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DRUG MONITORING BY A FULLY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUE, INVOLVING DIRECT INJECTION OF PLASMA

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SUMMARY

A procedure involving direct injection of whole plasma for analyses of drugs by an automated high-performance liquid chromatograph was developed. This system comprised two columns, two pumps, one detector, two programmable switching valves, an automatic sample injector with a cooling device for sample tubes and a microprocessor. Effluents from the first column, containing a drug of interest, were selectively introduced into the second column for further separation. The columns used were an aqueous gel chromatography column (column 1) and an ODS column (column 2). The solvent for column 1 must be weaker than that for column 2, so that the solutes from the former will be enriched at the top of the latter. The validity and applicability of this procedure for the study of drug metabolism were demonstrated with the antibiotic cefmetazole, the anticoagulant warfarin, the antitumour agent carboquone and the anaesthetic ketamine.

INTRODUCTION

The recent refinements of high-performance liquid chromatography (HPLC) have made it easy to determine many compounds in biological fluids. Fortunately, since many drugs contain UV-absorbing moieties, concentrations down to the ng level can be monitored in biological materials. However, with the increased demand for analyses and the development of automation and data processing, sample preparation has become the limiting step in the analysis of biological samples. The most common method for preparing sample solutions may involve deproteinization by adding precipitating agents[1], such as trichloroacetic acid, extraction by organic solvents, clean-up by disposable short columns, etc. However, these methods are not only time-consuming but often result in low recoveries and thus may not be suitable as sample clean-up procedures.

The column-switching technique or coupled column chromatography[2–9] is known to increase separation selectivity. In this technique, an effluent from one column is transferred to a second column, usually having a different separation mode.

In this report we describe an on-line column-switching procedure which en-

TABLE I

ANALYTICAL CONDITIONS FOR THE DETERMINATION OF CEFMETAZOLE, WARFARIN, CARBOQUONE AND KETAMINE IN PLASMA

TBAB = tetrabutylammonium bromide; MES = 2-(N-morpholino)ethanesulphonate.

Drug	Column and mobile phase		Flow-rate ($ml\ min^{-1}$)	Detection, UV (nm)	Programmed time (min)		
	Column 1	Column 2			Injection in column 1	Introduction from column 1 to column 2	Stop
Cefmetazole	TSK G2000 SW (300 × 7.5 mm); 0.005 M TBAB, 0.025 citrate (pH 5)	ERC-ODS-1262 (100 × 6.0 mm); 0.005 M TBAB, 0.025 M citrate (pH 5)–acetonitrile (78:22)	1.0	254	0	13.0–15.0	30.0
Warfarin	TSK G2000 SW (300 × 7.5 mm); 0.15 M sodium chloride, 1/15 M phosphate (pH 7)–acetonitrile (95:5)	μBondapak C$_{18}$ (300 × 3.9 mm); 0.15 M sodium chloride, 1/15 M phosphate (pH 7)–acetonitrile (7:3)	1.0	310	0	16.0–22.0	30.0
Carboquone	TSK G2000 SW (300 × 7.5 mm); 0.1 M MES (pH 7)–acetonitrile (9:1)	μBondapak C$_{18}$ (300 × 3.9 mm); 0.1 M MES (pH 7)–acetonitrile (7:3)	1.0	331	0	10.0–13.0	20.0
Ketamine	TSK G2000 SW (300 × 7.5 mm); 0.05 M potassium dihydrogen phosphate	ERC-ODS-1272 (200 × 6.0 mm); 0.05 M potassium dihydrogen phosphate–acetonitrile (3:1)	1.0	210	0	16.0–19.5	30.0

ables drug levels to be monitored in blood by direct injection of plasma without any pre-treatment, such as deproteinization or extraction. The columns used were an aqueous gel column for the pre-separation of drugs from major plasma components and an ODS-silica column for the determination of the drug concentration. The validity of our procedure has been verified by application to the determination of cefmetazole in plasma, of warfarin, a strongly protein-bound drug; of carboquone, an unstable drug, and of ketamine, a weakly UV-absorbing drug.

EXPERIMENTAL

Apparatus

The HPLC system used in this study consisted of a Model 635 liquid chromatograph (Hitachi, Tokyo, Japan), equipped with a Model 638 variable-wavelength detector (Hitachi), a Model 833 microprocessor (Hitachi) and a Model 6A pump (Shimadzu, Kyoto, Japan) as an extra pump. The column-switching valve system, Model PT-8000 (Toyo Soda, Tokyo, Japan), consisted of two six-port switching valves, capable of operating with a programmable timer. The automatic sample injector was a Model 710B (Waters Assoc., Milford, MA, U.S.A.) equipped with a cooling system for sample tubes. The columns used were: TSK gel G2000SW (300 × 7.5 mm I.D., Toyo Soda), ERC-ODS-1262 (100 × 6.0 mm I.D.; Erma Optical Works, Tokyo, Japan), ERC-ODS-1272 (200 × 6.0 mm I.D., Erma Optical Works) and μBondapak C_{18} (300 × 3.9 mm I.D., Waters Assoc.). The liquid chromatograph, switching valves and sample injector could be operated as a fully automated system.

Reagents and chemicals

Cefmetazole, carboquone and ketamine hydrochloride were obtained from Sankyo (Tokyo, Japan) and warfarin was purchased from Sigma (St. Louis, MO, U.S.A.).

The mobile phases were of either HPLC or analytical grade, from various sources, and were used without further purification.

Animals

Spraque-Dawley male rats, weighing 200–250 g, were used.

Preparation of blood samples

Blood samples were withdrawn from the jugular veins of the rats, using heparinized syringes, and centrifuged at 8000 g for 30 s. All plasma samples were put into the autosampler without any pre-treatment.

Analytical conditions

Table I shows the analytical conditions for cefmetazole, warfarin, carboquone and ketamine hydrochloride in plasma.

RESULTS AND DISCUSSION

Analytical system and procedure

The operating procedure and its characteristics as well as the methods for

optimizing the separation conditions are described below, using cefmetazole, a ce-
phamycin antibiotic, as an example.

Fig. 1 shows a schematic diagram of the column-switching valve system used
for direct injection of plasma. Two valves were automatically rotated from the posi-
tions indicated by the heavy lines to those indicated by dotted lines or in the reverse
direction, by using the programmable timer, In the pre-separation step (A) plasma
samples were injected into column 1 at time zero, while column 2 was being flushed
or conditioned, using pump 2. An effluent from either column 1 or 2 can be passed
through the detector. The next step (B) was the introduction of the effluent from
column 1 into column 2. The minimum volume of the effluent containing the solute
of interest was introduced by switching valve 1. In step C, cefmetazole was deter-
mined on column 2, and column 1 was flushed and prepared for the next injection.
Columns 1 and 2 contained TSK gel G2000SW and ERC-ODS-1262, respectively.
A TSK gel G2000SW column was effective in the separation of water-soluble poly-
mers, such as proteins. Gel chromatography allows sample separation in a short time,
based on the different sizes of the solutes. The selection of the mobile phase for this
column is simpler than for columns with other separation modes. This was very
advantageous in the selection of the primary column.

The next problem which had to be solved was the selection of the mobile
phases. One of the most important requirements for effective chromatographic sep-
aration is the injection of sample solutions in as small a volume as possible, which
is clearly incompatible with the column-switching technique. In the present proce-
dure, 2–5 ml of an effluent from column 1 were introduced into column 2, which
corresponds to the injection of 2–5 ml of a sample solution into an analytical column.
To eliminate peak broadening caused by the introduction of a large volume of ef-
fluent into column 2, a mobile phase with a high polarity must be selected for this
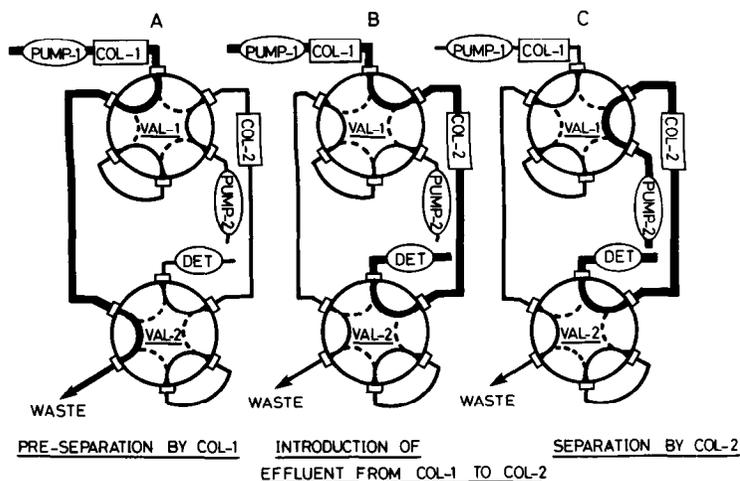column.

Fig. 1. Schematic diagram of the column switching valve system used for direct injection of plasma. For
a description see the text. COL = column, DET = detector, VAL = valve.

Cefmetazole could be eluted at a reasonable retention time by 5–10% acetonitrile solution in buffer when an ODS column was used. The polarity difference was not enough to prevent peak broadening. As already reported[10], cefmetazole was successfully chromatographed by using an ion-pairing mode and with a 20% acetonitrile solution as the mobile phase. When column 1 was eluted with a 100% aqueous buffer and a 20% acetonitrile solution was used as a mobile phase for column 2, solutes from column 1 could be enriched at the top of column 2, even though a large volume of effluent from column 1 had been introduced. It was found that a difference of at least 20% was necessary in the organic solvent concentrations of the two mobile phases in order to enrich solutes on top of column 2.

Table II shows a comparison of the peak height of cefmetazole obtained with the coupled columns, *i.e.*, column 1 plus column 2, and with column 2 only. No loss of peak height by the coupled column method compared to that obtained with column 2 only could be seen, even though cefmetazole was eluted at almost double the retention time when the coupled system was used. This suggests that no migration of the solute, cefmetazole, had occurred from the top of column 2 before passage of the aqueous acetonitrile mobile phase.

TABLE II

COMPARISON OF RETENTION TIME AND PEAK HEIGHT OF CEFMETAZOLE ON COUPLED COLUMNS AND A SINGLE COLUMN

Coupled system (column 1 + 2)		Single column (2)	
t_R (min)	Peak height (mm)	t_R (min)	Peak height (mm)
25.4	217	11.0	222
26.8	208	11.4	215
26.1	213	11.1	212
25.3	211	11.3	220
26.1	220	11.9	225
Mean: 25.9 ± 0.6	213.8 ± 4.8 (C.V. = 2.2%)	11.1 ± 0.2	218.8 ± 5.3 (C.V. = 2.4%)

Fig. 2 shows typical chromatograms obtained by the present procedure. A, B and C illustrate chromatograms obtained by injecting a standard solution into column 1, column 2 and the coupled system, respectively. D and E show chromatograms obtained by injecting blank and spiked plasma respectively into the coupled system. No interfering peaks could be seen near the retention time of cefmetazole when a blank plasma was injected. Calibration graphs for cefmetazole showed good linearity both for standard solution and plasma spiked in the range 1–10 μg/ml.

As the recovery with spiked plasma was quantitative, in practice, standard solutions could be prepared simply by dissolving cefmetazole in water instead of using spiked plasma. The reproducibility, as given by the relative standard deviation, was 2.6% for five replicate analyses of spiked samples containing 5 μg/ml.
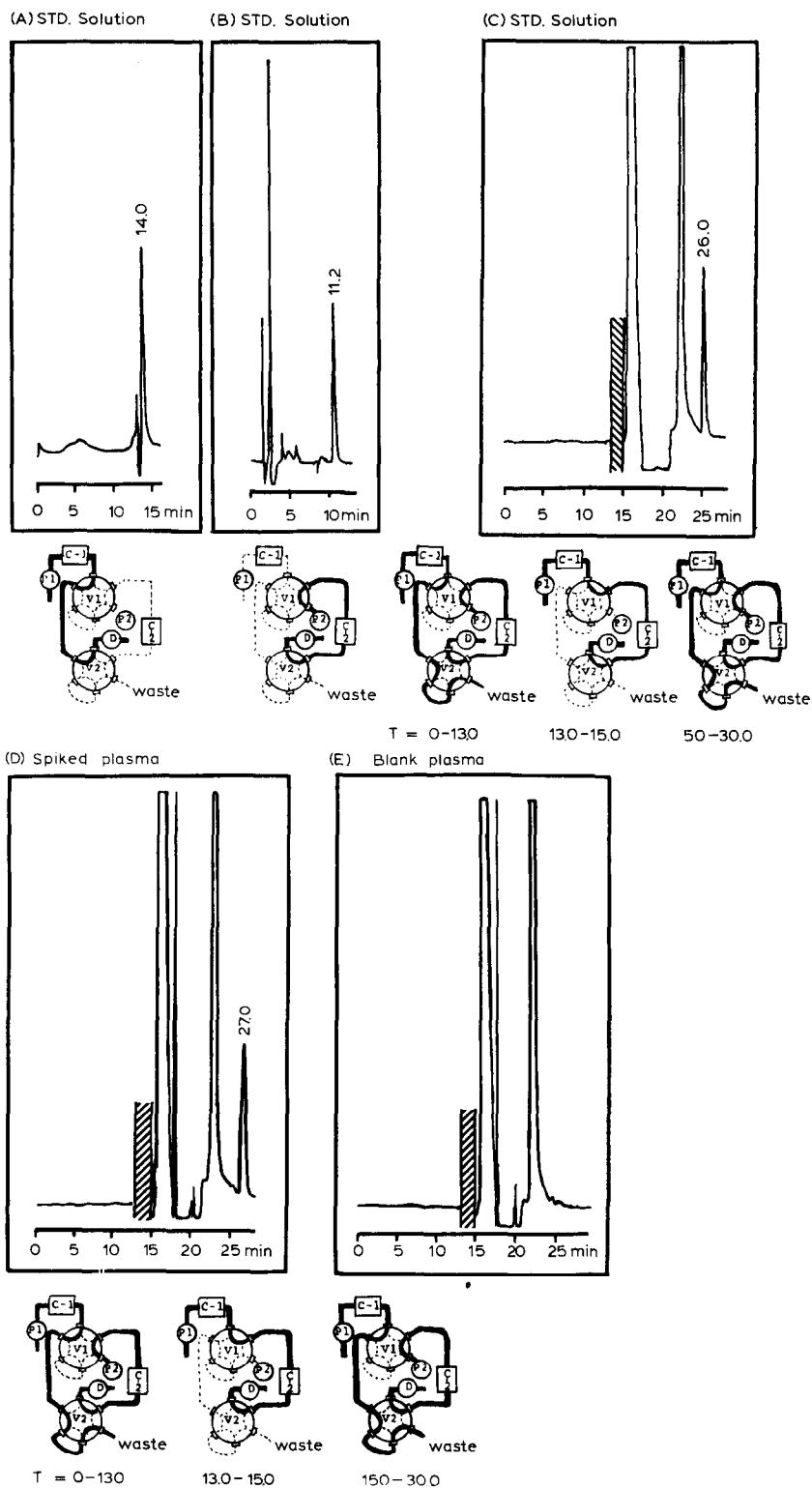
Fig. 2. Typical chromatograms obtained by the described procedure. For a description see text. Conditions as in Table I.

The stability of both columns was extremely good. However, the packing material used for column 1 is so delicate that it has a pretty short life when determining molecular weights. Since the role of this column was only to separate low-molecular-weight drugs from plasma protein, it had enough separation efficiency even after prolonged use. In fact, it has not been changed after 1 year in daily use. The ODS materials used for column 2 were stable and had longer lives than in conventional use because they were not deteriorated by plasma components.

Application to a drug with strong protein binding

When the present procedure was applied to a drug with strong protein binding, we were concerned that the recovery of the drug might be low if a part of the drug was discarded with the plasma protein. Warfarin[11-13], which is known to exist mostly in protein-bound form in plasma, was investigated by the column-switching technique. Some of the ways to displace a drug from its binding site on plasma proteins are: to dilute the plasma in excess of buffer, to change its pH or to add salts, organic solvents, surface-active agents[14], etc. Using these various methods, the separation of warfarin from plasma on column 1 was carried out by adding acetonitrile and various concentrations of sodium chloride to the mobile phase. As shown in Table III, the recovery of warfarin from plasma protein was low when the separation was carried out with mobile phases having no or a low sodium chloride concentration. However, the addition of > 0.1 M sodium chloride resulted in quantitative recovery of warfarin. In routine analyses, a sodium chloride concentration of 0.15 M was employed. Fig. 3 shows a typical chromatogram for warfarin in plasma.

TABLE III

RECOVERY OF WARFARIN FROM RAT PLASMA

Warfarin was added to rat plasma and incubated at 37°C for 30 min. The mobile phase for column 1 was 0.05 M phosphate buffer (pH 7.0) (including the indicated concentration of sodium chloride)–acetonitrile (95:5).

Concentration of sodium chloride (M)	Recovery (%)
0	93.0
0.05	96.0
0.10	101.1
0.20	100.9

Application to an unstable drug

Carboquone, an antitumour agent, is unstable due to the two aziridinyl groups in the molecule. For example, when carboquone was extracted by chloroform and then evaporated to dryness, either *in vacuo* or under nitrogen gas, more than 50% of it was decomposed[15]. Another example of the instability is that when carboquone was eluted with 0.05 M phosphate buffer (pH 7) it was decomposed and eluted as three peaks.
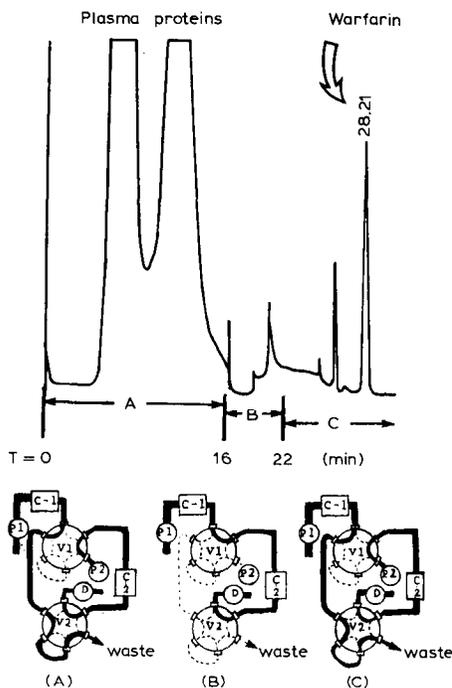
Fig. 3. Typical chromatogram for warfarin in plasma. Conditions given in Table I.

The automated analysis of plasma carboquone after intravenous administration to rats was carried out using TSK gel G2000SW in column 1 and μBondapak C_{18} in column 2. The selection of the buffer components and pH as well as of the methanol ratio for both eluents was very critical for a successful determination. Carboquone is most stable at neutral pH. Among the various kinds of buffers tested, morpholinoethanesulphonate was the best as regards the stability of carboquone.

Although 3 ml of the effluent from column 1 had been introduced into column 2, carboquone was eluted as a sharp peak, since the difference in methanol concentration of the two eluents was as much as 20%. Detailed analytical conditions are given in Table I. Typical chromatograms for a standard solution and a spiked plasma sample are shown in Fig. 4. Calibration plots obtained for both standard solutions and spiked plasma showed almost the same straight line, over the range 10–1000 ng/ml, passing through the origin.

Application to a weakly UV-absorbing drug

Ketamine, as anaesthetic agent, has been determined exclusively by using gas chromatography or gas chromatography–mass spectrometry (GC–MS)[16,17] due to its weak UV absorption and low clinically effective concentration. The therapeutic level of ketamine in plasma could be determined by our procedure without clean-up using the UV absorption at 210 nm. The results obtained by our procedure were compared with a GC method. The analytical conditions are shown in Table I.
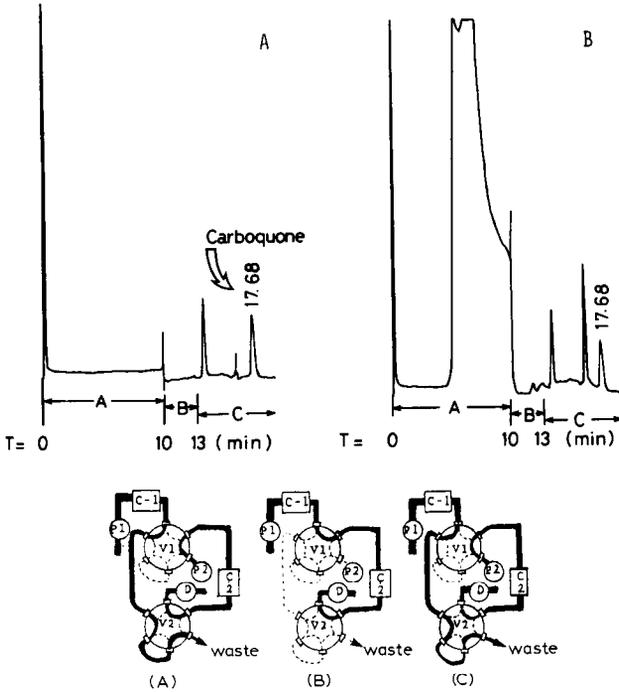
Fig. 4. Typical chromatograms for a standard solution of carboquone (A) and plasma spiked with car-boquone (B). Conditions in Table I.

In order to provide a difference of at least 20% in the acetonitrile concentrations of the two mobile phases, pure buffer for column 1 and buffer–acetonitrile (3:1) for column 2 were chosen as the mobile phases. Detection was carried out at a wavelength of 210 nm since the absorption coefficient at this wavelength is 8100, 15 times larger than that (550) at 268 nm.

With the increased sensitivity at 210 nm, the noise and interference from plasma components made it difficult to determine the blood level of ketamine by conventional extraction methods. With our column-switching procedure, ketamine could be separated from plasma components, as shown in Fig. 5. The linearity of the detector response was established under the assay conditions from 50 to 2000 ng/ml ($r = 0.9990$).
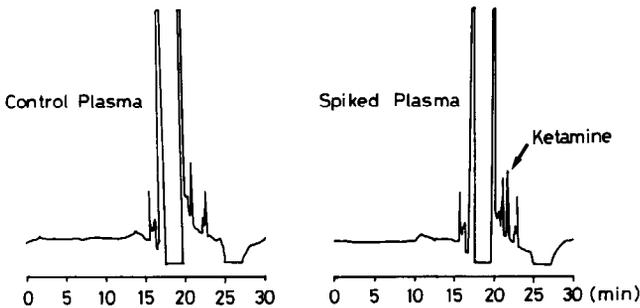

Fig. 5. Separation of ketamine in human plasma. Conditions in Table I.

Spiked plasma samples were analyzed by the present procedure and by the GC method of Stillert *et al.*[16]. The detection limit and the coefficient of variation with the present method were about 50 ng/ml and 2.1% (1 μg/ml, $n = 5$), respectively, while with the GC method they were about 5 ng/ml and 14.6%. Note also that the GC method involves extraction, derivatization, washing and concentration, is very tedious and time-consuming and requires skill and experience. Table IV gives the assay results for spiked plasma obtained by the GC and HPLC procedures. The present procedure compared well with the GC method.

TABLE IV

ASSAY RESULTS FOR KETAMINE-SPIKED HUMAN PLASMA

Concentration (ng/ml)	Found (ng/ml)	
	GC	HPLC
100	121 ± 12	91 ± 8
200	199 ± 4	201 ± 4
500	467 ± 33	497 ± 4
1000	1070 ± 43	1024 ± 10

CONCLUSION

A procedure involving direct injection of whole plasma for the determination of various kinds of drugs by an automated column-switching technique was developed. This technique combines sample clean-up, concentration and chromatographic separation in one process. It is suitable for the determination of various kinds of drugs that exhibit strong protein binding, lack of chemical stability and UV absorption.

ACKNOWLEDGEMENTS

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