Journal of Pharmaceutical & Biomedical Analysis
Vol. 4, No. 2, pp. 181–189, 1986
Printed in Great Britain

0731–7085/86 $3.00 + 0.00
Pergamon Journals Ltd.

Coupled columns in bioanalytical work

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Abstract: The bioanalytical laboratory giving a service in the development of new drugs has to provide flexibility as well as routinely assay a large number of samples, preferably with automated procedures. Liquid chromatography with coupled columns can be most useful for this purpose, as exemplified in the present paper, where a coupled-column configuration, which has been used for automation, screening and method validation, is described.

Keywords: *Coupled columns; liquid chromatography; bioanalysis.*

Introduction

The bioanalytical laboratory involved as a service function in the development of new drugs has to cope with many different analytical problems. Methods have to be developed, starting with the first exploratory work on research animals, through to bioanalytical studies for final registration. Different research laboratories have to be serviced during the course of this work as indicated in Fig. 1.

The bioanalytical laboratory has thus to be flexible and also able to assay routinely a large number of samples, preferably with automated procedures. In the author's laboratory, liquid chromatography (LC) with coupled columns can be most useful in bioanalytical work, providing flexibility as well as the possibility for automated analysis. In the present paper, this is exemplified with a coupled column system which has been used for (a) automation, (b) screening and (c) method validation, as briefly summarized below.

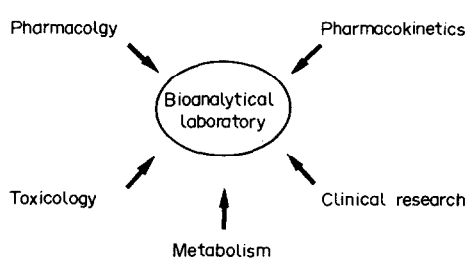

Figure 1
The bioanalytical laboratory working with development of drugs provides analytical services for several research laboratories.

* Presented at the 'Symposium on Liquid Chromatography in the Biomedical Sciences', June 1984, Ronneby, Sweden.

(a) *Automation*

Enprofylline (3-propylxanthine) is a novel xanthine derivative with antiasthmatic properties and subject to clinical trials. In early investigations, automated LC with direct injection was used for quantitative analysis of enprofylline in urine samples. However, selectivity problems made it necessary to find a more definite solution which could also be run automatically on a 24-h basis.

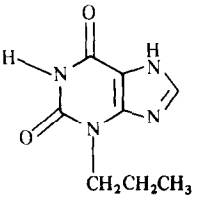

Figure 2
Structural formula of enprofylline.

(b) *Screening*

It was necessary to measure the excretion of the unchanged drug for a number of xanthines in rat urine. Therefore there was a demand for a flexible, selective and labour-saving analytical procedure.

(c) *Method validation*

Bambuterol ((5-[2-*tert*-butylamino)-1-hydroxyethyl]-m-phenylene-bis(dimethyl-carbamate)) (Fig. 3) is a prodrug that has been developed to produce prolonged therapeutic effect and to reduce side-effects of terbutaline, the active principle of Bricanyl®. For pharmacokinetic evaluation in man it was necessary to determine plasma concentrations of terbutaline. To study the accuracy of the results obtained by a GC–MS procedure [1], a highly selective analytical procedure was needed. For this purpose, a method was used based on the LC-system described earlier, with coupled columns and electrochemical detection [2] for the determination of terbutaline in human plasma.

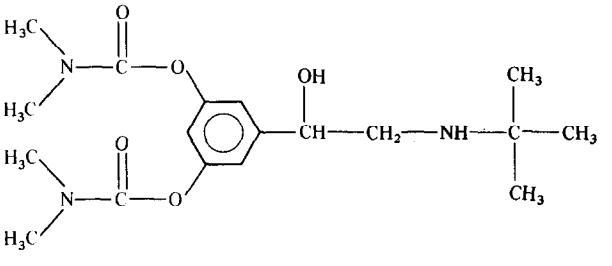

Figure 3
Structural formula of bambuterol.

Experimental

Coupled-column configuration

Figure 4 illustrates the coupled-column system used. Samples are injected with an Auto Sampler and transferred by mobile phase 1 to column 1 where analytes are preseparated. By switching the valve under microprocessor control, part of the eluate (4–7 ml) from this column containing the analyte and an internal standard is trapped on column 2. This column also serves to compress the band in order to reduce peak broadening effects. As large particles (see Table 1) are used in column 2, pressure fluctuations on valve switching are negligible. The rest of the eluate from column 1 is

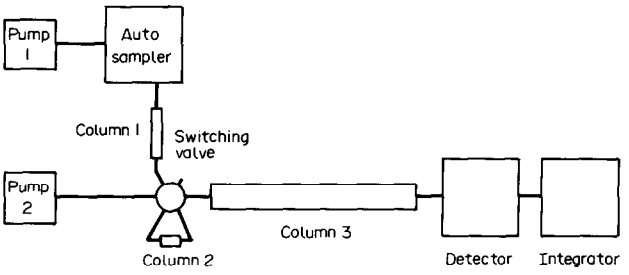

Figure 4
Schematic diagram of the coupled-column system used.

directed to waste. The sample is then backflushed from column 2 to column 3 by means of mobile phase 2. The final separation is performed on column 3.

The fraction of the eluate to be trapped on column 2 is determined by connecting column 1 to the detector and injecting pure standards of the analyte and the internal standard. For detection either a UV or an amperometric detector was used. Table 1 summarizes all columns and mobile phases used in the applications described in this paper.

Assay procedure

To 100 μl of urine was added 100 μl of an internal standard solution and the sample was diluted ten times with buffer. A 100 μl sample was used for injection. For calibration, standard and internal standard were added to blank urine.

Equipment

The pumps used were the Waters Model M 45 or M 6000 A solvent delivery systems (Milford, MA, USA); the flow-rate was 1–2 ml min^{-1}. The detectors used were a Waters M 440 UV detector (λ = 280 nm) and an amperometric detector comprising the following parts: a LC-4B potentiostat with a TL-5 glassy carbon electrode and a Ag/AgCl (3M NaCl) reference electrode, all from Bioanalytical Systems. The working potential was +0.90V. A Waters model 710 B Auto Sampler was employed, the injection volume being 100–200 μl. The Hewlett–Packard 3388 A integrator was used for valve switching, for recording chromatograms and for quantitation. The valve used was a Valco model CV-6-UHPa-N60, equipped with either a pneumatic actuator or motordriven.

Results

(a) *Automated quantitative analysis of enprofylline in human urine*

Enprofylline (E) is a novel type of antiasthmatic drug which has been selected from careful structure–activity studies on a great number of xanthine derivatives.

Given orally or intravenously to man, about 90% is recovered unchanged in the urine. The high concentrations (1–500 μg ml^{-1}) found in urine together with its high molar absorptivity ($\epsilon_{280} \sim 10^4$ $M^{-1} \cdot cm^{-1}$) made it possible to analyse enprofylline with an automated LC system after direct injection of the sample [L.-E. Edholm, L. Heintz and L. Ögren, unpublished results].

However, this approach was not sufficient for further work as selectivity problems soon became apparent. By using the above coupled-column system (cf Fig. 4 and Table 1), direct injection was still possible with full automation.

Table 1
Columns and mobile phases used for quantitative analysis of enprofylline, other xanthines and bambuterol in urine and plasma

Substance	Column 1	Column 2	Column 3	Mobile phase 1	Mobile phase 2
Enprofylline and other xanthines	50 × 50 mm Nucleosil 10 SA	Waters guard column, Sep Pak C_{18} (23 × 3.9 mm)	200 × 5 mm 5 μm Nucleosil C_{18},	Sodium acetate buffer (0.06 M) pH 4.6–4.7	Methanol phosphoric acid buffer 1 g orthophosphoric acid/litre, pH ~ 2.5*
Bambuterol	150 × 4.6 mm 7-μm Nucleosil $7C_6H_5$	ditto	200 × 4.6 mm Nucleosil 10 SA	ditto	Acetonitrile–sodium acetate–citric acid buffer, pH ~ 6.0

*See text for xanthines.
All columns were packed in stainless steel tubes with a polished inner surface.

Analytical characteristics. Figure 5a shows a chromatogram obtained after direct injection of urine onto column 3. For comparison chromatograms 5b and 5c show what is obtained after direct injection of urine using the coupled-column system.

Table 2 summarizes data on precision that were obtained on spiked urine samples. The system has been running for several years in the author's laboratory on a 24-h basis without any interference problems due to other compounds.

Control of retention and selectivity. Detailed descriptions of the chromatographic behaviour for enprofylline as well as other xanthine derivatives will be given elsewhere (L.-E. Edholm, L. Heintz and L. Ögren, to be published; L.-E. Edholm and C. Bodenäs, to be published).

Due to the widespread consumption of caffeine (1,3,7-trimethylxanthine) in food and beverages, human urine frequently contains caffeine and its metabolites. As these are closely related to E, they are also a potential source of interference.

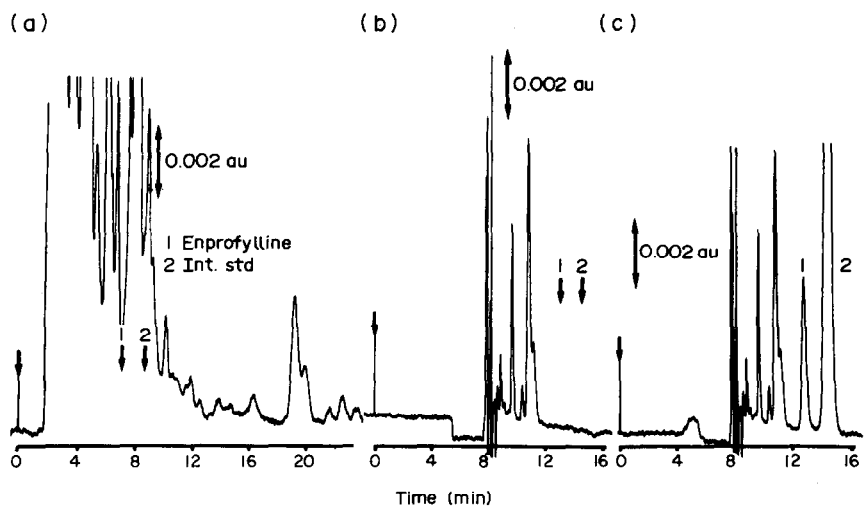

Figure 5
Analysis of urine samples after direct injection (see Fig. 4). *Without coupled columns* (a) Urine blank injected onto column 3. *With coupled columns* (b) Urine blank; (c) urine containing enprofylline 8 μg/ml (1) and internal standard (2).

Table 2
Data obtained for spiked urine samples*

		Relative standard deviation (%)		
	n	8 μg ml^{-1}	16 μg ml^{-1}	82 μg ml^{-1}
Within-assay	47	0.8		
Between-assay	7		0.9	1.7

* Absolute recovery ~ 100%.
Interassay calibration: $y = 0.0225\ (\pm 0.0005)\ x + 0.010\ (\pm 0.010)$; $n = 7$; correlation coefficient $r = 0.9999$.

As the pK_a of enprofylline is 8.2, and the pK_a values for many of the xanthines generated from caffeine metabolism are similar, pH has little effect on retention and selectivity for these substances in the pH region most frequently used in reversed-phase chromatography.

However, it has been shown that it is possible to separate caffeine and all of its known metabolites in man on column 3 (L.-E. Edholm, L. Heintz and L. Ögren, to be published). However, batch-to-batch variations in the packing material have made it necessary to carefully test every new batch. On the ion-exchange column, sodium acetate buffer pH 4.6 is used as mobile phase. Retention of enprofylline and the internal standard is thought to be predominantly dependent on hydrophobic interactions with the stationary phase. In this system pH also has little effect on the retention of the xanthines. The background interference, however, is dependent on pH (L.-E. Edholm and C. Bodenäs, in preparation).

It was found that a pH of 4.6 was optimal. Figures 6a and b show chromatograms obtained with the ion-exchange column connected directly to the UV detector. Generally the ion exchange material was chosen so that enprofylline and the internal standard eluted as closely as possible, with sufficient separation from caffeine. The ion-exchange material also suffers from batch-to-batch variations, but this does not interfere with the overall selectivity. In fact, batch-to-batch variations on column 3 are of less importance as caffeine, for example, and a lot of other compounds are eliminated on column 1.

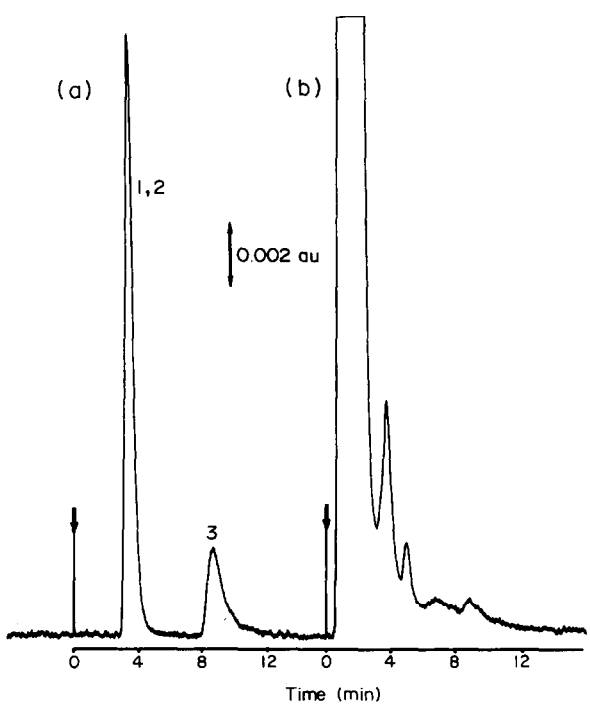

Figure 6
Chromatograms obtained with the ion-exchange column directly connected to the UV detector (see Fig. 4). (a) Standard mixture containing enprofylline (1), internal standard (2) and caffeine (3). (b) Urine blank.

(b) *Screening of xanthines*

The following example demonstrates how the coupled-column system could be used very efficiently in a bioanalytical situation, where a great deal of flexibility is needed and at the same time a lot of work could be saved.

Information was needed regarding the excretion in the unchanged form of several different new xanthine derivatives in rat urine. Preliminary studies showed that direct injection of samples on to a single column was not possible. Some kind of selective work-up step was thus necessary. The same set-up as used for enprofylline in urine could be used to solve this problem. Only slight modifications of the organic modifier in mobile phase 2 had to be made, because of the differing lipophilicities of the xanthines. In Figs 7a and b an example is given again showing the efficiency of the system.

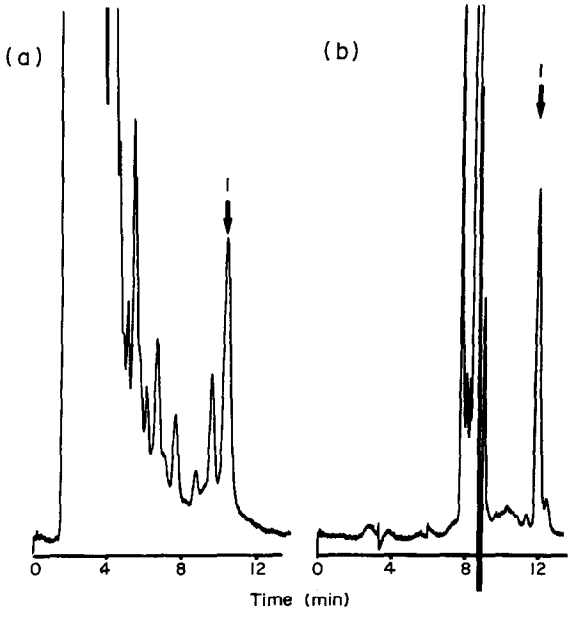

Figure 7
Chromatograms obtained after direct injection of rat urine containing a xanthine derivative. (a) Without coupled columns. Onto column 3. (b) With coupled columns.

(c) *Method validation*

The plasma concentrations of terbutaline were to be determined after administration of the prodrug bambuterol (B). For this purpose a method based on GC–MS [1] for determining terbutaline was originally chosen. It had previously been shown that this method and an LC method with coupled columns and electrochemical detection gave the same results on human plasma samples after terbutaline administration [2]. However, there were indications that the GC–MS procedure gave higher values on plasma samples after administration of B.

A comparative study on human plasma samples after administration of B to man was undertaken using the two methods. It was found that the GC–MS method in general gave higher results (cf Fig. 8). Investigations have shown that one possible explanation for the high values obtained by the GC–MS procedure is that hydroxylated metabolites

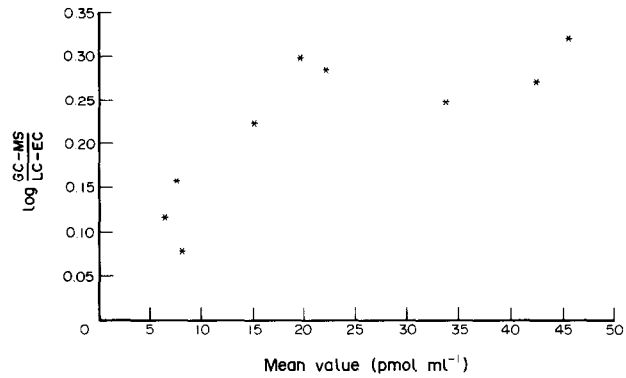

Figure 8
Comparison of GC–MS and LC–EC for the analysis of terbutaline in human plasma after administration of bambuterol.

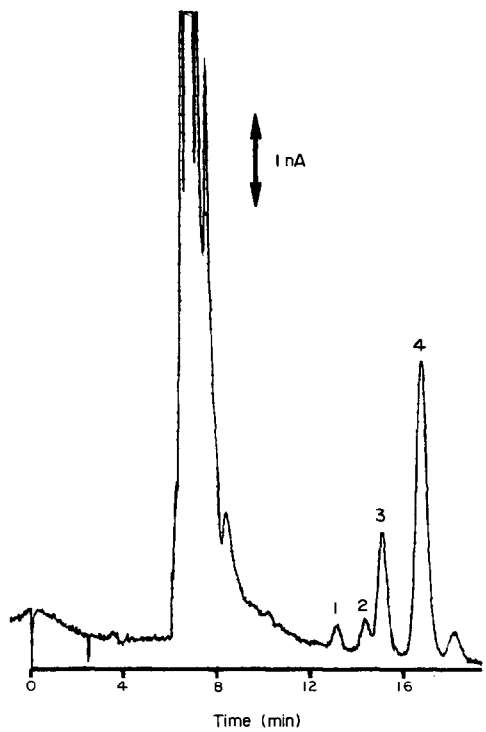

Figure 9
Chromatogram of a plasma sample after administration of bambuterol. Peaks 1 and 2 are of unknown identity, but are generally found in samples after administration of bambuterol. Peaks 3 and 4 are terbutaline (12 pmol ml^{-1}) and internal standard (50 pmol ml^{-1}), respectively.

of B are transformed into terbutaline in the derivatization step (A. Tunek and C. Lindberg, personal communication).

Selectivity of the LC-system

Figure 9 shows a chromatogram of a plasma sample using the coupled-column system after administration of bambuterol. Peaks designated 1 and 2 are of unknown identity, but are generally found in samples after administration of the drug.

By combining the trapping of analyte with two modes of chromatography (see Table 1 and Fig. 4), i.e. reversed-phase chromatography with ion-exchange chromatography and electrochemical detection, a highly selective system is obtained. This is the method of choice for quantitative analysis of terbutaline in human plasma after administration of bambuterol, and the method is in use for pharmacokinetic evaluation of the drug.

Conclusion and future work

The benefit of using liquid chromatography with coupled columns in bioanalytical work could be considerable, as exemplified in this paper. Many different configurations are possible [3] and the choice would be dependent upon the problem. Extreme selectivity could be obtained if different modes of chromatography were combined with selective detectors.

With the use of automated samplers and microprocessor controlled valves, automation is easily obtained. Work is now in progress in the author's laboratory to further evaluate the use of coupled columns in metabolism studies and as a means to increase sample throughput.

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[*Received for review 15 June 1985*]