ELSEVIER

Journal of Chromatography B, 655 (1994) 73–81

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Novel precolumn deproteinization method using a hydroxyapatite cartridge for the determination of theophylline and diazepam in human plasma by high-performance liquid chromatography with ultraviolet detection

Hiroshi Iwase*, Keiko Gondo, Toshio Koike, Ichiro Ono

Ajinomoto Co., Inc., Central Research Laboratories, 1-1 Suzuki-cho Kawasaki-ku, Kawasaki, 210, Japan

(First received September 7th, 1993; revised manuscript received January 25th, 1994)

Abstract

The deproteinization of human plasma was carried out using a hydroxy apatite cartridge as a precolumn. After human plasma had been passed through the cartridge followed by suitable elution, protein-free eluate was obtained within only 1 min without the need for centrifugation. Deproteinization was evaluated by the determination of albumin, γ-globulin and transferrin in the eluate by high-performance liquid chromatography (HPLC) with UV detection. Determination of theophylline and diazepam in human plasma was performed by HPLC with UV detection. The proposed method was suitable for the determination of these two drugs in human plasma, because it is simple and rapid (retention time of each drugs ≈ 15 min) and microamounts of sample are required (50–100 μl). The calibration graphs for theophylline and diazepam were linear in the range 0.1–10 μg and 0.1–65 ng, respectively. Recoveries of both drugs were over 90% by the standard addition method.

1. Introduction

The protein present in human plasma exerts a considerable influence on the determination of physiologically important compounds such as amino acids, vitamins, drugs and their metabolites. Deprotenization methods for handling biological fluids, including chemical methods (organic solvents, acids or ion exchangers) and physical methods (thermal fixation, ultrafiltration or ultracentrifugation) have been reviewed by Deyl *et al.* [1] and Sarwar and Boung [2]. Deproteinization of plasma or serum should be carried out within 30 min after sample collection and the supernatant should be analysed as soon as possible [1]. Of the chemical deproteinization methods, precipitation, elution and centrifugation procedures are most frequently used. Usually, the protein was removed by the addition of acetonitrile, alcohols, perchloric acid, metaphosphoric acid, trifluoroacetic acid or sulphosalicylic acid [3–12] to biological samples prior to analysis. More than 50% of some amino acids were trapped in the protein precipitate. The protein precipitate must be washed several times [1]. However, these methods are tedious and time

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00055-A

consuming, because they require the addition of reagent to sample followed by centrifugation to obtain a clear supernatant.

A hydroxyapatite (HAP) analytical column has been used in analyses for proteins, monoclonal antibodies and naturally occurring glycosides and saponins [13–16]. An HAP cartridge has not been used for the simple, rapid and microsample deproteinization. In this paper, we report a deproteinization method for human plasma using an HAP cartridge (PCPure cartridge) for the determination of theophylline (therapeutic blood level 5–25 μg/ml [17]), which is used therapeutically to treat reversible airway obstruction and apnea of infancy, and the muscle relaxant diazepam (therapeutic blood level 0.1–1 μg/ml [17]) in human plasma. Determinations were performed by high-performance liquid chromatography (HPLC) with UV detection.

Various methods have been developed for the determination of theophylline [11,12,18–21] and diazepam [22–24] in biological samples by HPLC after clean-up, including deproteinization followed by centrifugation, solid-phase extraction and column switching.

This paper deals with the optimization of the deproteinization and elution conditions of standard theophylline and diazepam using a PCPure cartridge for the simple, rapid and microsample determination of two drugs in human plasma by HPLC with UV detection. Further a comparison of the analytical data for the two drugs in human plasma by the proposed method and published methods is presented.

2. Experimental

2.1. Reagents and materials

Theophylline, 7-(2-hydroxyethyl)theophylline and 4,4′-difluorobenzophenone were purchased from Tokyo Kasei (Tokyo, Japan) and diazepam from Wako (Osaka, Japan). Acetonitrile was of HPLC grade. Other reagents were all of analytical grade. Albumin, γ-globulin and transferrin were purchased from Sigma (St. Louis, MO, USA). PCPure cartridges (0.4 g) were obtained from Moritex (Tokyo, Japan) and Koken (Tokyo, Japan). After washing the cartridges with acetonitrile (10 ml) followed by removal of acetonitrile by evaporation, the cartridges were ready for use.

2.2. Preparation of theophylline and internal standard [7-(2-hydroxyethyl)theophylline] standard solutions

Theophylline (50 mg) and 7-(2-hydroxyethyl)theophylline (50 mg) were each dissolved in and diluted to 100 ml with aqueous 5% acetonitrile solution in a volumetric flask, then each solution (10 ml) was further diluted to 100 ml with 0.5% aqueous acetonitrile solution in a volumetric flask, giving concentrations of 50 μg/ml each.

2.3. Preparation of diazepam and internal standard [4,4′-difluorobenzofenone] standard solutions

Diazepam (10 mg) was diluted to 100 ml with 10% aqueous acetonitrile solution in a volumetric flask, then this solution (10 ml) was further diluted to 100 ml with 1% aqueous acetonitrile solution in a volumetric flask, giving a concentration of 10 μg/ml.

4,4′-Difluorobenzofenone (10 mg) was diluted to 100 ml with 5% aqueous acetonitrile solution in a volumetric flask, giving a concentration of 100 μg/ml.

2.4. Preparation of albumin, γ-globulin and transferrin standard solutions

Albumin (800 mg) and transferrin (60 mg) were diluted to 20 ml with 0.9% NaCl solution and γ-globulin (600 mg) was diluted to 20 ml with sodium phosphate buffer (pH 7) in volumetric flasks.

2.5. Plasma collection

Blood was freshly collected in a heparin tube from healthy individuals and drug-free plasma

was obtained by centrifugation at 1700 *g* for 15 min at 5°C. The plasma obtained was then used immediately.

2.6. Sample preparation for evaluation of deproteinization

In the dilution method, plasma was diluted with various eluents followed by passing through PCPure cartridges. In the injection method, a 20–300-μl aliquot of plasma was directly injected into PCPure cartridges after the eluent had passed through the cartridge. Each fraction 1–5 (300 μl) obtained by the above two methods was tested for the evaluation of deproteinization by HPLC with detection at 250 nm.

Deproteinization was calculated from following equation:

$$\text{deproteinization } (\%) = [(A - B)/A] \cdot 100$$

where A is the content (mg) of albumin, γ-globulin or transferrin in the amount of plasma used and B is the content (mg) of albumin, γ-globulin or transferrin in each eluate (300 μl).

2.7. Sample preparation for determination of theophylline

To drug-free plasma (2 ml) in a centrifuge tube, 1 ml of standard theophylline (50 μg) was added. The mixture was vortex-mixed for 10 s and then this solution was used as the sample for the determination of the theophylline in human plasma.

In the dilution method, to an aliquot of 750 μl of the above plasma sample, 250 μl (12.5 μg) of 7-(2-hydroxyethyl)theophylline and 1500 μl of 0.9% NaCl solution were added. The mixture was vortex-mixed for 10 s and passed through a PCPure cartridge. The protein-free eluate (first elution of 300 μl) was used as the test solution.

In the injection method, an aliquot of 75 μl of the above plasma sample and 25 μl (1.25 μg) of 7-(2-hydroxyethyl)theophylline were injected into a PCPure cartridge and then 10% aqueous acetonitrile solution was passed through. The protein-free eluate (first elution of 600 μl) was used as the test solution.

2.8. Sample preparation for determination of diazepam

To a drug-free plasma (100 μl) in a centrifuge tube, 10 μl of standard diazepam solution (0.1 μg) was added. The mixture was vortex-mixed for 10 s and this solution was used for the determination of diazepam in human plasma.

An aliquot of 110 μl of the above plasma and 10 μl (1 μg) of 4,4′-difluorobenzofenone were injected into PCPure cartridges and then 50% aqueous acetonitrile solution was passed through. The protein-free eluate (first elution of 600 μl) was used as the test solution.

2.9. Apparatus and conditions for the determination of albumin, γ-globumin and transferrin

A Model 576 high-performance liquid chromatograph equipped with a Model 502U UV detector (GL Sciences, Tokyo, Japan) set at 250 nm was used. The samples were applied with an LC-Robo Model APS-220 (Ajinomoto, Tokyo, Japan) with an effective volume of 10 μl. HPLC was carried out on a 50 × 0.7 cm I.D. column of Asahipak G-520 (5 μm) (Asahikasei, Tokyo, Japan) using 30 m*M* sodium phosphate–150 m*M* sodium sulphate (pH 7, adjusted with phosphoric acid) as eluent. The flow-rate was 1.0 ml/min at 40°C.

2.10. Apparatus and conditions for the determination of theophylline and diazepam

A Model 655 A-11 high-performance liquid chromatograph (Hitachi, Tokyo, Japan) equipped with a Model L-4000 UV detector (Hitachi) set at 280 nm for theophylline and 240 nm for diazepam was used. Each sample was applied with a Rheodyne Model 7125 sample-loop injector with an effective volume of 20 μl. HPLC was carried out on a 15 × 0.46 cm I.D. column of Inertsil ODS-2 (5 μm) (GL Sciences) using acetonitrile–water (5:95) for theophylline and acetonitrile–water (50:50) for diazepam. The flow-rate was 1.0 ml/min at 40°C.

3. Results and discussion

3.1. Deproteinization

In order to examine the different effects of the eluents in deproteinization, deproteinization of human plasma was evaluated using PCPure cartridges with various eluents. Deproteinization of protein was evaluated by the determination of albumin, γ-globulin and transferrin in the eluate by HPLC with UV detection at 250 nm. Calibration graphs were constructed by plotting the peak heights against the amounts of albumin, γ-globulin and transferrin, respectively. Satisfactory linearity was obtained in the ranges 0–400 μg for albumin and γ-globulin and 0–60 μg for transferrin. The detection limits (at a signal-to-noise ratio of 2) of albumin, γ-globulin and transferrin were each evaluated as *ca.* 0.5 μg.

The effects of the amount of human plasma and the choice of eluents on removing of human plasma by both the dilution method and the injection method were studied. It can be seen in Figs 1–3 and Table 1 that these factors have an effect on the deproteinization.

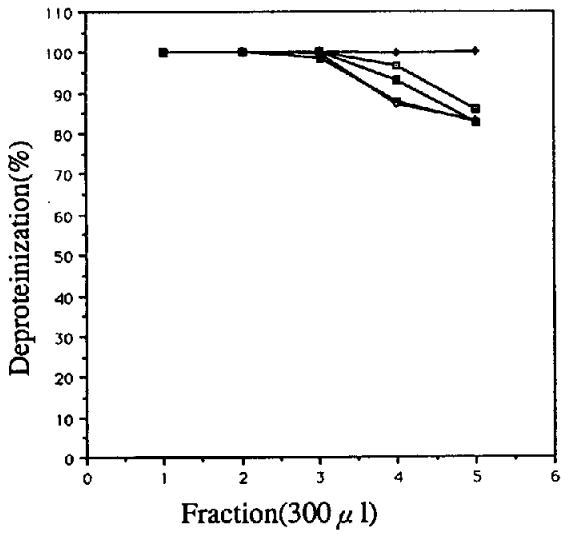

Fig. 1. Effect of pH of eluent on deproteinization (dilution method). Eluent, 10 m*M* sodium phosphate buffer; sample, plasma–eluent (1:3). pH: □ = 6.0, ◆ = 6.5; ■ = 6.8; ◇ = 7.7; ● = 8.5; (last two sets of data are almost identical).

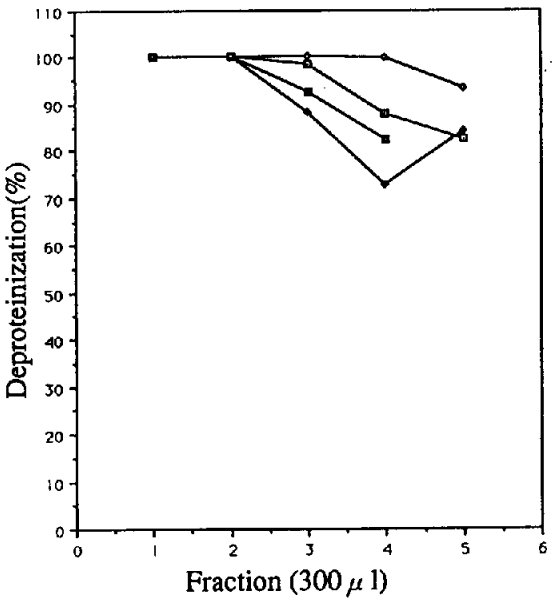

Fig. 2. Effect of salt of phosphate buffer and 0.9% sodium chloride on deproteinization (dilution method). Eluent, 10 m*M* phosphate buffer (pH 6.8); sample, plasma–eluent (1:3). Phosphate salt: □ = Na; ◆ = NH_4; ■ = K. ◇ = 0.9%NaCl.

When the ratio of plasma to 10 m*M* sodium phosphate buffer was 1:3, the deproteinization efficiency was over 99% in fractions 1–3. Compounds in human plasma may be eluted completely in the above fraction. In particular, buffer (pH 6.5) was the most suitable eluent, because the deproteinization efficiency was 100% in fractions 1–3 and 5 and 99.7% in fraction 4. A comparison of sodium, ammonium and potassium salts of 10 m*M* phosphate buffer (pH 6.8) showed that the sodium salt was a suitable component in the three eluents. Further comparison of 10 and 50 m*M* sodium phosphate buffers and the amount of plasma showed that a higher deproteinization efficiency was obtained when the concentration of the eluent was lower and the amount of plasma was smaller; 0.9% NaCl solution was suitable as the eluent in the same way as described for 10 m*M* phosphate buffer (pH 6.5) because the deproteinization efficiency was almost the same in fractions 1–4.

In the injection method, aqueous solutions [water, 0.9% NaCl and 10 m*M* sodium phos-

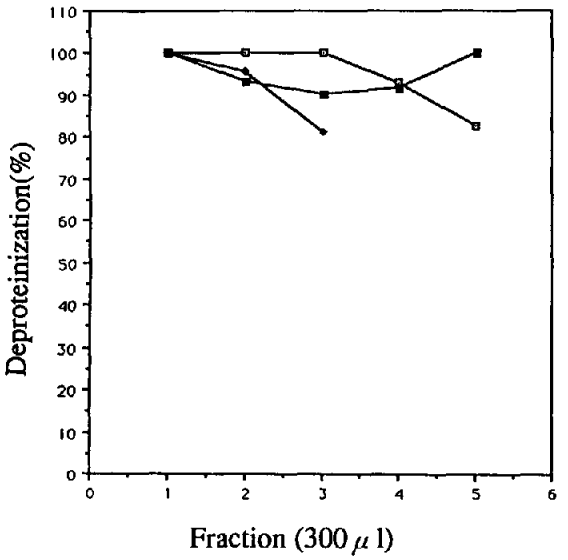

Fig. 3. Effect of amount of plasma and concentration of eluents on deproteinization (dilution method). □: Eluent, 10 m*M* sodium phosphate buffer (pH 7.7); sample, plasma eluent (1:3). ■: Eluent, 10 m*M* sodium phosphate buffer (pH 7.7); sample, plasma–eluent (1:1). ◆: Eluent concentration, 50 m*M* sodium phosphate buffer (pH 7.7); sample, plasma–eluent (1:1).

phate buffer (pH 6.8 and 8.6)] and organic solvents (acetonitrile and methanol) were examined as eluents using 20–300 μl of plasma.

The deproteinization efficiency was about 99–100% in fractions 1–5 for any plasma samples using both the aqueous and organic solvents.

Plasma protein was deproteinized within only 1 min by the dilution method or the injection method. The injection method was simpler and more convenient than the dilution method because it required no dilution of samples prior to passing through the cartridge and was usable with both aqueous and organic solvents.

3.2. Effect of eluent on recoveries of theophylline and the internal standard (I.S.)

Elution of standard theophylline and the I.S. from PCPure cartridges was examined (Table 2). Theophylline and the I.S. were determined in fractions 1–5 (each 300 μl).

In the dilution method, water, 0.9% NaCl solution and 10 m*M* sodium phosphate buffer (pH 6.8) were examined as eluents. It can be

Table 1
Deproteinization of fractions using a PCPure cartridge with the injection method

Plasma (μl)	Eluent	Deproteinization (%) in fraction (300 μl)				
		1	2	3	4	5
100	H_2O	100.0	99.0	99.4	99.4	100.0
50	10 m*M* PB[a] (pH 6.8)	100.0	100.0	99.3	99.3	98.8
100	10 m*M* PB (pH 6.8)	100.0	100.0	99.2	99.1	100.0
50	10 m*M* PB (pH 8.6)	100.0	100.0	99.3	99.3	98.8
100	10 m*M* PB (pH 8.6)	100.0	100.0	99.2	99.1	100.0
50[b]	0.9% NaCl	100.0	100.0	100.0	100.0	100.0
300	0.9% NaCl	100.0	98.1	98.7	99.9	99.9
100	5% CH_3CN	100.0	99.9	99.5	99.9	100.0
100	10% CH_3CN[c]	100.0	100.0	100.0	100.0	100.0
300	50% CH_3CN	98.8	100.0	99.8	100.0	100.0
100	10% CH_3OH	100.0	100.0	99.7	99.8	99.8
100	10% C_2H_5OH	100.0	100.0	100.0	100.0	100.0
20	10 m*M*PB(pH 6.8)[d]	100.0	100.0	100.0	100.0	100.0

[a] PB = sodium phosphate buffer.
[b] 100 and 200 μl of plasma: deproteinization in all fractions = 100.0%.
[c] 25%, 50%, 75% and 100% CH_3CN: deproteinization in all fractions = 100.0%.
[d] Containing 0.25% L-cysteine.

Table 2
Effect of eluent on recoveries of theophylline, diazepam and the I.S. (dilution method)

Fraction	Sample	Recovery (%)		
		Theophylline		Diazepam
		H_2O	0.9% NaCl	0.9% NaCl
1	Drug	80.5	100	86.1
	I.S.	92.8	100	71.3
2	Drug	87.6	100	87.4
	I.S.	94.6	100	75.3
3	Drug	94.4	100	87.5
	I.S.	94.6	100	78.6
4	Drug	95.5	100	89.2
	I.S.	94.6	100	80.0
5	Drug	96.5	100	100.0
	I.S.	94.6	100	101.0

Sample concentration: theophylline, 25 μg/ml and I.S., 25 μg/ml; diazepam, 1 μg/ml and I.S., 10 μg/ml.

Table 3
Effect of eluent on recoveries of theophylline and the I.S. (injection method)

Eluent	Recovery in fraction (300 μl) (%)			
	1		2	
	Theophylline	I.S.	Theophylline	I.S.
H_2O	99.3	100	0.7	0
10 m*M* PB[a] (pH 6.8)	98.3	100	1.7	0
0.9% NaCl	97.1	100	2.9	0
5% CH_3CN	97.4	100	2.6	0
10% CH_3CN	97.5	100	2.5	0
25% CH_3CN	97.1	97.1	2.9	2.9
50% CH_3CN	100	100	0	0
75% CH_3CN	100	100	0	0
100% CH_3CN	94.4	90.6	5.6	9.4
10% CH_3OH	96.2	100	3.8	0
25% CH_3OH	95.9	99.3	4.1	0.7
50% CH_3OH	100	100	0	0
75% CH_3OH	100	100	0	0
100% CH_3OH	86.7	92.6	13.3	7.4

Amounts injected: theophylline, 1.25 μg; I.S., 1.25 μg.
[a] PB = sodium phosphate buffer.

seen in Table 2 that the recoveries of both compounds in fractions 1 and 5 were about 95% using water as the eluent. On the other hand, both compounds were recovered completely in fractions 1–5 using 0.9% NaCl solution. Phosphate buffer was not suitable as the eluent, because both compounds showed peaks with inflections on the chromatograms for all fractions, and thus could not be measured. The 0.9% NaCl solution was selected as a suitable eluent, considering the complete elution of both compounds and deproteinization of protein (Fig. 3).

In the injection method, water, 0.9% NaCl solution, 10 m*M* sodium phosphate buffer (pH 6.8), acetonitrile and methanol were examined as eluents (Table 3). It was found that 10% aqueous acetonitrile solution was a suitable eluent, considering the removal of protein and complete elution of theophylline and the I.S.

3.3. Effect of eluent on recoveries of diazepam and the I.S.

Elution of standard diazepam and the I.S. from PCPure cartridges was examined (Table 4). Diazepam and the I.S. were determined in fractions 1–5 (each 300 μl).

In the dilution method, 0.9% NaCl solution was examined. However, unsatisfactory recoveries of both diazepam and the I.S. were obtained (Table 2), because of their solubility in the eluent. Thus, the dilution method might not be suitable for compounds with some solubility in the eluent.

In the injection method, water, 0.9% NaCl solution, 10 m*M* sodium phosphate buffer (pH 6.8), acetonitrile and methanol were examined as eluents. It was found (Table 4) that complete and rapid elution (first elution of 300 μl) of both diazepam and the I.S. was obtained by using 50% and 60% aqueous acetonitrile solution. In subsequent work, 50% acetonitrile aqueous solution was adopted as the eluent, considering the deproteinization of protein, complete and rapid elution of diazepam and the I.S. and the solvent front on the chromatogram.

Table 4
Effect of eluent on recoveries of diazepam and the I.S. (injection method)

Eluent	Recovery in fraction (300 μl) (%)					
	1		2		3	
	Diazepam	I.S.	Diazepam	I.S.	Diazepam	I.S.
H_2O	79.5	95.5	20.5	4.5	0	0
10 m*M* PB[a] (pH 6.8)	54.3	83.6	41.2	16.4	4.5	0
0.9% NaCl	87.8	91.9	9.7	8.1	2.4	0
10% CH_3CN	93.9	97.2	6.1	2.8	0	0
25% CH_3CN	94.6	96.4	5.4	3.6	0	0
50% CH_3CN	100	100	0	0	0	0
60% CH_3CN	100	100	0	0	0	0
75% CH_3CN	91.7	94.3	8.3	5.7	0	0
100% CH_3CN	73.0	85.5	27.0	14.5	0	0

Amounts injected: diazepam, 0.1 μg; I.S., 1 μg.
[a] PB = sodium phosphate buffer.

3.4. Chromatography of theophylline and diazepam

Theophylline and diazepam were determined in human plasma by HPLC with UV detection using PCPure cartridges and 10% or 50% aqueous acetonitrile solutions.

Typical chromatograms of theophylline (detection limit *ca.* 2.9 ng at a signal-to-noise ratio of 2) and diazepam (detection limit *ca.* 0.25 ng at a signal-to-noise ratio of 2) obtained by the injection method are shown in Fig. 4. These drugs and the I.S. were detected without interference from coexisting compounds. Both drugs and both I.S. of blank plasma (drug-free) were not detected on the chromatograms.

3.5. Determination of theophylline and diazepam

Calibration graphs for theophylline and diazepam were constructed by plotting the peak-height ratio (y) with respect to the I.S. against the amount of theophylline and diazepam (x, μg). Satisfactory linearity was obtained in the ranges 0.1–10 μg ($y = 0.068x - 0.001$) for theophylline and 0.1–65 ng ($y = 0.494x - 0.004$) for diazepam.

Known amounts of theophylline and diazepam were added to drug-free plasma and the overall recoveries were calculated by the standard addition method. The recoveries of theophylline and diazepam were over 90% (Table 5).

Analytical data for theophylline and diazepam obtained using the proposed method and pub-

Table 5
Recoveries of theophylline and diazepam added to drug-free plasma by injection method

Concentration (μg/ml)		R.S.D. ($n = 5$) (%)	Recovery (%)
Added	Found		
Theophylline			
0	0	–	
5	4.6	1.25	92
10	9.7	1.23	97
25	24.5	1.24	98
50	50.3	1.21	100.6
Diazepam			
0	0	–	
0.25	0.23	3.3	92
0.5	0.47	3.4	94
1	0.94	3.3	94
2	1.87	3.2	93.5

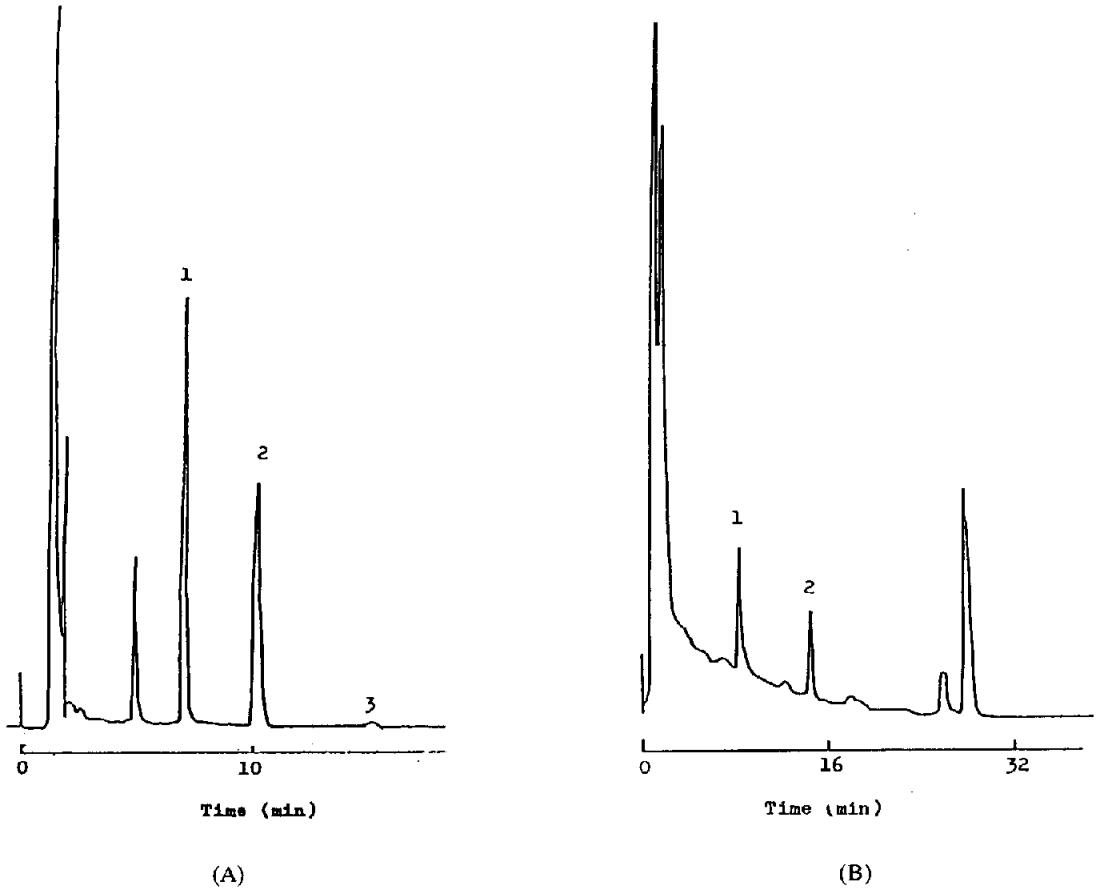

Fig. 4. (A) Chromatogram of theophylline in human plasma obtained by HPLC with UV detection at 280 nm after deproteinization using a PCPure cartridge. Amount of theophylline injected, 62.5 ng in 20 μl. HPLC was carried out on a 15 × 0.46 cm I.D. column of Inertsil ODS-2 (5 μm) using acetonitrile–water (5:95) as the eluent at a flow-rate of 1.0 ml/min at 40°C with 0.05 AUFS. Peaks: 1 = theophylline; 2 = 7-(2-hydroxyethyl)theophylline (I.S.); 3 = caffeine. (B) Chromatogram of diazepam in human plasma obtained by HPLC with UV detection at 240 nm after deproteinization using a PCPure cartridge. Amount of diazepam injected, 3.33 ng in 20 μl. HPLC was carried out on a 15 × 0.46 cm I.D. column of Inertsil ODS-2 (5 μm) using acetonitrile–water (50:50) as the eluent at a flow-rate of 1.0 ml/min at 40°C with 0.005 AUFS. Peaks: 1 = diazepam; 2 = 4,4′-difluorobenzophenone (I.S.).

lished methods were compared (Table 6). To examine plasma protein binding, plasma samples were incubated at 37°C for 30 min.

The results in Table 6 show that the analytical data for theophylline and diazepam in human plasma were almost identical, irrespective of incubation at 37°C or not. These results might suggest that PCPure cartridges can be applied to protein-binding compounds. The relative standard deviations (R.S.D.) of the theophylline and diazepam recoveries were 1.23% and 3.2% (n = 5), respectively.

Hence the simple and rapid determination of theophylline and diazepam in human plasma was achieved by the proposed method using both dilution and injection methods.

Table 6
Comparison of analytical data for theophylline and diazepam in human plasma using the proposed method (injection method) and published methods

Method	Recovery (%)	
	RT[a], 10 s	37°C, 30 min
Theophylline		
Proposed method		
Dilution method	99.7	98.8
Injection method	99.6	99.1
Published methods		
6% $HClO_4$ [11]	100.5	99.5
20% $HClO_4$ [12]	99.4	99.4
Diazepam		
Proposed method		
Injection method	93.5	93.7
Published methods		
Acetonitrile [22]	95.5	95.6
Methanol [23]	96.0	95.0

Sample: theophylline, 1.25 μg in human plasma (50 μl); diazepam, 0.1 μg in human plasma (100 μl).
[a] Room temperature.

4. Conclusion

The use of commercially available PCPure cartridges for the simple, rapid and micro-sample deproteinization of human plasma seems very useful for the determination of theophylline and diazepam in human plasma by HPLC with UV detection.

After human plasma was passed through the cartridge, followed by suitable elution, the protein-free compounds were obtained within only 1 min in the eluate without the need for centrifugation.

The proposed method is satisfactory with respect to simplicity, rapidity and accuracy in comparison with published methods [10,11,18–24]. It is simple and convenient, and therefore applicable to the automated routine determination of theophylline and diazepam in human plasma. Application of the proposed method to the determination of drugs and their metabolites, vitamins and amino acids in biological fluids is being studied. PCPure cartridges could also be applied for the purification of proteins.

5. References

[1] Z. Deyl, A. Hyanek and M. Horakova, *J. Chromatogr.*, 375 (1986) 177.
[2] G. Sarwar and H.G. Boung, *J. Chromatogr.*, 615 (1993) 1.
[3] K. Iriyama, M. Yoshiura, T. Iwamoto and Y. Ozaki, *Anal. Biochem.*, 141 (1984) 238.
[4] M.A. Kotnink, J.H. Skala, H.E. Sauberrlich and S.T. Omaye, *J. Liq. Chromatogr.*, 8 (1985) 31.
[5] L.S. Liau, B.L. Lee, A.L. New and C.N. Ong, *J. Chromatogr.*, 612 (1993) 63.
[6] T.L. Perry and S. Hansen, *Clin. Chim. Acta*, 25 (1969) 53.
[7] H. Godel, T. Graser, P. Foldi, P. Pfaender and P. Furst, *J. Chromatogr.*, 297 (1984) 49.
[8] Y. Yamakawa and J. Chiba, *J. Liq. Chromatogr.*, 11 (1988) 663.
[9] A.M. Uhie, E.A. McIernan, J.T. Tucher and K. Odea, *J. Chromatogr.*, 564 (1991) 81.
[10] J. Schmitt and C.J. McClain, *J. Chromatogr.*, 568 (1991) 207.
[11] J. Blanchard, S. Harvey and W.J. Morgan, *J. Chromatogr. Sci.*, 28 (1990) 303.
[12] J. Moncrieff, *J. Chromatogr.*, 568 (1991) 177.
[13] T. Kadoyaa, T. Isobe, M. Ebihara, T. Ogawa, M. Sumita, H. Kuwahara, A. Kobayashi, T. Ishikawa and T. Okuyama, *J. Liq. Chromatogr.*, 9 (1986) 3543.
[14] S. Hjerten, J. Lindererg and B. Shopova, *J. Chromatogr.*, 440 (1988) 305.
[15] Y. Yamakawa a . Chiba, *J. Liq. Chromatogr.*, 11 (1988) 663.
[16] T. Okuyama, M. Tanaka and K. Takahashi, *J. Chromatogr.*, 466 (1989) 390.
[17] M. Saito and Z. Tamura, *Drug Level Monitoring*, Hirokawa, Tokyo, 1982, p. C-37.
[18] K.T. Muir, J.H.G. Jonkman, D. Tang, M. Kunitani and S. Riegelman, *J. Chromatogr.*, 221 (1980) 85.
[19] R. Chiou, R.J. Stubbs and W.F. Bayne, *J. Chromatogr.*, 422 (1987) 281.
[20] S. Hotchkiss and J. Caldwell, *J. Chromatogr.*, 423 (1987) 179.
[21] K. Matsumoto, H. Kikuchi, H. Iri, H. Takahashi and M. Umino, *J. Chromatogr.*, 425 (1988) 323.
[22] M.J. Koenigbauer, S.P. Assenza, R.C. Willoughby and M.A. Curtis, *J. Chromatogr.*, 413 (1987) 161.
[23] C.E. Lau, S. Dolan and M. Tang, *J. Chromatogr.*, 416 (1987) 212.
[24] C.D. Gaitonde and P.V. Pathak. *J. Chromatogr.*, 528 (1990) 407.