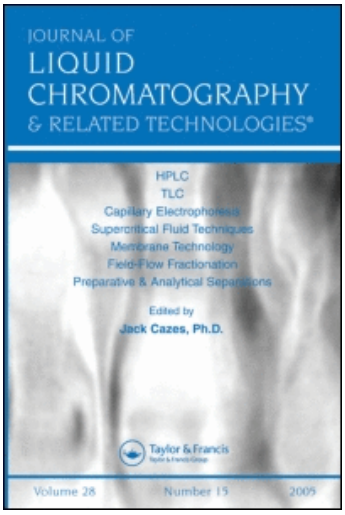

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Micellar Liquid Chromatography of Adenosine in Cacao

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MICELLAR LIQUID CHROMATOGRAPHY OF ADENOSINE IN CACAO

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ABSTRACT

A rapid isocratic micellar HPLC procedure for the separation of adenosine from theobromine in cacao was developed. The adenosine peak was identified by using the enzymatic peak shift technique with adenosine deaminase (ADA). Separation was performed on a polyvinyl alcohol (PVA) column using a mobile phase containing 0.012 M sodium dodecyl sulfate (SDS) and 0.005 M phosphate buffer (pH 11.5). Quantitation and detection limits were determined. In addition the separations obtained on a PVA column with the micellar mobile phase were compared to separations obtained on a C_{18} column using both isocratic and gradient elution.

INTRODUCTION

In the food, candy and soft drink industries, cacao is used extensively. The major component of cacao is theobromine (1). Present in smaller concentrations are other purines such as caffeine and 7-methyl

xanthine. Recently it has been reported that trace amounts of adenosine are also present in cacao (2). To determine the amount of adenosine present using the reversed phase mode of HPLC with a hydroorganic mobile phases is difficult because of the interference caused by the theobromine and other purines (3-15).

In this paper , we demonstrated the usefulness of a micellar mobile phase for the separation of adenosine from theobromine on a PVA column. We also compared our micellar HPLC method with RP-HPLC methods for the determination of adenosine concentration in cacao extracts.

MATERIALS

Apparatus

For the micellar work, a Waters M 6000A pump (Waters Associates, Milford, MA, U.S.A.), equipped with a Rheodyne 7125 injector (Berkeley, CA, U.S.A.) connected to a Waters 440 dual-wavelength detector was used. To avoid contamination in the reversed phase, two Waters M 6000A pumps equipped with a M 660 solvent programmer and a U6K injector were used. The columns used were a 9 µm Asahipak GS 320H polyvinyl alcohol column (25 cm x 7.6 mm I.D.) (Asahi Chemical Industry, Kawasaki, Japan) and a 5 µm Partisphere C_{18} column (11 cm x 4.7 mm I.D.) (Whatman Inc., Clifton, NJ, U.S.A.). A Hamilton cartridge guard column (Hamilton, Reno, NV,

U.S.A.) was used for the PVA column and a guard column (5 cm x 4.6 mm I.D.) filled with Whatman Co:Pell ODS (30-38 μm) was used for the C_{18} column. Chromatograms were recorded on an Omniscribe strip chart recorder (Houston Instruments, Austin, TX, U.S.A.). Peak areas and retention times were obtained with an HP 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Reagents

Theobromine (Thb), 7-methylxanthine (7-MeXan), theophylline (Thp), caffeine (Caf), adenine (Ade), adenosine (Ado), and adenosine deaminase (ADA) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Stock solutions were prepared in doubly-distilled, deionized water, and the pH was adjusted to 7.3 with phosphate buffer. In order to dissolve each compound, several drops of 2 M NaOH solution were added. All stock solutions were stored at -20°C. Electrophoresis grade sodium dodecyl sulfate (SDS) was obtained from Bio-Rad (Richmond, CA, U.S.A.) and was used as received. Defatted cacao sample was obtained from Hershey Foods Co. (Hershey, PA, U.S.A.).

METHODS

The micellar mobile phase was 0.012 M SDS containing 0.005 M Na_2HPO_4 and its pH was adjusted to 11.5 with NaOH. For the work done with the C_{18} column

using an isocratic mode, the methanol mobile phase consisted of 15% methanol and 1% acetic acid (pH 2.8 and 4.8). For the linear gradient mode, 0.02 M KH_2PO_4 containing 0.1% methanol, pH 5.5 (solution A) and 100% methanol (solution B) were used. All the mobile phases were filtered through 0.45 µm Nylon-66 membrane filters (Rainin Instruments, Ridgefield, NJ, U.S.A.).

The sample preparation used by Ritter et al. (2) was adapted with a minor modification. Five grams of defatted cacao sample was added to 60 ml of distilled water. The solution was gently boiled with stirring for 30 min and then cooled to room temperature. The solution was diluted up to 100 ml with distilled water. An aliquot of the solution was centrifuged and the supernatent was filtered through 0.45 µm membrane filters. The extracts were stored at -20°C and thawed before use. A 50 µl aliquot of the extracts was injected for the quantitation. The recovery of Ado was determined by adding 1 µmole of an Ado standard to the extracts. The average recovery of Ado was 91%. For the enzymatic peak shift technique, 1 µl of ADA (2000 Units) was added to 100 µl of the extracts. The solution was buffered with 10 µl of 0.02 M phosphate (pH 7.5), and vortexed for 1 min. The sample mixture was incubated for 5 min at 37°C. Blank tests were also performed under the same conditions.

RESULTS AND DISCUSSION

Kreiser et al. developed a HPLC procedure for determining theobromine and caffeine in cacao products on a μBondapak C_{18} column with the isocratic elution using a mobile phase of 20% methanol containing 1% acetic acid (16); however when we used these conditions, Ado and Thb eluted together. Figure 1 shows representative chromatograms for the standard mixture and the cacao extracts. Although increasing pH resolved Ado and Thb, other closely eluting peaks interfered with the Ado peak in the cacao extracts (Figure 2). Thus the determination of the concentration of Ado in the cacao extracts is difficult even when the methanol composition or pH are varied in the isocratic mode.

With the gradient mode using the same C_{18} column, better resolution of Ado and Thb was observed (Figure 3a). In the separation of the cacao extracts, however, a very closely eluting peak interfered with the Ado peak (Figure 3b).

In the previous paper, we reported that the retention behavior of nucleosides and bases on a PVA column using a micellar mobile phase was quite different from that on RP-HPLC silica-based columns using a hydroorganic mobile phase (17). In addition, with the PVA column a much wider pH range of 3-13 can be used compared to the pH limitation of silica-based columns (pH 2-7.5). With the PVA column Ado was

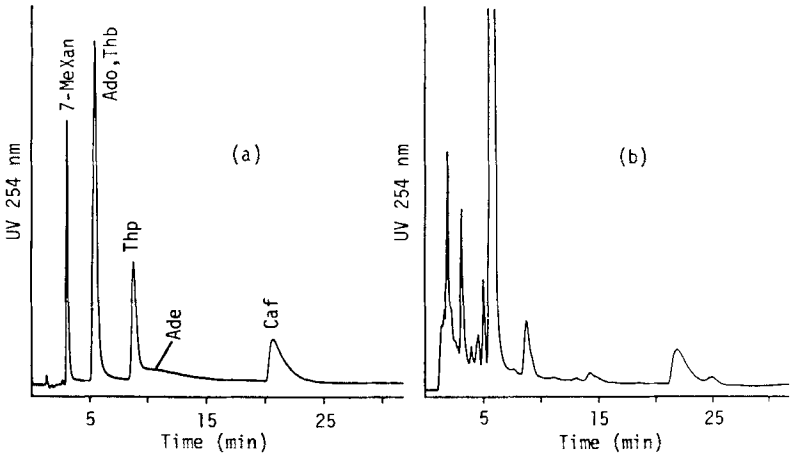

FIGURE 1. Chromatograms of (a) standard mixture and (b) cacao extracts in the isocratic mode. Column, 5 µm Partisphere C_{18} (11 cm x 4.7 mm I.D.); mobile phase, 15% methanol in 1% acetic acid (pH 2.8); flow rate, 1 ml/min; sensitivity, 0.02 AUFS; temperature, ambient.

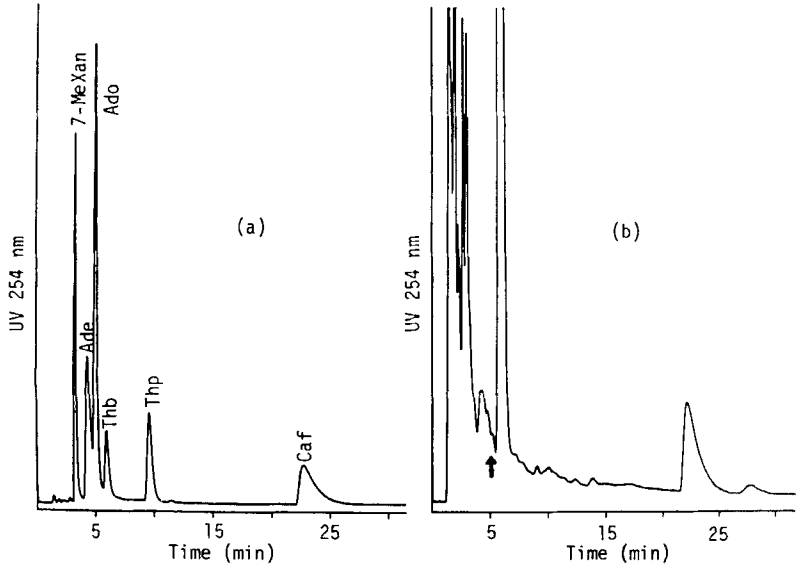

FIGURE 2. Chromatograms of (a) standard mixture and (b) cacao extracts in the isocratic mode. Mobile phase, 15% methanol in 1% acetic acid (pH 4.8). Other chromatographic conditions are the same as in FIGURE 1. The arrow indicates the retention time of Ado.

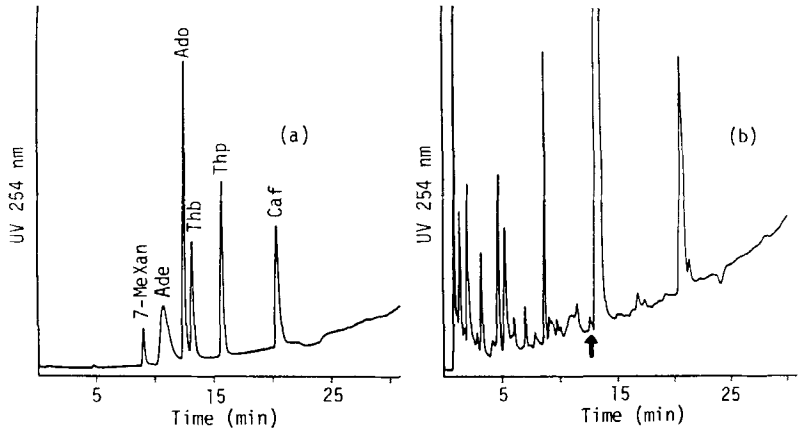

FIGURE 3. Chromatograms of (a) standard mixture and (b) cacao extracts in the linear gradient mode. Mobile phase, 0.1% methanol in 0.02 M KH_2PO_4 (pH 5.5) to 45% methanol, 30 min gradient; flow rate, 1.5 ml/min. Other chromatographic conditions are the same as in FIGURE 1. The arrow indicates the retention time of Ado.

successfully separated from Thb at pH 11.5 with a micellar mobile phase and the total analysis time for the separation of the cacao extracts was reduced (Figure 4). Good reproducibility of retention time was obtained with 0.25% relative standard deviation (n = 9).

Two different techniques were used for the identification of the Ado peak; co-injection of the Ado standard with cacao extracts and the enzymatic peak shift technique with ADA. The retention time of Ado by the former technique was the same as that in the elution of the standard mixture (Figure 4b).

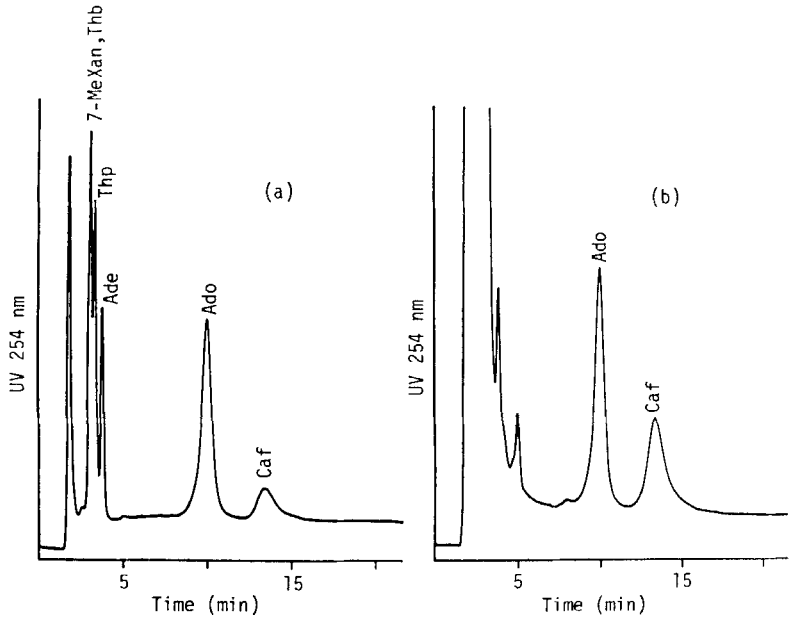

FIGURE 4. Chromatograms of (a) standard mixture and (b) cacao extracts spiked with Ado in the isocratic mode. Column, 9 µm Asahipak GS 320H (25 cm x 7.6 mm I.D.); mobile phase, 0.012 M SDS in 0.005 M Na_2HPO_4 (pH 11.5); flow rate, 2 ml/min. Other chromatographic conditions are the same as in FIGURE 1.

Previously, our laboratory reported the usefulness of the enzymatic peak shift technique for the identification of Ado in human serum with the isocratic mode (3). Also, Ratech et al. demonstrated the applicability of the enzymatic peak shift technique for identifying modified adenine nucleosides in mouse urine with the gradient mode (18). These methods are useful if the Thb level is negligible or if the Ado and Thb peaks are well resolved.

Since Thb is a major xanthine base in cacao (1), a large amount of Thb in the cacao extracts interferes with the identification of the Ado peak when the reversed phase was used. On the contrary, the enzymatic peak shift technique could be used for peak identification with the micellar chromatography since the Ado was completely separated from the amount of Thb in the cacao extracts. Thus the Ado peak disappeared when an aliquot of the cacao extracts was reacted with the ADA (Figure 5a and 5b). The enzyme, itself, did not interfere with the identification of the Ado peak (Figure 5c). Thus the enzymatic peak shift technique with ADA is useful for the identification of Ado in cacao with the micellar liquid chromatographic separation of Ado from the other constituents in the cacao.

Quantitation and detection limits of Ado in cacao extracts were investigated. The calibration curve showed a good linearity (r^2 = 0.999) over the concentration range of 0.1-1 μmole of Ado. The Ado concentration in cacao was found to be 0.029 ± 0.001 mg/g. The detection limit of Ado was 10 nmole at 0.005 AUFS when the signal-to-noise ratio was equal to 2.

In summary, Ado in cacao was successfully separated from other components in cacao by HPLC using a PVA column with a micellar mobile phase. The Ado peak was unambiguously identified and very low

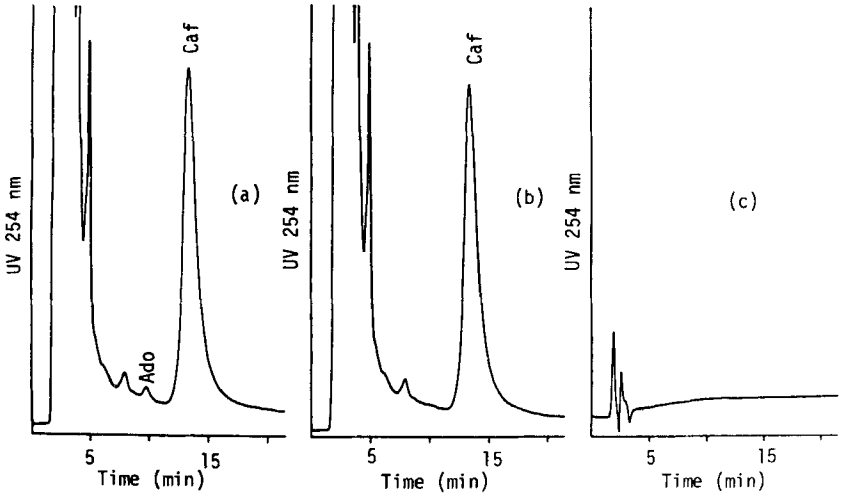

FIGURE 5. Chromatograms of (a) cacao extracts without ADA and (b) with ADA, and (c) ADA only. All chromatographic conditions are the same as in FIGURE 4.

concentrations of Ado in the sample determined. The advantages of using a micellar mobile phase on a PVA column over that of hydroorganic mobile phases on a C_{18} column for the separation of Ado in cacao are; (1) shorter analysis time, (2) isocratic separation of Ado in the presence of a large amount of Thb, (3) low detection limits, (4) unambiguous identification of the Ado peak, and (5) column stability at high pH.

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