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Use of 3-(1,8-naphthalimido)propyl-modified silyl silica gel as a stationary phase for the high-performance liquid chromatographic separation of purine derivatives

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Abstract

The use of a packing material, 3-(1,8-naphthalimido)propyl-modified silyl silica gel (NAIP), as a stationary phase for high-performance liquid chromatography, has been studied. NAIP behaved like a reversed-phase stationary phase with some π–π interaction. Purine derivatives, i.e., xanthine, hypoxanthine, uric acid, theobromine, theophylline and caffeine, were separated by a column packed with NAIP using an eluent of borate solution (pH 6.4)–MeOH (50:50, v/v). Of these, caffeine was selected as the target of the subsequent investigation and its determination was examined in commercially available medicinal drinks and pharmaceutical preparations. The average recoveries of caffeine were 98.0–107.4% for five drinks and 99.6–107.8% for five tablets and one powder. Subsequently, determination of caffeine and its metabolites in human plasma was examined. In twelve normal human plasma, caffeine levels ranged from 0.24 to 4.26 μg/ml. Time curves of plasma caffeine concentrations and those of its demethylated metabolite, 1,7-dimethylxanthine (1,7-DMX), after an oral ingestion of caffeine (200 mg) were measured by the proposed method and it was found that the maximum concentrations of caffeine and 1,7-DMX were obtained at 1–1.5 h and 3–6 h after ingestion, respectively.

1. Introduction

In the course of the study aimed at the development of new column packing materials for high-performance liquid chromatography (HPLC), we prepared a series of 3-(N-substituted)aminopropylsilyl silica gels and evaluated them for their ability to separate biologically important compounds, such as, for example, water-soluble vitamins [1] and sugars [2]. Recently, we have developed two types of gels: 3-(1-naphthoylamino)- and 3-(1-anthroylamino)-propyl-modified silyl silica gels. Having an aromatic moiety, these were shown to behave in a separation mode by a π–π or hydrophobic interaction, and were used for the separation of nucleic acids [3]. Further, five more 3-(N-substituted)aminopropyl-modified silyl silica gels immobilizing organic dyes or aromatic dicarboxylic anhydrides were prepared in anticipation of a π–π interaction and examined for their ability to separate purine derivatives related to nucleic acids [4]. Of these, 3-(1,8-naphthalimido)propyl-modified silyl silica gel (NAIP) was shown to be a useful gel for this purpose. Caffeine, having a purine skeleton, is known as a central nervous

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system stimulant and has been used as a cardiac and respiratory stimulant and as a diuretic. Thus a simple and precise method for the determination of caffeine is required for pharmaceutical and biomedical studies, and many methods have been developed, including HPLC with ultraviolet (UV) detection [5–7] and with diode-array detection [8], micellar electrokinetic capillary chromatography [7,9], and gas chromatography and fluorescent polarization immunoassay [10]. In this study, we have evaluated the NAIP column for the separation of purine derivatives and subsequently applied it to the determination of caffeine in commercially available medicinal drinks and pharmaceutical tablets. Finally, we applied the NAIP column to the determination of caffeine and its major metabolite, 1,7-dimethylxanthine, in human plasma after ingestion of caffeine.

2. Experimental

2.1. Chemicals

Xanthine, hypoxanthine, uric acid, theobromine, theophylline, and caffeine were purchased from Wako Pure Chemicals (Tokyo, Japan). 1,3-Dimethyl-7-(2-hydroxyethyl)xanthine and 1,7-dimethylxanthine (1,7-DMX) were obtained from Sigma Chemical (St. Louis, MO, USA). Pentoxifylline and propentofylline were kindly supplied as gifts by Hoechst Japan (Tokyo, Japan). The stock solutions ($1 \cdot 10^{-4}$ M) of these compounds were prepared by dissolving with water, except for uric acid and xanthine, which were dissolved in 0.05 M borate solution (pH 8.0).

NAIP (Fig. 1) was prepared from 3-aminopropylsilyl silica gel and 1,8-naphthalic anhydride by the method described previously [4]: particle size, 5 μm; pore diameter, 120 Å; elemental analysis (C, 11.69; H, 1.20; N, 1.29%). NAIP was packed by a slurry method with a mixture of glycerine and methanol (50:50, v/v) in a stainless-steel column (150 × 6 mm I.D.).

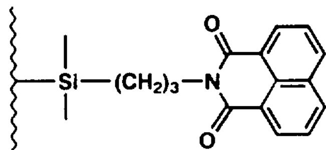

Fig. 1. NAIP structure.

Other reagents were analytical grade. Methanol was redistilled and passed through a membrane filter (0.2 μm). Water was deionized and distilled once.

Borate solutions were prepared as follows: H_3B0_4 (6.183 g) and NaCl (1.461 g) were dissolved in 500 ml water. The solution was brought to the appropriate pH by adding of $Na_2B_4O_7$/H_2O in 250 ml water.

2.2. Sample preparation

Pharmaceutical preparations and drinks containing caffeine were commercially available medicines. Drinks were diluted 4–25 times with water to prepare samples. Five tablets were accurately weighed and finely powdered by a mortar. A part of the resultant powder was accurately weighed, dissolved in appropriate volumes of water, and filtered. The filtrate was used as sample solution. To prepare the powder sample, a powder preparation was accurately weighed, dissolved in water and filtered to prepare the sample solution.

2.3. HPLC apparatus and conditions

The HPLC system consists of a Tosoh CCPD pump (Tokyo, Japan), a Hitachi 638-0410 variable-wavelength UV monitor (Tokyo, Japan), a Rheodyne 7125 loop injector (Cotati, USA) with a 20-μl sample injector, a NAIP column (5 μm, 150 × 6 mm I.D.) in a column oven (Tosoh, CO 8010), and a Rikadenki R-01 recorder (Tokyo, Japan). The column temperature was maintained at 30°C. The eluent was borate solution (pH 6.4)–methanol at a flow-rate of 1.0 ml/min. The eluate was monitored at 270 nm.

2.4. Subjects and blood specimens

Samples were obtained from twelve healthy volunteers (mean age 24.8 years, range 20–39 years). Venous blood was collected in an EDTA Vacutainer tube and centrifuged at 1500 *g* for 20 min at 4°C. The supernatant was immediately treated or frozen and stored at −20°C. For the time curve measurements, three volunteers took decaffeinated food for the 24 h preceding ingestion of caffeine. After ingestion of caffeine (200 mg), venous blood was collected at regular intervals.

2.5. Determination of caffeine and 1,7-DMX in human blood plasma

Working curves for the determination of caffeine in plasma were derived as follows. To each 100 μl of plasma was added 10 μl each of a known concentration of caffeine anhydride and 1,3-dimethyl-7-(2-hydroxyethyl)xanthine as an internal standard (I.S.) in water. After vortex mixing, 1 ml of acetone was added, vortex-mixed for 1 min and centrifuged for 5 min at 2500 *g*. The supernatant was passed through a membrane filter (0.45 μm) and an aliquot (20 μl) was injected onto the HPLC system.

3. Results and discussion

3.1. HPLC conditions

The separation properties of the NAIP packed column were examined using theobromine (2 nmol/injection) and borate solution (pH 7.0)–methanol (50:50, v/v) as a sample and an eluent, respectively. At 20°C the retention time (t_R, in min), theoretical plate number, and tailing factor were 16.9, 5400, and 1.58, respectively. For six of the purine derivatives, i.e., xanthine, hypoxanthine, uric acid, theobromine, theophylline, and caffeine, the effect of salt species in eluents (50% methanol) on their separation was first examined; among the three solutions examined [borate solution (pH 7.0), 0.05 *M* phosphate solution (pH 7.0), and 0.05 *M* acetate solution (pH 7.0)], borate solution gave the preferable separation. In this study, we selected borate solution and examined its effects on the separation.

The content of borate solution (pH 7.0) in an eluent affected the t_R to an appreciable extent. The t_R's for all compounds increased with an increase of borate solution content. This might mean that the separation is achieved in a mode similar to the reversed-phase one. In the pH range of 6.0–7.5, a relatively good separation was obtained with a borate solution with pH on the low side; pH 6.4 was selected. The capacity factor (k') for the separation of purine derivatives including two vasodilators, propentofylline, and pentoxifylline was affected appreciably by the column temperature; k' decreased with an increase in temperature (Fig. 2). Consequently, the column temperature was kept at 30°C for subsequent experiments.

The durability of the column was tested by continuous flow of the eluent [borate solution (pH 6.4)–methanol (60:40, v/v)]; at least for 500 h, the column was able to separate four purine

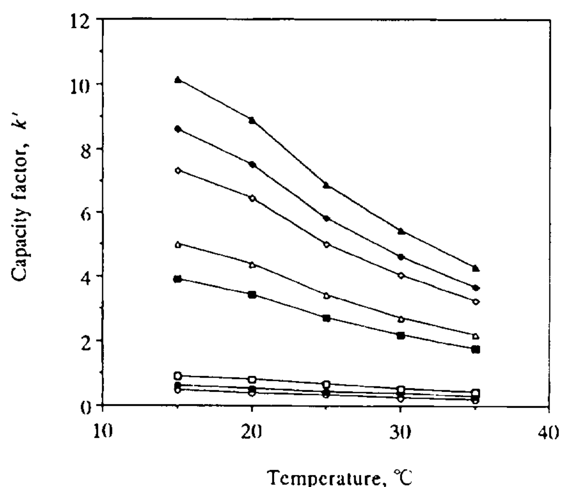

Fig. 2. Effect of column temperature on capacity factor. Sample ($1 \cdot 10^{-4}$ *M*): (○) uric acid; (●) hypoxanthine; (□) xanthine; (■) theobromine; (△) theophylline; (◇) propentofylline; (◆) pentoxifylline; (▲) caffeine. Eluent: borate solution (pH 6.4)–methanol (50:50, v/v); flow-rate: 1.0 ml/min.

derivatives. Fig. 3 shows a representative chromatogram of purine derivatives. The differences in t_R's for these compounds on NAIP might be based on the reversed-phase-like separation with some $\pi-\pi$ interaction and also seemed to be affected by the substituted group, CH_3, on the purine skeleton. Uric acid, hypoxanthine, and xanthine, having no CH_3 group, were eluted fast, and caffeine, with three CH_3 groups, was eluted the most slowly. However, the retention mechanism with $\pi-\pi$ interaction has not been clarified.

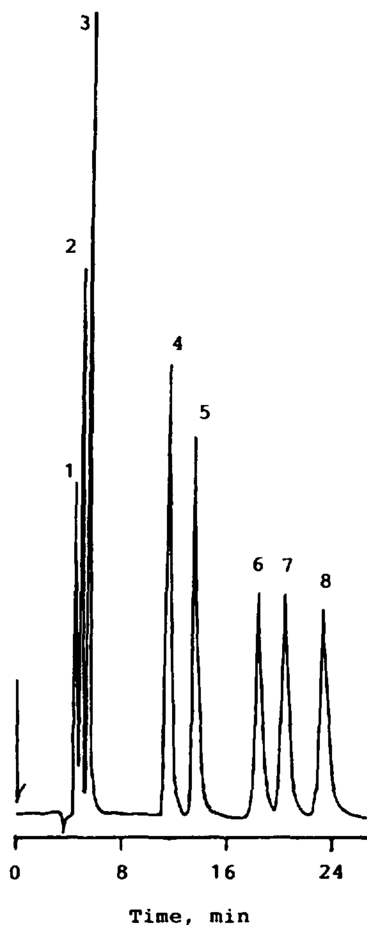

Fig. 3. Chromatogram of purine derivatives. Sample ($1 \cdot 10^{-4}$ *M*). Peaks: 1 = uric acid; 2 = hypoxanthine; 3 = xanthine; 4 = theobromine; 5 = theophylline; 6 = propentofylline; 7 = pentoxifylline; 8 = caffeine. Eluent: borate solution (pH 6.4)–methanol (50:50, v/v); flow-rate: 1.0 ml/min.

3.2. Determination of caffeine in drinks, tablets, and powder

Caffeine is not only an important pharmaceutical compound as a central nervous system stimulant, but also a widely consumed compound present in tea, coffee, and drinks. In this work, therefore, we tried to determine caffeine in commercially available medicinal drinks and pharmaceutical preparations. Rapid separation is an advantage in analysis. The chromatograms of these samples, obtained under the same elution conditions as Fig. 3, were relatively simple and shortening of retention of caffeine seemed to be possible. Thus we changed the composition of the eluent and obtained favorable separations of caffeine from other components in drinks and pharmaceutical preparations by using borate solution (PH 6.4)–methanol (35:65, v/v); the resultant t_R of caffeine was ca. 15 min. The calibration curve for caffeine anhydride was linear over the range from 4.86 to 242.7 μg/ml with correlation coefficient (r) of 0.999. The average contents of caffeine in five drinks and six preparations (tablets and powder) were 98.0–107.4% and 99.6–107.8%, respectively (Table 1). Fig. 4 shows representative chromatograms for drinks and tablets.

3.3. Determination of caffeine and 1,7-DMX in plasma

The determination of caffeine and its metabolite, 1,7-DMX, in human plasma was further investigated. These compounds and the I.S. in plasma could not be separated from the plasma components by an elution system such as the one used to produce Fig. 4. Therefore the composition of the eluent was examined again and it was found that borate solution (pH 6.4)–methanol (50:50, v/v) is suitable as an eluent for this purpose. By the eluent system, the peak of theophylline (t_R = 13.5 min) could be separated from that of 1,7-DMX (t_R = 12.6 min). The peaks were identified by co-chromatography and diode-array detection. Working curves prepared by an internal standard method with the peak-height ratio using the spiked plasma were linear over

Table 1
Contents of caffeine in drinks and preparations

	Indicated (mg)	n[a]	S.D. (mg)	Mean found (mg)	Found/indicated (%)
Drink					
a	50	5	0.34	49.1	98.2
b	50	5	0.71	49.0	98.0
c	50	5	1.30	53.7	107.4
d	30	5	0.46	30.9	103.0
e	200	5	2.88	203.5	101.8
Tablet					
f	30	5	0.47	30.6	102.0
g	40	5	1.27	41.2	103.2
h	12.5	5	0.53	13.0	104.3
i	8.3	5	0.81	8.9	107.8
j	8.3	5	0.26	8.9	104.8
Powder					
k	70	5	2.81	69.7	99.6

[a] The number of samples.

the range of 0.6–19.4 μg/ml ($r = 1.000$) for caffeine and 0.6–18.0 μg/ml ($r = 0.998$) for 1,7-DMX, with detection limits of 0.14 and 0.12 μg/ml at a signal-to-noise ratio of 3, respectively.

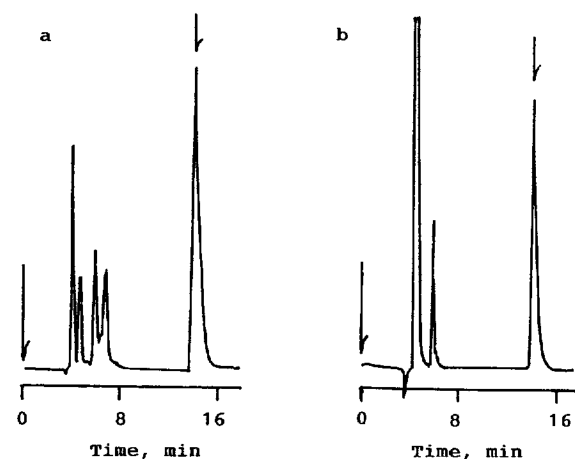

Fig. 4. Chromatograms of caffeine in (a) drink and (b) tablet. Sample: (a) 3 ml of drink (50 ml) was diluted with water to 25 ml, and injected onto the HPLC system; (b) 2.4 mg of powdered tablets were dissolved in 25 ml of water, filtered and injected onto the HPLC system. Eluent: borate solution (pH 6.4)–methanol (35:65, v/v); flow-rate: 1.0 ml/min.

The within-one-day precision was determined five times on the same day with a spiked plasma. The between-day precision was measured once per day for five days. The relative standard deviations of the within-one-day and the be-

Table 2
Concentration of caffeine in normal human blood plasma

Age	Gender	Concentration[a] (μg/ml)
34	m	n.d.[b]
26	m	1.64
22	m	3.79
22	m	n.d.[b]
20	m	1.59
20	m	0.24
39	f	4.26
24	f	2.76
23	f	2.48
23	f	n.d.[b]
23	f	n.d.[b]
22	f	0.93
Mean	1.74	
S.D.	1.55	

[a] Mean of duplicate measurements.
[b] Not detected.

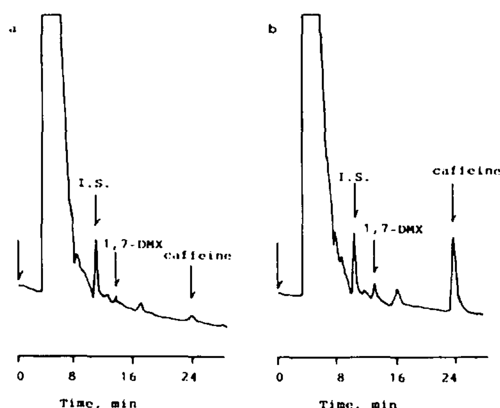

Fig. 5. Chromatograms of plasma sample before and after ingestion of caffeine. Sample: (a) before and (b) 1.5 h after ingestion of a drink containing caffeine (200 mg); I.S.: 2.8 μg/ml. Eluent: borate solution (pH 6.4)–methanol (50:50, v/v); flow-rate: 1.0 ml/min.

tween-day precision were 1.8% and 2.9% for caffeine (9.7 μg/ml), and 0.5% and 2.8% for 1,7-DMX (5.4 μg/ml), respectively. The recoveries were $102.8 \pm 2.6\%$ ($n = 5$) for caffeine (9.7 μg/ml) and $98.7 \pm 2.8\%$ ($n = 5$) for 1,7-DMX (5.4 μg/ml). The concentrations of caffeine in twelve normal human plasma samples were measured; the levels ranged from 0.24 to 4.26 μg/ml (Table 2).

Time curves of caffeine and 1,7-DMX levels in plasma after ingestion of caffeine (200 mg) were measured on three healthy volunteers by the proposed method. Fig. 5 shows the representative chromatograms of plasma before and after ingestion of caffeine. However, the peak for theophylline could not be found because the concentration was lower than the detection limit of the proposed method. As shown in Fig. 6, the highest concentrations were obtained at 1–1.5 h for caffeine and 3–6 h for 1,7-DMX after ingestion.

The proposed method has some advantages. The pretreatment of plasma sample is simpler than those used for extraction methods [8,10], and only requires deproteinization with acetone. Separation with isocratic elution with UV detection is simpler than methods comprising gradient elution with diode-array detection [8] and elution with a change of flow-rate [5]. However, the sensitivity of the method is not sufficient to determine theophylline as a caffeine metabolite, which is a disadvantage compared to the diode-array detection method [8] and fluorescent polarization immunoassay [10].

4. Conclusions

In this work we have successfully used a NAIP column for the separation of purine derivatives and determination of caffeine in medicinal drinks and pharmaceutical preparations. As the proposed method for the determination of caffeine

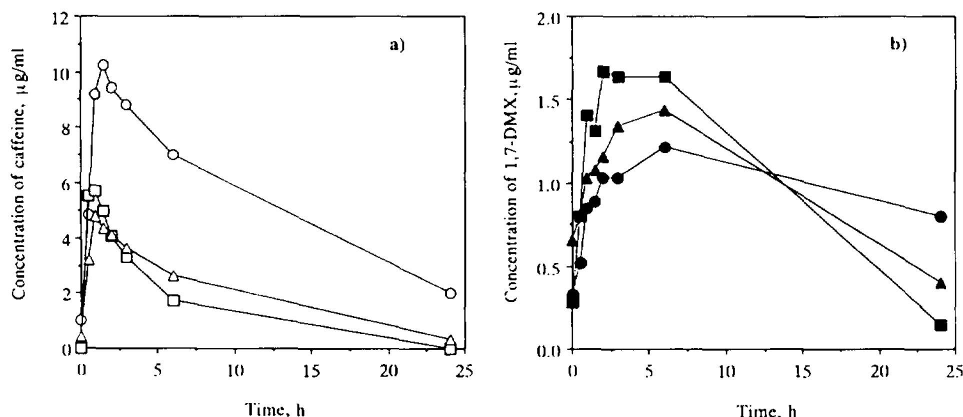

Fig. 6. Time curves of plasma levels of (a) caffeine and (b) 1,7-DMX after ingestion of caffeine. Three volunteers (A: ○ and ●; B: □ and ■; C: △ and ▲) each took a drink containing caffeine (200 mg).

and 1,7-DMX in plasma was simple and precise, it should be useful for biomedical and pharmaceutical studies. With a view to increasing the applicability of a NAIP column having a reversed-phase-like mode with some $\pi-\pi$ interaction, we shall test it for the separation of other biologically important substances such as barbiturates.

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