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RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR SALIVARY AND SERUM CAFFEINE FOLLOWING AN ORAL LOAD

AN INDICATOR OF LIVER FUNCTION

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SUMMARY

A rapid isocratic reversed-phase high-performance liquid chromatography (HPLC) system for the quantitative measurement of serum and salivary caffeine is described. The best separation of caffeine from other methylxanthines was achieved by chromatography on an ODS-Hypersil column using a solvent system of 0.1 *M* ammonium acetate pH 4.6–acetonitrile (85:15, v/v). The effluent was monitored at 280 nm. Caffeine was extracted from diluted serum and saliva samples (10–500 μl) by adsorption on a small Bond-Elut C_{18} cartridge and recovered by elution with methanol. Thermospray HPLC–mass spectrometry conditions were optimized to afford a means of directly identifying caffeine in samples. The positive-ion mass spectrum was characterized by an intense protonated molecular ion, MH^+, at m/z 195 and negligible fragmentation. When the mass spectrometer was operated in selected ion monitoring mode, caffeine could be detected in less than 1 μl of serum and saliva at a concentration of 1 μg/ml. Caffeine (3.5 mg/kg body wt.) was administered orally to healthy adults, children, and newborn infants, and to patients with liver disease. The clearance rate and half-life were determined as a test of liver function. A prolongation in the elimination of caffeine was observed in patients with liver disease and, although there was some overlap in the values obtained for patients with noncirrhotic liver desease and healthy persons, the oral caffeine load test may usefully serve as a dynamic assessment of liver function in the serial follow-up of patients with liver disease.

INTRODUCTION

Measurement of drug clearance has been proposed as a technique for the quan-

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titative assessment of liver function, and many drugs have been previously evaluated in adults[1,2]. Due to the restrictions on the use of radioactivity or because of potential toxicity, only minimal evaluation of these tests has been performed in pediatric patients.

Caffeine is generally regarded as safe in the range of usual dietary consumption[3,4], and when given orally, is rapidly absorbed, undergoes demethylation via the hepatic mixed-function oxidase system, and is excreted in the urine[5–7]. A prolongation of the caffeine clearance rates has been previously observed in adults with liver diseases[8–12]. Since comparisons of plasma and salivary caffeine concentrations have previously demonstrated good correlations[13], the measurement of salivary caffeine was investigated as a non-invasive alternative to assaying blood levels for the assessment of liver function in pediatric patients. A rapid extraction technique and a highly sensitive high-performance liquid chromatography (HPLC) method were developed, which are suitable for routine use. In addition, direct HPLC–mass spectrometric (MS) analysis by means of the thermospray interface was applied to the definitive identification of caffeine in biological samples.

MATERIAL AND METHODS

Chemicals and reagents

The methylxanthines, caffeine, theophylline, and theobromine, were obtained from Sigma (St. Louis, MO, U.S.A.), and acetonitrile and ammonium acetate (HPLC grade) from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Methanol (Mallinckrodt, St. Louis, MO, U.S.A.) was redistilled before use. All other reagents and chemicals were of reagent grade or better. Reversed-phase octadecylsilane-bonded silica cartridges (Bond-Elut C_{18}, capacity 100 mg/1 ml) were obtained from Analytichem (Harbor City, CA, U.S.A.) and washed with a volume (*ca.* 1 ml) of distilled water, methanol, and distilled water prior to use.

Oral caffeine load test

Caffeine (food grade from Sigma, 3.5 mg/kg body wt.) was dissolved in a small volume of water, fruit juice, or milk and administered orally. Blood and saliva samples were obtained before and at timed intervals after the administration (generally, 1, 2, 4, 6, 8 or 10, and 12 or 14 and 24 h).

Extraction of methylxanthines from serum and saliva

Serum and saliva samples (10–500 μl) were diluted with an equal volume of 0.1 *M* ammonium acetate (pH 4.6) and vortex-mixed for 30 s. The sample was passed through a Bond-Elut C_{18} cartridge to extract methylxanthines. The cartridge was washed with 500 μl of 0.1 *M* ammonium acetate (pH 4.6), followed by 500 μl of water. The methylxanthines were recovered by elution with methanol (1 ml) and evaporated to dryness under nitrogen. Immediately prior to analysis the extract was dissolved in 50 μl methanol thereby avoiding evaporation and possible errors in quantitation.

HPLC of caffeine

HPLC was performed on a Varian Model 5000 chromatograph (Varian, Palo

Alto, CA, U.S.A.), equipped with a Rheodyne injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 10-μl sample loop. Chromatographic separation of methylxanthines was achieved on a 5-μm particle size ODS-Hypersil column, 25 cm × 4.6 mm I.D. (Shandon Southern, Sewickley, PA, U.S.A.). A mobile phase of 0.1 *M* ammonium acetate pH 4.6–acetonitrile (85:15, v/v) was used at a flow-rate of 1.5 ml/min. The methylxanthines were detected at 280 nm.

Quantification of caffeine

Quantification of caffeine in serum and saliva samples was achieved from the peak-height response and comparing this with the peak-height response for a series of caffeine standards of known concentrations in the range (0.2–1.6 μg/ml). The external calibration standards were analyzed with each batch of samples and a linear relationship between peak-height response and caffeine concentration was observed within this calibration range (correlation coefficient $r = 1.00$, slope = 1.009, and intercept = 0.0003).

Thermospray HPLC–MS

The Varian 5000 series high-performance liquid chromatograph was coupled to a Finnigan 4635 quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, U.S.A.) via a thermospray interface. The mass spectrometer was operated in (i) continuous scanning mode over the mass range 110–300 dalton (2 s/cycle) and (ii) selected ion monitoring of the protonated molecular ion for caffeine at m/z 195. Thermospray conditions were: vaporizer temperature 150°C and jet block temperature 210°C. HPLC conditions were as described above.

RESULTS

HPLC analysis of serum and saliva

Fig. 1 indicates typical HPLC chromatograms for serum and salivary samples, collected 2 and 6 h after the administration of an oral dose of caffeine to a healthy child. Under these chromatographic conditions, caffeine was eluted reproducibly within 5 min of injection and was adequately separated from other methylxanthine metabolites or UV-absorbing components in the samples. The precision of the method was determined from replicate analysis of serum and saliva samples with a caffeine concentration of 1.6 μg/ml. The intra-assay coefficient of variation ($n = 5$) for both fluids was 2.5% and the inter-assay precision ($n = 5$) was 5%. The recovery of added amounts of caffeine to serum and saliva samples was found to be quantitative (in excess of 95%). The sensitivity of the method (signal-to-noise ratio, 2.5:1) was sufficient to detect caffeine at a level of 0.005 μg/ml serum or saliva when 0.5 ml of sample volume was taken for analysis.

The relationship between salivary and serum samples collected simultaneously from subjects at intervals following an oral caffeine load is shown in Fig. 2. A linear relationship and good agreement between the two values ($r = 0.98$) was found.

Thermospray HPLC–MS

The optimum thermospray interface temperatures at the flow-rate (1.5 ml/min) used for the UV detection of caffeine were determined by multiple injections of a

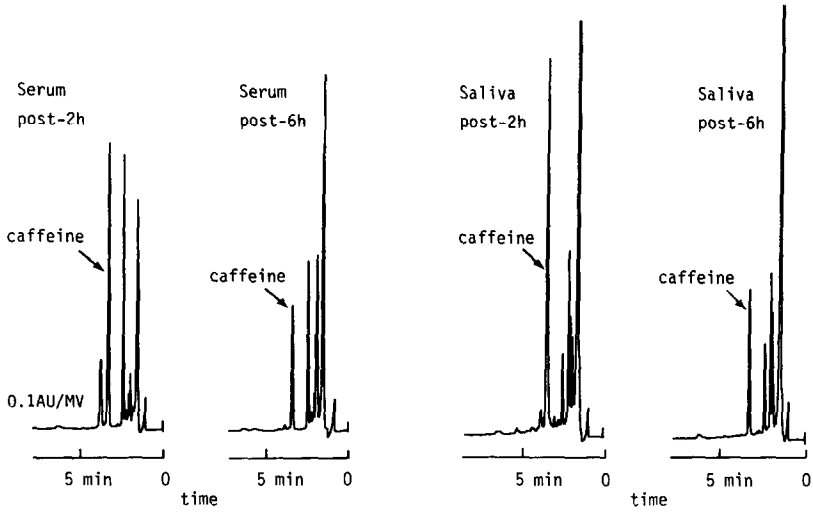


Fig. 1. Typical HPLC chromatograms obtained for serum and saliva samples at 2 and 6 h following an oral dose of caffeine 3.5 mg/kg body weight to a healthy child.

standard solution of caffeine and varying the heater temperatures and repeller voltage. The conditions giving the maximum ionization were found to be a vaporizer temperature of 150°C and block temperature of 210°C.

Fig. 3 illustrates the total ion current chromatograms obtained by continuous scanning over the mass range *m/z* 110–300 for the equivalent of 90 μl of a serum sample (caffeine concentration *ca.* 1.5 μg/ml), injected into the column, and the responses obtained for a mixture of 100 ng each of the methylxanthine standards, theophylline, and caffeine. There was no apparent loss of chromatographic resolution as a result of interfacing the HPLC column with the mass spectrometer. In the full-scan mode the sensitivity was sufficient to detect 10 ng of caffeine. The thermospray positive-ionization mass spectrum for the authentic caffeine standard and the caffeine peak in the HPLC chromatogram of the serum sample were identical (Fig. 4). The soft ionization process of this technique resulted in a positive-ion mass spectrum

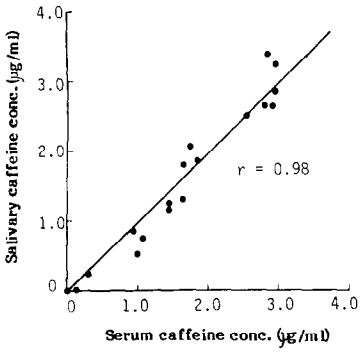
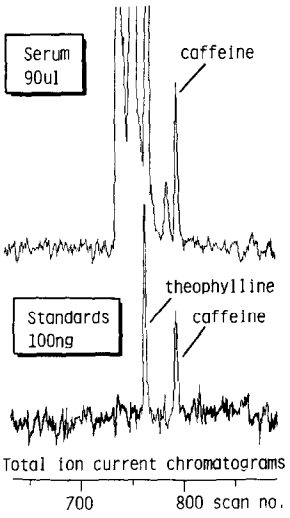


Fig. 2. Correlation between salivary and serum caffeine concentrations.



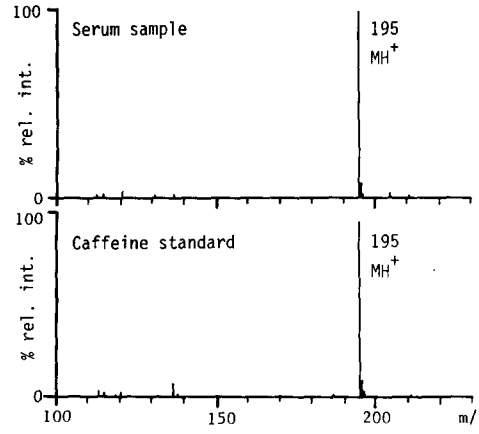


Fig. 3. Total ion current chromatograms obtained in continuous scanning mode (m/z 110–300) from the thermospray HPLC–MS analysis of (i) a serum sample the equivalent of 90 μl injected on column (caffeine concentration approx. 1.5 μg/ml) and (ii) a standard mixture of 100 ng of theophylline and caffeine. The vaporizer temperature was 150°C and jet block temperature 210°C. HPLC conditions were exactly as described above.

Fig. 4. Thermospray ionization positive-ion mass spectra obtained for the peak eluting at the HPLC retention time equivalent to that of caffeine in the serum sample shown in Fig. 3 with that of an authentic caffeine standard. Spectra are characterized by an intense protonated molecular ion (MH^+) and negligible fragmentation.

characterized by an intense ion at m/z 195 corresponding to the protonated molecular ion (MH^+) and negligible fragmentation. When the mass spectrometer was operated in the selected ion monitoring (SIM) mode for the ion m/z 195, significantly enhanced sensitivity, *ca.* 100-fold, was attained. Fig. 5 illustrates SIM chromatograms obtained for the equivalent of 0.45 and 0.50 μl of serum and saliva, respectively (concentrations approx. 1.0 μg/ml), and 500 pg of caffeine injected on-column. The limit of sensitivity of this approach was approximately 200 pg, at a signal-to-noise ratio of 2.5:1.

Caffeine elimination following oral load in normal adults and children and in patients with liver disease

Pharmacokinetic studies of caffeine elimination showed that caffeine clearance in healthy persons is rapid with half-lives ($t_{1/2}$) less than 4 h, while in cholestatic patients the half-lives were markedly prolonged (Fig. 6).

In healthy adults and children the half-lives of caffeine were 5.38 ± 1.22 h (mean ± S.D.) and 2.89 ± 0.46 h, while clearance values were 1.92 ± 0.45 ml/min/kg and 10.49 ml/min/kg respectively. A prolongation of the elimination of caffeine was observed for patients with liver disease, where for cirrhosis the half-life and clearance values were 23.3 ± 14.06 h and 1.18 ± 1.23 ml/min/kg, while in non-cirrhotic liver disease these values were 6.08 ± 4.40 h and 4.72 ± 2.65 ml/min/kg respectively. While some overlap was observed between groups these data reveal a trend, in which

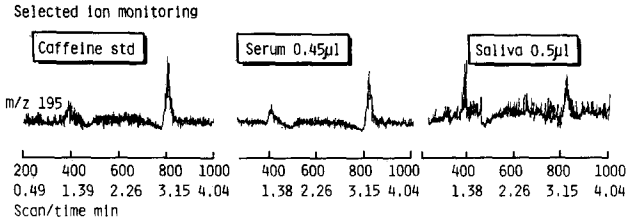


Fig. 5. Thermospray HPLC–MS selected ion current chromatograms of the ion m/z 195 characteristic of the MH^+ of caffeine recorded for the on column injection of 0.45 µl serum and 0.5 µl of saliva taken from a healthy infant 6 h following an oral caffeine load. For comparison the response obtained for 500 pg of a caffeine standard is shown.

the children with liver disease generally had a prolongation of the half-life and lower clearance rates than healthy controls.

DISCUSSION

Many methods have been used for the measurement of serum caffeine concentrations, including gas chromatography[10,14], radioimmunoassay[15], enzyme immunoassay[16], and high-performance liquid chromatography[17–26].

For routine purposes, HPLC offers the most attractive technique for the determination of caffeine, and consequently many different methods have been described. Many of these have focussed on the separation of caffeine from other methylxanthines. Reversed-phase HPLC has been generally adopted; the differences between methods relate to variations in the mobile phase or wavelengths of detection.

The method we describe here specifically for the rapid determination of serum and salivary caffeine for use in an oral caffeine load test for liver function, is similar to the one described by Blanchard *et al.*[19]. These authors used a mobile phase of acetonitrile–sodium acetate buffer. However, a significant improvement in peak

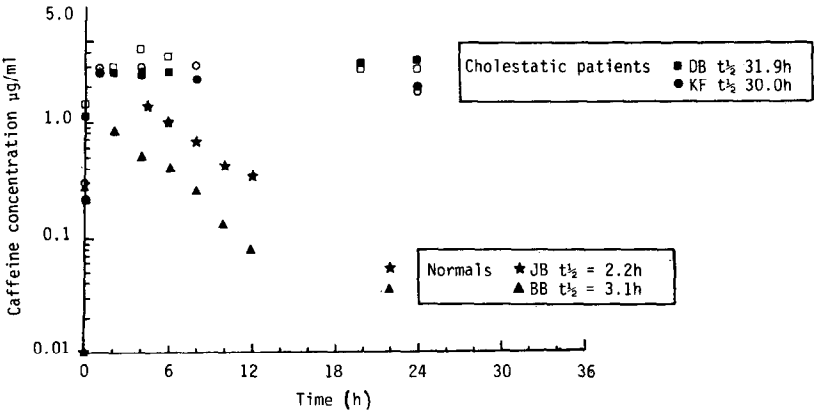


Fig. 6. Salivary (closed symbols) and serum (open symbols) caffeine concentrations determined in serial samples over a 24-h period following an oral caffeine load (3.5 mg/kg body wt.) for two healthy children and two patients with cholestatic liver disease.

shape, resolution, and analysis time was obtained by using a simple isocratic solvent system of 0.1 *M* ammonium acetate pH 4.6–acetonitrile (85:15, v/v). With this system caffeine was eluted within 3–4 min of injection and completely separated from other methylxanthines or components in the sample extracts.

Most of the earlier procedures for the determination of caffeine in serum or saliva employed time-consuming solvent extraction steps, although direct injection of the sample has also been advocated with the use of a pre-column[19]. Solid-phase extraction techniques have been available now for some time, and in most instances they are preferable to liquid–liquid extraction because of their simplicity, speed, and efficiency. The method we describe utilizes a simple and rapid quantitative extraction of caffeine from the samples, following their dilution and passage through a Bond-Elut cartridge. With the use of a Vac-Elut vacuum box, (Analytichem) up to 60 samples/h can be extracted[27].

The application of these cartridges has been reported in recent HPLC methods for caffeine[26,28] and it is evident that this type of procedure will supersede the more conventional methods of liquid–liquid extraction. Indeed, with the recent introduction of automated solid-phase HPLC injection systems (AASP-Varian Instruments, Sunnyvale, CA, U.S.A.), the extracted sample can be inserted directly in-line with the HPLC column and the entire sample can be injected, thereby decreasing analysis time by eliminating the need for sample elution, concentration, and reconstitution, and effectively improving sensitivity and precision. This system is currently under evaluation in our laboratory.

Definitive identification of caffeine in biological samples has generally relied upon MS analysis with introduction of the sample via a gas chromatograph[29] or by direct probe, following purification and separation of caffeine by thin-layer chromatography[30,31] or HPLC[32]. The recent introduction of the thermospray ionization HPLC–MS interface[33–35] has expanded the scope of MS applications and, in particular, permits the direct analysis of HPLC effluents with polar mobile phases at flow-rates of 1–2 ml/min. In addition to acting as an HPLC interface the thermospray affords a soft ionization of solute molecules during their passage through the ion source. Vaporization of the mobile phase takes place in a critically heated and constricted stainless-steel coil, and charge interaction of the solute molecules with salts present in the mobile phase results in the formation of protonated molecular ions and/or adduct ions, depending upon the conditions employed. The mass spectra recorded are similar to those produced with the soft ionization techniques of fast atom bombardment or chemical ionization.

Thermospray HPLC–MS conditions were optimized to obtain a mass spectrum for caffeine which was characterized by an intense ion at *m/z* 195, corresponding to the protonated molecular ion, and virtually no fragmentation of the molecule was seen. No apparent loss in chromatographic resolution was observed, and in the continuous scanning mode, satisfactory spectra were obtained on as little as 10 ng of caffeine injected. Since most of the ionization resided in the $[MH^+]$ ion, selected ion monitoring of *m/z* 195 afforded a highly sensitive and specific HPLC–MS detection system for caffeine. This is evident from the SIM profiles (Fig. 5) obtained where as little as 0.5 μl of serum or saliva was injected on column. In this mode, the limit of detection was at the pg level, and significantly better than that attainable by HPLC with UV detection.

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