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Journal of Chromatography A, 797 (1998) 203–209

JOURNAL OF CHROMATOGRAPHY A

Determination of drugs in biological fluids by high-performance liquid chromatography with on-line sample processing

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Abstract

An automated two column HPLC system with the new packing material LiChrospher RP-18 ADS (alkyl-diol-silica) was tested for the determination of several drugs and metabolites (talinolol, celiprolol, metoprolol, oxprenolol, triamterene, trimethoprim, tiracizine, articaine, detajmium, ajmaline, lamotrigine) in various biological fluids (serum, urine, intestinal aspirates, supernatants of cell cultures and supernatants after protein denaturation). The method allows the direct injection of biological fluids into a reversed-phase HPLC system and on-line clean-up and sample enrichment by a column-switching technique. Precision, accuracy and sensitivity were similar to conventional assays as described in the literature. With this new method it was possible to measure drug concentrations in various biological fluids without changing the sample preparation procedure. In some cases an additional sample preparation like protein denaturation or solid-phase extraction was advantageous to enhance the sensitivity of the method and the life-time of the ADS column.

Keywords: Sample handling; Column switching; Drugs

1. Introduction

The sample preparation for chromatographic analysis, whether by liquid–liquid or solid-phase extraction (SPE), is often the most time-consuming step in an analysis. Furthermore, there are additional disadvantages of these methods. Liquid–liquid extraction cannot be easily automated and often requires large quantities of high-purity solvents. Traditional SPE is extremely flexible, but automation of the technique requires expensive robotic devices and often large quantities of high-purity solvents. Usually macromolecular compounds (e.g. proteins) have to be removed from a sample prior to HPLC analysis, as they are precipitated by larger amounts of organic solvents in the mobile phase or non-specifically and irreversibly bound or denatured by residual silanol groups on the surface of the chromatographic support. The recent development of special column packing materials allows a direct and repetitive injection of untreated biofluids, i.e. an automatic LC-integrated sample preparation in bioanalysis. The sample is first fractionated into sample matrix and analytes by the use of a special extraction column. In the next step, the analyte fraction is transferred in a back-flush mode onto an analytical column where it is separated and quantified in a conventional manner [1–3].

The on-line liquid–solid extraction with the new bimodal restricted access material (RAM) is based on the complete non-adsorptive size-exclusion ($M_r >$ 15 000) of macromolecules and on the simultaneous extraction and enrichment of low-molecular weight

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0021-9673/98/$19.00
PII S0021-9673(97)00966-7

target compounds (such as drugs). LC-integrated sample clean-up of fluids using such materials like LiChrospher RP-18 ADS (alkyl-diol-silica) and coupled-column switching has some advantages: repeated direct injection of untreated fluids, quantitative removal of the matrix, on-column enrichment of analytes, quantitative, matrix-independent analyte recovery, no requirement for addition of an internal standard, total automation, safe handling of hazardous or infectious samples, high number of analysis cycles and low costs per analysis [4–8]. Unfortunately it is impossible to get all advantages in a single method. For example a higher injection volume implies a higher sensitivity but it means also a lower number of analyses and higher costs.

An automated two column HPLC system with the new packing material LiChrospher RP-18 ADS was tested for the determination of several drugs and metabolites (talinolol, celiprolol, metoprolol, triamterene, trimethoprim, tiracizine, articaine, detajmium, ajmaline, lamotrigine, oxprenolol) in various biological fluids (serum, urine, intestinal aspirates, supernatants of cell cultures and supernatants after protein denaturation).

2. Experimental

2.1. Chemicals

Talinolol, triamterene, tiracizine, detajmium and ajmaline were kindly provided by Arzneimittelwerk (Dresden, Germany). The other drugs were obtained from: lamotrigine: Welcome (Burgwedel, Germany), oxprenolol: Sigma (St. Louis, MO, USA), celiprolol: Hafslund Nycomed Pharma (Linz, Austria), trimethoprim: Berlin-Chemie (Germany), articaine: Hoechst Marion Roussel (Bad Soden, Germany). Acetonitrile LiChrosolv (for chromatography), methanol LiChrosolv (for chromatography), triethylamine, disodium hydrogenphosphate, and potassium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany). Pure water (20 MΩ) was obtained using an ion-exchange system RS 40 E, SG Ionenaustauscher (Barsbüttel, Germany).

2.2. Standard solutions

Stock solutions of drugs were prepared by dissolving the salts in methanol to a final concentration of 1 mg/ml. Working solutions were obtained by further dilution of the stock solutions with methanol or water.

2.3. Chromatographic system

The employed column-switching system was adopted from [2] and is illustrated in Fig. 1. The samples were automatically injected onto one of both extraction columns (ECs). The clean-up of the first sample, which was injected onto EC1 in position A of the valve, was performed with pump A. The EC1 was then washed with the clean-up solvent (EC eluent) and transferred onto the analytical HPLC column by switching the valve in position B (back-flush mode). The transfer step could only take place after complete elution of the protein matrix from the precolumn. Otherwise, the analytical column may become contaminated with protein, leading to an irreversible increase in back-pressure and a decrease of capacity and selectivity. Additionally it is possible, that residuals not removed of the matrix influence the chromatograms (peak height, retention time). The analytical chromatography was performed using pump B and analytical eluent (AC eluent). During the chromatography the EC2 was equilibrated with the clean-up solvent. The second sample was injected onto EC2. All subsequent analyses were performed alternating the whole procedure.

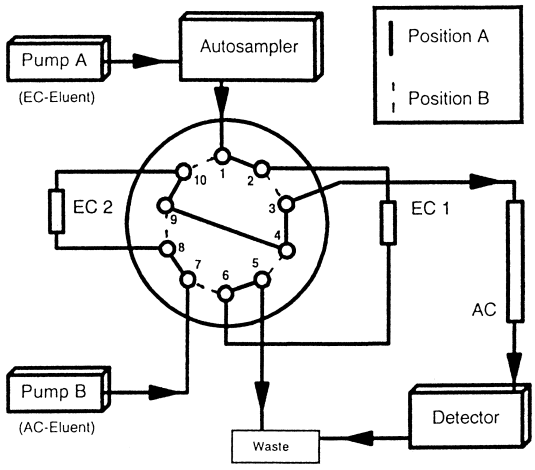

Fig. 1. Schematic flow diagram of the employed column-switching system

The equipment consisted of an autosampler Varian 9100 (Walnut Creek, USA) injecting different aliquots into a 500 μl-sample loop. The extraction-column eluent delivered by an HPLC pump LC 6A (Shimadzu, Kyoto, Japan) with a flow-rate of 1.0 ml/min was used to transfer the sample onto two SPE cartridges [LiChroCART 25 - 4 packed with LiChrospher RP-18 ADS (spherical particles 25 μm, pore diameter approximately 6 nm) and sealed with special manu-CART endfittings (Merck)]. Both cartridges are fitted to a 10-port valve E C10W (Valco, Schenkon, Switzerland). The chromatographic column [RP 18, 125 mm×4 mm, 5 μm; endcapped, LiChroCART HPLC-cartridge (Merck)] was maintained at a temperature of 40°C. For the various drugs several mobile phases, detectors and wavelengths were used:

Talinolol, celiprolol, metoprolol, oxprenolol and tiracizine: The mobile phase consisted of acetonitrile–potassium phosphate buffer (0.05 mol/l, pH 4) (27:73, v/v) with a flow-rate of 1 ml/min. The UV-detector (Shimadzu LD 6A) was set at a wavelength of 242 nm or 230 nm, respectively [9–13]. Retention times were: talinolol 6.1 min, celiprolol 3.6 min, metoprolol 3.0 min, oxprenolol 4.7 min, tiracizine 8.1 min and for the tiracizine metabolites M2, M1, M3: 2.9, 3.1 and 7.0 min.

Triamterene and trimethoprim: The mobile phase consisted of methanol–phosphate buffer (0.02 mol/l, pH 4) (38:62, v/v) with a flow-rate of 1 ml/min; Retention times were 2.7 min for triamterene and 1.9 min for trimethoprim. UV detector: Varian 9050, wavelength 245 nm. Fluorescence detection: Shimadzu RF 10A; excitation 270 nm and emission 389 nm [14] Detajmium and ajmaline: The mobile phase consisted of a mixture of methanol–phosphate buffer (0.1 mol/l) adjusted to pH 3.5 with H_3PO_4 (26:74, v/v) with a flow-rate of 1 ml/min; retention times 3.5 min (ajmaline) and 7.0 min (detajmium). Fluorescence detection: Shimadzu RF 10A; excitation 247 nm and emission 353 nm [15].

Lamotrigine and oxprenolol: The mobile phase consisted of acetonitrile and phosphate buffer (0.05 mol/l, pH 4) (20:80, v/v) with a flow-rate of 1 ml/min. Retention time of lamotrigine was 3.1 min and of oxprenolol was 5.7 min. The UV detector (Shimadzu LD 6A), set at a wavelength of 280 nm [16,17].

Articaine: The mobile phases consisted of acetonitrile–phosphate buffer (0.05 mol/l, pH 5) (14:86) with a flow-rate of 1 ml/min; retention time 5.6 min. UV Detector: Varian 9050, wavelength 274 nm [18].

The detectors were connected to a data system (MT2, Kontron).

It was easy and advantageous for method development and for measurement of a small series of samples to reduce the HPLC equipment to a simpler system with only one extraction column and a six-port valve [2,3].

To determine the elution profile of sample matrix and analytes, the ADS extraction column was directly coupled to an appropriate UV or fluorescence detector. The flow-rate was 1 ml/min. After injection of a sample the elution profile of the matrix at 220 nm was registered using an integrator. The fractionating step was complete (t_M) when the matrix peak reached the baseline (Fig. 2a–d). Elution of the analytes took place with the same mobile phase and flow-rate as used for sample matrix elution and with an analyte concentration of 50 μg/ml The time t_A means the beginning of analyte elution.

Subsequent to complete elution of the sample

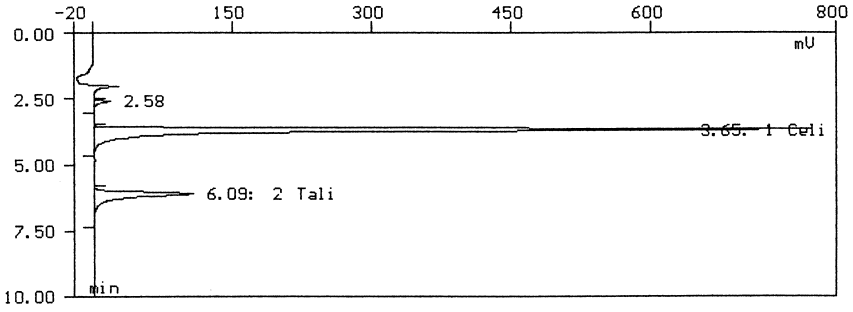

Fig. 2. Chromatogram of an intestinal aspirate sample. Peaks: 3.6 min celiprolol (internal standard; 10 μg/ml); 6.1 min talinolol (2 μg/ml).

matrix, the 10-port valve was switched and the stronger elution power of the AC eluent caused the desorption of the analytes from the extraction column. For the analysed drugs, a special transfer eluent or a gradient of the AC eluent were not necessary.

2.4. Samples

Analyses were carried out with serum, urine or intestinal fluid of young healthy volunteers or with pooled drug free samples from healthy volunteers [13,14,19,20]. The pooled samples were spiked with various concentrations of several drugs. Transport experiments of drugs were made in cell cultures (Caco-2 monolayers) and the concentrations had to be measured in the supernatants above or below the cell monolayer [21]. To denature plasma proteins 1.5 ml of acetonitrile was added to 0.5 ml of the sample, the supernatant was dried and redissolved in 200 μl of water. An alternative was protein denaturation with perchloric acid. But the injection of this supernatant was not suitable because the analytes were not retained on the ADS column. The SPE of ajmaline and detajmium from serum was carried out with Oasis cartridges HLB 1 cc (Waters, Milford, MA): condition 1 ml of methanol and 1 of ml water; load, 1 ml of serum; wash, 1 ml of methanol 5% in water; elute, 1 ml of methanol; evaporated and redissolved in 200 μl of phosphate buffer (0.1 mol/l) with H_3PO_4 adjusted to pH 3.5.

3. Results and discussion

3.1. Determination of t_M

To determine the elution profile of the sample matrix and t_M the extraction column was directly connected to an UV detector. The fractionation step was influenced by the volume and the kind of biological sample, the composition and the flow-rate of the mobile phase (Table 1). The wavelength of the detector played a role in the determination of t_M. Subsequent to the injection of 200 μl of the original or diluted biological sample (1:2 or 1:4 with water) the elution profile of the matrices was registered. The high variation of t_M-values especially for urine and intestinal fluid samples was possibly caused by the variable consistency of these fluids.

A complete elution of the sample matrix with water was possible for urine, supernatants of cell cultures and serum only after protein denaturation with acetonitrile. The addition of methanol or acetonitrile to the eluent improved the clean-up of samples (Table 1). However, in order to avoid undesired precipitation and irreversible adsorption of proteins onto the packing material of the ADS column, the content of organic modifier in the mobile phase should be less than 20% vol. methanol and less than 10% (v/v) acetonitrile. For the fractionation of serum and intestinal aspirates it was necessary to add buffer to increase the ionic strength and organic modifier (Table 1). For serum samples it

Table 1
The time t_M (in min) for the complete elution of the sample matrix

	Mobile phase								
	Aqua dest.			Buffer A		Buffer B		Buffer C	
	100%	80%	90%	100%	90%	100%	90%	100%	90%
Methanol		20%							
Acetonitrile			10%		10%		10%		10%
50 μl serum	>20	>20	>20	>20	>20	>20	>20	>20	>20
50 μl intest. fluid	10	4	5	5	7	8	6	7	6
200 μl intest. fluid	15	8	15	12	12	13	14	12	10
200 μl urine	8	5	5	7	6	9	8	8	7
200 μl cell buffer	6	3.5	5	3	3	4	3	4	3
200 μl prot. denat.	12	6	8	8	6	12	12	15	13

Flow-rate 1 ml/min, injection volume 200 μl, original or diluted with water.
Buffer A: 0.05 *M* phosphate buffer pH 5; Buffer B: 0.05 *M* phosphate buffer pH 7.8; Buffer C: 0.01 vol% phosphoric acid+triethylamine pH 5.

was difficult to achieve a complete elimination of all matrix components. The serum matrix is a mixture of proteins and other endogenous non-proteinic substances. Either a very long time (more than 20 min) or a strong eluent was necessary. For serum samples of more than 50 μl it was better to use an additional sample preparation method (protein denaturation, SPE) or conventional sample preparation.

Other authors have suggested column switching after elution of the proteins but prior to complete elution of all matrix components [4,8]. This procedure increases the risk of interference in the chromatogram or block of the column and was not used in the present study.

3.2. Determination of t_A

To examine the complete retention of the analytes the ADS column was directly connected to an appropriate detector. Elution of the analyte took place with the same mobile phases and the flow-rate used for elution of the sample matrix. The elution profile was registered for 20 min, since a later beginning of elution is uncritically for quantitative extraction and recovery.

Using pure water or water with less than 20% methanol or less than 10% acetonitrile none of the drugs were eluted from the ADS column within 20 min (Table 2). Addition of buffer lead to a faster elution of the analytes. The buffer played an important role, since the retention was influenced by the kind and concentration of ions and especially by the pH value. Phosphate buffer was a stronger eluent than triethylamine buffer.

To guarantee quantitative extraction and recovery of the analyte t_A must be larger than t_M. Switching times of 12 min or less are required, i.e. $t_A > 12$ min and $t_M < 12$ min, to get five or more analyses per hour.

3.3. Determination of drug concentrations in biological fluids

The completion of a simple HPLC equipment with an automated integrated sample processing using LiChroCART 25-4 cartridge, packed with LiChrospher RP-18 ADS was possible for already existing HPLC methods except for normal-phase systems. It is necessary to use a reversed-phase system and mixable eluents for AC and EC. The sample volume is limited by the sample loop and by the capacity of the ADS column to separate analytes and matrix.

The manual sample preparation with liquid–liquid extraction or SPE was substituted with a fully automated HPLC system for talinolol, celiprolol, metoprolol, tiracizine and metabolites; chromatograms are shown in Figs. 2 and 3. The used injection volume was 200 μl. Original samples or diluted

Table 2
The time t_A (in min) for the start of elution analyte

	Mobile phase								
	Aqua dest.			Buffer A		Buffer B		Buffer C	
	100%	80%	90%	100%	90%	100%	90%	100%	90%
Methanol		20%							
Acetonitrile			10%		10%		10%		10%
Talinolol	>20	>20	>20	>20	>20	>20	>20	>20	21
Celiprolol	>20	>20	>20	>20	4	>20	20	>20	7
Metroprolol	>20	>20	>20	>20	3	>20	5	>20	4
Tiracizin	>20	>20	>20	>20	>20	>20	>20	>20	>20
Triampterene	>20	>20	>20	12	3	>20	4	>20	3
Ajmalin	>20	>20	>20	>20	4	>20	18	>20	20
Articaine	>20	>20	>20	5	2	>20	4	>20	2.5
Lamotrigine	>20	>20	>20	>20	4	19	2	>20	9

Flow-rate 1 ml/min, injection volume 200 μl, analyte concentration 50 μm/ml.
Buffer A: 0.05 *M* phosphate buffer pH 5; Buffer B: 0.05 *M* phosphate buffer pH 7.8; Buffer C: 0.01 vol% phosphoric acid+triethylamine pH 5.

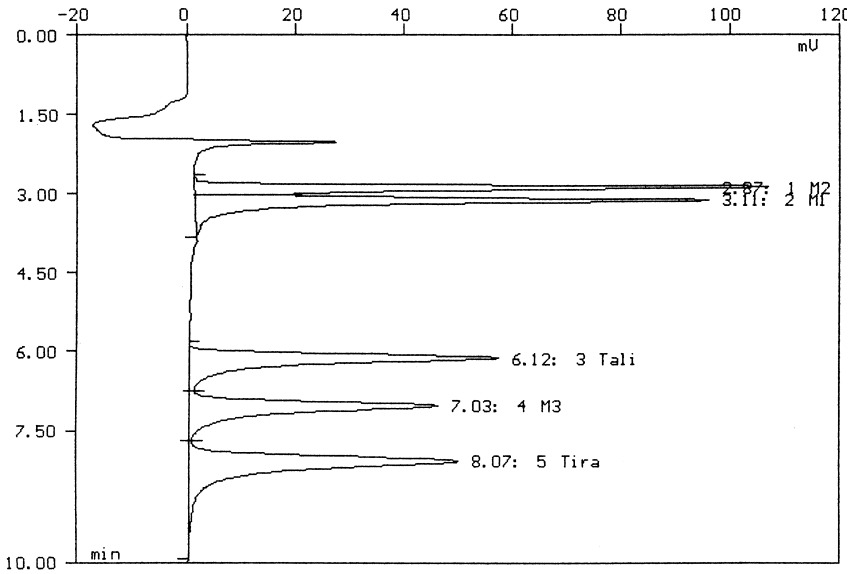

Fig. 3. Chromatogram of a cell supernatant sample. Peaks: tiracizine 7.0 min, and its metabolites M2 2.9 min, M1 3.1 min, M3 7.0 min and the internal standard talinolol 6.1 min.

samples (1:2 or 1:4 with water) were injected. For measurements in urine, supernatants after protein denaturation or elutes of SPE sample matrixes were eluted with a mixture of 90–95% water and 5–10% methanol or acetonitrile without buffer. The matrices of supernatants of cell cultures and diluted urine samples were eluted only with water. Precision, accuracy and sensitivity of the column-switching method were similar to conventional assays as reported in the literature [9,12,13]. For six repeated independent measurements of samples with 100 ng/ml, a coefficient of variation of $<10\%$ was found for talinolol, celiprolol, metoprolol, triamterene, tiracizine and its metabolites. The method was also used for trimethoprim, articaine, detajmium, ajmaline, lamotrigine and oxprenolol (Fig. 4). A validation of these methods was not made yet.

Satisfyingly sensitive methods for the direct determination of drug concentrations in serum samples could not be found for any drug used in this study with the described equipment. An appropriate mobile phase could not be found to separate the drugs in aliquots of more than 50 μl of serum. In all cases elution of the analytes started before the elution of the sample matrix was complete. Column switching after elution of the proteins but prior to complete elution of all matrix components were not made in order to avoid a blocking of the columns.

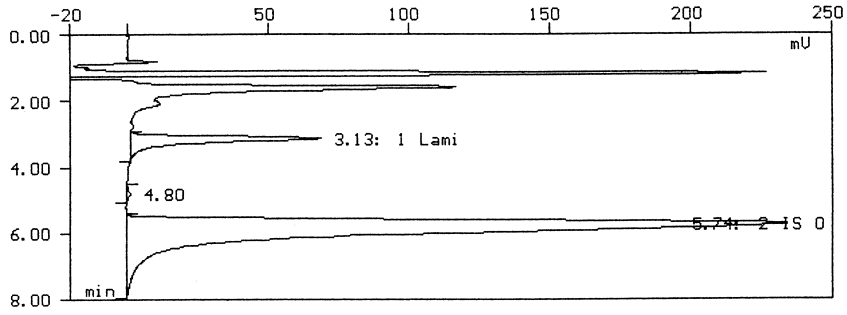

Fig. 4. Chromatogram of an urine sample. Peaks: lamotrigine 3.1 min (2 μg/ml) and internal standard oxprenolol 5.7 min (10 μg/ml).

For a direct measurement of drugs in serum or plasma, a drug has to be more lipophilic for a better retention on the ADS column or highly concentrated or has to have a higher coefficient of extinction, that a sample volume of less than 50 μl is sufficient.

For the determination of triamterene, tiracizine and its metabolites in serum a single-step conventional sample preparation procedure was not sufficient. Therefore a combination of protein denaturation with acetonitrile, drying and redissolving in water and the described HPLC system with automated integrated sample preparation were used. Detajmium and ajmaline in serum were determined with a combination of a SPE and ADS column. In comparison with extraction columns filled with conventional silica based reversed-phase materials the robustness of RP-18 ADS and the very high number of analysis cycles were the main advantages [12–14].

4. Conclusions

In conclusion the new reversed-phase sorbent LiChrospher RP-18 ADS has been demonstrated to be suitable for LC-integrated sample preparation of biological fluids. Most of the time-consuming manual sample preparation with solid-phase extraction or liquid–liquid extraction could be substituted with an on-line liquid–solid extraction. The methodology is a good completion in our clinical laboratory and it was easy to adapt conventional methods for the automated column-switching HPLC system. Precision, accuracy and sensitivity of the column-switching method are similar to conventional assays. With this new method it was possible to measure drug concentrations in various biological fluids without changing the sample preparation procedure. In comparison with extraction columns filled with conventional silica-based reversed-phase materials, the robustness of RP-18 ADS and the very high life-time were the main advantages. For serum or plasma samples with small quantities of polar analytes, additional sample preparation like protein denaturation or SPE, have been necessary to enhance the sensitivity of the method and the life-time of the ADS column.

Acknowledgements

The authors acknowledge the technical assistance of Ms. G. Grosse, Ms. M. Pescheck and Ms. K. Wagner.

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