Journal of Chromatography, 573 (1992) 59–64
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6114

Fast liquid chromatography for the determination of drugs in plasma and combination with liquid–solid extraction in a fully automated system

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(First received November 15th, 1990; revised manuscript received August 16th, 1991)

ABSTRACT

Fast liquid chromatography was applied to the assay of several drugs in plasma. Short columns, 3.3–4 cm long, packed with C_{18} material, 3 μm particle size, were used. The peaks were little subject to extra-column band-broadening because the investigated drugs were eluted with high capacity factors in order to obtain an adequate separation from plasma components. The main influences on efficiency were the response time of the detector and the solvent composition of the injected sample. Conventional apparatus was used. A fully automated analytical system combining liquid–solid extraction via disposable extraction columns and fast liquid chromatography on a small-dimensioned 3 μm particle size column is described for the assay of drugs in plasma. Automation was accomplished by using the Automatic Sample Preparation with Extraction Columns system.

INTRODUCTION

Since the first use of 3-μm particles in reversed-phase high-performance liquid chromatography (RP-HPLC) [1,2], the availability of columns filled with 3-μm particle materials has been increasing. The reduction in particle size from 10 or 5 μm to 3 μm leads to an increase in both column efficiency [3,4] and optimum mobile phase flow-rate [4,5]. The column length can then be reduced and the flow-rate increased. Separations on short 3 μm particle size columns can be achieved with reduced analysis time and little or no compromise in performance [4–6]. Nevertheless, such columns are rarely used for the routine analysis of drugs in body fluids.

In this study, a practical evaluation of short columns packed with 3-μm particles was performed so as to establish the best conditions of use for sensitive analysis of numerous samples. Liquid–liquid extraction from plasma was applied to the determination of two new antiepileptic drugs prior to chromatography. Liquid–solid extraction via disposable extraction columns (DECs) was applied to the determination of carbamazepine and its epoxide and *trans*-diol metabolites. As liquid–solid extraction requires only liquid transfers, it is easier to automate. Therefore, the assay of carbamazepine involving liquid–solid extraction was automated. The Automatic Sample Preparation with Extraction Columns (ASPEC) system [7,8] was used.

EXPERIMENTAL

Materials

Carbamazepine (5*H*-dibenz[*b*,*f*]azepine-5-carboxamide), a tricyclic antiepileptic drug, its epoxide metabolite (10,11-epoxycarbamazepine), its *trans*-diol metabolite (10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine) and the internal standard (5,6-dihydro-11-oxo-11*H*-dibenz [*b*,*e*]azepine-5-carboxamide) were provided by Ciba-Geigy (Basle, Switzerland).

Two new structurally related antiepileptic drugs, ATE I and ATE II (CGP 33 101), and two

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analogue compounds used as internal standards, ATE III and IV, were also provided by Ciba-Geigy. The four compounds are triazole–carboxamide derivatives with the triazole ring attached to a benzyl moiety. Propyphenazone, triclabendazole and its sulfone and sulfoxide metabolites were also provided by Ciba-Geigy.

Reagents

Potassium dihydrogenphosphate, dipotassium hydrogenphosphate and pH 12 buffer (Titrisol) were purchased from Merck (Darmstadt, Germany). Diethyl ether and dichloromethane (Pestipur quality) were purchased from SdS (Peypin, France). Acetonitrile (HPLC quality) and methanol (Royal Society American Chemical Society quality) were from Carlo Erba France (Puteaux, France). Water was purified and deionized using a Milli-Q reagent-grade water system (Millipore, Bedford, MA, USA).

Apparatus

The chromatographic system consisted of a Model 303 pump (Gilson, Villiers-le-Bel, France), a SEDERE Model Sedex 100 (Touzart et Matignon, Vitry-sur-Seine, France) or a WISP Model 710 B (Waters, Milford, MA, USA) injector, an ASPEC system (Gilson) instead of the injector for the assay of carbamazepine and a Model Spectroflow 783 or 773 variable-wavelength UV detector (Kratos, Ramsey, NJ, USA). The wavelength was set at 210 nm for the assay of carbamazepine and its metabolites and at 230 nm for the assay of ATE I and ATE II. The response time was set at 1 s. A Model C-R3A integrator recorder (Shimadzu, Kyoto, Japan) was used. Connecting tubes with an internal diameter of 0.25 mm were used.

Columns and filter

A guard column (stainless-steel tube, 3.3 cm × 4.7 mm I.D.) was placed between the pump and the injector. It was filled in our laboratory. A 0.8-g aliquot of Partisil 10 ODS-3, 10 μm particle size (Whatman, Clifton, NJ, USA), was suspended in 27 ml of isopropanol–methanol (80:20, v/v). Methanol was used as the pressurizing solvent. The filling pressure was set at 450 bar for 30 min with a Haskel pneumatic pump.

Prepacked analytical columns (stainless-steel tube, 4.7 mm I.D.), filled with 3-μm particles, were used: Hypersil ODS, 4 cm long (Société Française Chromato Colonne, Neuilly-Plaisance, France); Pecosphere C_{18}, 3.3 cm long (Perkin-Elmer France, Montigny-le-Bretonneux, France); Supelcosil LC-18, 3.3 cm long (Supelco France, St-Germain-en-Laye, France).

A filter (No. FL 01, Société Française Chromato Colonne) with a replaceable 2-μm-pore frit was inserted between the injector and the analytical column.

Disposable extraction columns (DECs)

C_{18} DECs of capacity 1 ml filled with 50 mg were used. They were manufactured by J. T. Baker (Deventer, Netherlands) and supplied by Sochibo (Vélizy-Villacoublay, France).

Sample handling

Liquid–liquid extraction. A 500-μl aliquot of plasma, 1 ml of pH 12 buffer and 50 μl of calibration solution (containing one of the two new antiepileptic drugs and the internal standard) were shaken for a few seconds on a vortex mixer in a 10-ml glass tube. A 7-ml aliquot of diethyl ether–dichloromethane (2:1, v/v) was added. The mixture was shaken for 15 min at 250 rpm and centrifuged for 5 min at 2200 *g*. A 6-ml sample of the organic phase was evaporated to dryness at 37°C under a stream of nitrogen. The residue was redissolved in 200 μl of a mixture of water and acetonitrile [containing between 6 and 10% (v/v) acetonitrile] and the tube was shaken for 5 min at 350 rpm. A 50-μl aliquot of the final sample was injected.

Liquid–solid extraction. The automated procedure of extraction of carbamazepine and its metabolites is described in detail in a separate paper [9]. A 100-μl aliquot of plasma was diluted manually with water. All the following operations were performed automatically by the ASPEC system. Diluted plasma was loaded onto an activated 50-mg DEC. After washing with 0.02 mol/l dipotassium hydrogenphosphate and water–methanol (95:5, v/v), the analytes were eluted with 250 μl of methanol. A 950-μl volume of water was added and the mixture was injected via a 500-μl sample loop. Each plasma sample was prepared during the chromatography of the previous sample.

Chromatography

The mobile phase was acetonitrile–methanol–0.05 mol/l potassium dihydrogenphosphate (15:5:80, v/v/v) for the determination of carbamazepine and its metabolites, and acetonitrile–0.01 mol/l potassium dihydrogenphosphate for the determination of ATE I (16:84, v/v) and ATE II (14:86, v/v). The aqueous part was filtered before use through a 0.45-μm filter (Millipore). The flow-rate of the mobile phase was 2 ml/min. The chromatography was carried out at room temperature.

RESULTS AND DISCUSSION

Mobile phase flow-rate

An increase in mobile phase flow-rate between 1 and 3 ml/min did not significantly modify the efficiency of the 3 μm particle size column. The baseline noise level measured on the chromatogram was not increased with increasing flow-rate. A flow-rate of 2 ml/min was selected for routine analysis in order to maintain an acceptable pressure drop across the analytical column (between 60 and 80 bar at the top of a 3.3-cm-long column).

Response time of the detector

A response time of the detector of 1 s was used as a compromise between efficiency and baseline noise level: when decreasing the response time from 1 to 0.5 s, the noise level (measured when the detector was set at a high sensitivity) was increased by a factor of 2, whereas the increase in efficiency was less than 5% (for ATE I and ATE III).

Solvent composition of the injected sample

It has been shown that the injected volume must be as small as possible to minimize band-broadening due to injection when using columns packed with 3-μm particles [10,11]. However, large volumes (50–100 μl) have been injected without loss of efficiency when the eluting strength of the injected sample was lower than that of the mobile phase [12]. Since 50-μl volumes of plasma extract or more are often injected onto conventional 5 μm particle size columns for pharmacokinetic studies, large volumes of extract have also been injected onto the 3-μm particle columns used in our laboratory so as to achieve a sufficient sensitivity. After liquid–liquid extraction and evaporation of the solvent and prior to injection, the residue was redissolved in a mixture containing less organic solvent than in the mobile phase. After liquid–solid extraction, the eluting strength of the extract was decreased by dilution with an aqueous solution prior to injection.

Equipment

Because analytes are rapidly eluted in small bands in fast LC, extra-column band-broadening can occur. This effect has been shown to become critical at k' values below 2 [3]. However, after extraction from plasma, drugs are generally eluted with relatively high k' values in order to obtain an adequate separation from co-extracted plasma components. For ATE I ($k' = 10$) and ATE III ($k' > 10$), no differences in efficiency greater than 10% were observed after addition of a filter and after substitution of either the 8-μl flow cell of the Kratos detector for a 2.4-μl flow

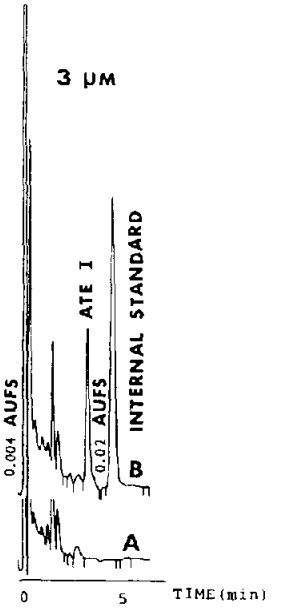

Fig. 1. Chromatograms corresponding to the determination of ATE I in plasma. Column, 4 cm long, Hypersil ODS, 3 μm particle size; mobile phase, acetonitrile–0.01 mol/l potassium dihydrogenphosphate (16:84, v/v); flow-rate, 2 ml/min; injected sample, (A) extract of a blank plasma sample, (B) extract of a spiked plasma sample containing 0.36 μmol/l ATE I and 3.6 μmol/l internal standard (ATE III).

cell, or the WISP injector for a SEDERE injector affording a low dead volume (equipped with a 50-μl sample loop). For all investigated drugs, good efficiencies were obtained with the conventional apparatus used: about 110 000 plates per meter for ATE I and ATE III for a 50-μl injected volume and about 80 000 plates per meter for carbamazepine and its less retained metabolite dihydroxycarbamazepine ($k' = 5$, $t_R = 1.3$ min) for a 500-μl injected volume. Chromatograms are shown in Figs. 1–3.

Column stability

A guard column was inserted between the pump and the injector and a filter with a low dead volume was inserted between the pump and the analytical column. When the filter frit (2-μm pores) was exchanged twice a week, no increase in pressure occurred. Pore frits of 0.5 μm were not used because increases in pressure occurred after only a few injections.

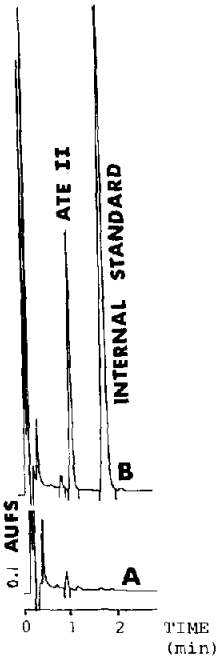

Fig. 2. Chromatograms corresponding to the determination of ATE II in plasma. Column, 3.3 cm long, Pecosphere C_{18}, 3 μm particle size; mobile phase, acetonitrile–0.01 mol/l potassium dihydrogenphosphate (14:86, v/v); flow-rate, 2 ml/min; injected sample, (A) extract of a blank plasma sample, (B) extract of a spiked plasma sample containing 10.5 μmol/l ATE II and 4.3 μmol/l internal standard (ATE IV).

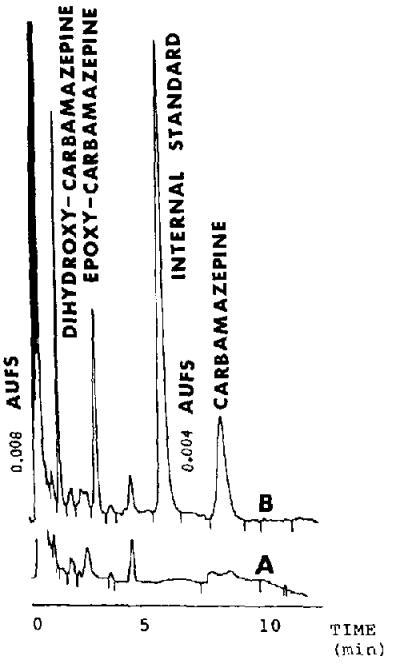

Fig. 3. Chromatograms corresponding to the determination of carbamazepine and its metabolites in plasma. Column, 3.3 cm long, Supelcosil LC-18, 3 μm particle size; mobile phase, acetonitrile–methanol–0.05 mol/l potassium dihydrogenphosphate (15:5:80, v/v/v); flow-rate, 2 ml/min; injected sample, (A) extract of a blank plasma sample, (B) extract of a spiked plasma sample containing 0.5 μmol/l carbamazepine and its metabolites and 5 μmol/l internal standard.

Automatic procedure

The ASPEC system reproduces automatically the operations of DEC conditioning, sample loading, washing and elution. Liquid transfers and mixings by bubbling can be accomplished [7,8], but evaporation of the eluate cannot be performed. Therefore, the eluate was directly injected in order to realize the automatic junction between extraction and HPLC. The eluting strength of the eluate was decreased before injection by addition of an aqueous solution. The weakest hydro-organic solvent that removed analytes from the sorbent in a volume as small as possible was determined in order to inject a reasonable sample volume without band-broadening and/or column damage.

For the assay of carbamazepine and its metabolites, a non-polar C_{18} DEC had to be used in order to trap the less retained metabolite dihydroxycarbamazepine. This metabolite could then

be eluted from the DEC with the mobile phase used for chromatography. But absolute methanol had to be used to remove the most hydrophobic analyte, carbamazepine. The overall recoveries were 70–80% when no washing was performed as compared with a direct injection. They were about 90% when two washing steps, necessary to eliminate interfering compounds, were applied. The analytes might consequently begin to migrate across the DEC during the rinsing steps, and this permitted reduction of the volume of methanol necessary to remove the analytes from the DEC.

With 50-mg DECs, the volume of methanol used for the elution of the analytes was only half of that required with the commonly used 100-mg DECs. As smaller solvent volumes were transferred, the time for sample preparation and injection was reduced from 12 to 10 min. Similar recoveries and chromatograms for an extract of blank plasma were obtained with 50- and 100-mg DECs.

TABLE I

WITHIN- AND BETWEEN-DAY PRECISION AND ACCURACY OF THE ASSAY

Nominal plasma concentration (μmol/l)	Within-day (n = 6)		Between-day (n = 4–6)	
	Nominal/found (mean) (%)	C.V. (%)	Nominal/found (mean) (%)	C.V. (%)
ATE I				
0.2	104	4	103	8
0.4	106	2	107	5
0.7	101	1	102	2
4	101	1	100	1
18	101	0.5	99	1
70	95	1	99	3
ATE II				
0.1	100	6	–	–
0.2	100	5	101	4
0.4	96	1	103	2
2	97	1	101	3
4	100	1	102	2
8	100	2	–	–

Reproducibility and limit of quantitation

Within- and between-day precision and accuracy for series of plasma samples spiked with ATE I or ATE II are shown in Table I. Reproducibility results for carbamazepine and its metabolites are given in a separate paper [9]. The limit of quantitation, established with a coefficient of variation lower than 10% and a mean recovery between 95 and 107% was 0.2 μmol/l (about 50 ng/ml) for ATE I and 0.1 μmol/l (about 25 ng/ml) for ATE II and carbamazepine and its metabolites. This limit was similar to that obtained with a 5 μm particle size column. The time of chromatography was between two and three times lower for the 3 μm than for the 5 μm particle size column when the resolution and the pressure at the top of the columns were similar.

Applications

Short 3 μm particle size columns were subsequently used as described above for the determination of other drugs in plasma.

Propyphenazone was determined in plasma following protein precipitation, dilution with water and injection of a 50-μl aliquot onto a Supelcosil LC-18-DB column, 3 μm particle size, 3.3 cm long [13]. About 350 deproteinized plasma samples have been injected per column without a significant change in the separation.

Triclabendazole and its sulfoxide and sulfone metabolites have been determined in plasma following liquid–solid extraction by using the ASPEC system and chromatography on a Supelcosil LC-18 column, 3 μm particle size, 3.3 cm long [13]. Non-polar 100-mg C_{18} DECs had to be used to trap triclabendazole and its metabolites as they were highly bound to plasma proteins and thus little retained on the sorbent during the loading step. A 1.5-ml aliquot of methanol was required to elute the compounds from the DEC after washing with water (Table II) and 1 ml only after washing with a mixture of water and methanol. A 1-ml sample of eluate diluted with water was injected in order to reach a sensitivity sufficient for pharmacokinetic purposes (10 ng/ml). An example of a chromatogram is given (Fig. 4).

CONCLUSION

Small-dimensioned 3 μm particle size columns should be useful to increase the work capacity of

TABLE II

INFLUENCE OF THE WASHING STEP ON THE OVERALL RECOVERY OF TRICLABENDAZOLE AND ITS METABOLITES FROM THE EXTRACTION COLUMN

Conditions: extraction column, Baker C_{18}, 100 mg; column, Supelcosil LC-18, 3 μm particle size, 3.3 cm long; mobile phase, acetonitrile–methanol–0.013 mol/l pH 5.2 phosphate buffer (8:47:45, v/v/v); flow-rate, 2 ml/min; injected sample volume, 1 ml.

Rinsing step	Elution step	Recovery[a] (n = 2) (%)		
		Sulphone metabolite	Sulphoxide metabolite	Triclabendazole
1 ml water	1 ml methanol	57	57	46
1 ml water	1.5 ml methanol	102	103	96
1 ml methanol–water (55:45, v/v)	1 ml methanol	98	92	95

[a] Calculated from the direct injection of a standard solution.

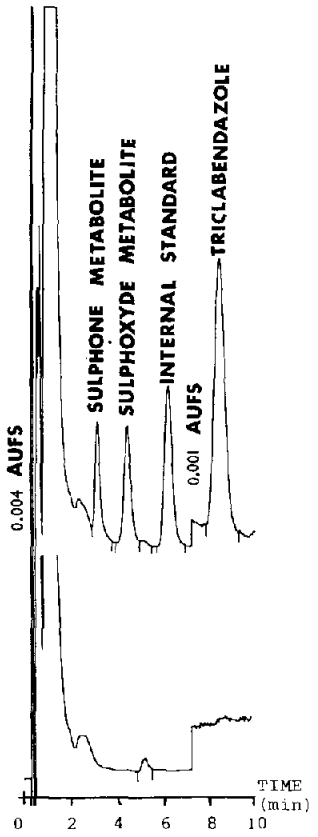

Fig. 4. Chromatograms corresponding to the determination of triclabendazole and its metabolites in plasma. Chromatographic conditions as in Table II. Injected sample. (A) extract of a blank plasma sample, (B) extract of a spiked plasma sample containing 0.3 μmol/l triclabendazole and its metabolites and 1.2 μmol/l internal standard.

LC for drug analysis. They can be used in conjunction with an automated system such as the ASPEC apparatus.

REFERENCES

1 N. H. C. Cooke and K. Olsen, *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, NJ, 1980*, Paper No. 021.
2 N. H. C. Cooke and K. Olsen, *J. Chromatogr. Sci.*, 18 (1980) 512.
3 N. Mellor, *Chromatographia*, 16 (1982) 359.
4 J. L. DiCesare, M. W. Dong and F. L. Vandemark, *Am. Lab.*, August (1981) 52.
5 N. H. C. Cooke, B. G. Archer, K. Olsen and A. Berick, *Anal. Chem.*, 54 (1982) 2277.
6 J. T. Työppönen, *J. Chromatogr.*, 413 (1987) 25.
7 F. Vérillon, B. Pichon and F. Qian, *Int. Lab.*, July/August (1987) 50.
8 M. C. Rouan, J. Campestrini, J. B. Lecaillon, J. P. Dubois, M. Lamontagne and B. Pichon, *J. Chromatogr.*, 456 (1988) 45.
9 M. C. Rouan, J. Campestrini, V. Le Clanche, J. B. Lecaillon and J. Godbillon, *J. Chromatogr.*, 573 (1992) 65.
10 J. L. DiCesare, M. W. Dong and J. G. Atwood, *J. Chromatogr.*, 217 (1981) 369.
11 M. W. Dong and J. L. DiCesare, *J. Chromatogr. Sci.*, 20 (1982) 49.
12 A. Rouchouse, D. Pauloin, J. T. Burke and B. Ferrandes, *J. Chromatogr.*, 353 (1986) 225.
13 M. C. Rouan, J. Campestrini, J. B. Lecaillon and J. Godbillon, *J. Chromatogr.*, submitted for publication.