because of the high degree of automation its transfer to other laboratories is facilitated.

Applications of this novel HPLC technique include drug development, clinical drug monitoring, pharmacokinetics, metabolism experiments, quality control, pharmaceutics research, forensic science and pollution analysis.

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