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Note

Comparison of thin-layer and gas chromatographic assays for caffeine in plasma

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Caffeine is one of the most widely ingested natural alkaloids and in some countries the per capita annual consumption may be as high as 100 g. Recently there has been an upsurge of interest in the clinical, toxicological and teratogenic effects of chronic caffeine consumption. Until recently the usual technique for the estimation of caffeine in biological fluids has been ultraviolet spectrophotometry [1]. This method is unsatisfactory and lacks the specificity and sensitivity required for pharmacokinetic studies. A radioimmunoassay procedure has been described for the measurement of caffeine in plasma and saliva but requires expertise for the preparation of the caffeine antibody. High-pressure liquid chromatographic (HPLC) methods for caffeine have also been described [2, 3] and one technique has been compared with radioimmunoassay and been found to give comparable results [2]. Thin-layer chromatographic (TLC) methods for caffeine have been developed for plant extracts [4, 5] and recently this method has been extended to measurements of caffeine in plasma [6, 7]. Gas—liquid chromatographic (GLC) procedures for estimation of plasma caffeine levels have also been described [8, 9]. One of these utilised an external standard (hexobarbital) added following the extraction step [8]. A GLC technique has also been developed for caffeine determination in combined analgesic formulations [10]. During the course of a study of caffeine kinetics in man we have had the opportunity to develop and compare TLC and GLC assays for caffeine in plasma.

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EXPERIMENTAL

Thin-layer chromatography

Materials and methods

Caffeine base was obtained from Sigma (London) Chemicals (Poole, Great Britain). All other chemicals were of analytical grade. Thin-layer plates were 10 cm × 20 cm high-performance TLC Fertigplatten (Merck Kieselgel 60 F 254, supplied by BDH, Poole, Great Britain).

Apparatus

Developed chromatograms were scanned on a Vitatron TLD 100 flying spot densitometer (MSE Instruments, Crawley, Great Britain). The instrument settings were excitation filter, UVB (mercury lamp); emission filter 523 nm; scanning speed, 3 cm/min; log-mode; level f; span 95; c = 4, and damping 1.

Extraction and development procedures

To 0.5 ml plasma in a ground-glass stoppered tube 5 ml chloroform, the extracting agent, were added and, after vortex mixing for 10 sec, the mixture was centrifuged for 5 min at 750 *g*. The chloroform layer was transferred to a conical tube and evaporated to dryness at 65° in a water-bath under a stream of nitrogen. The residue was dissolved in 100 μl acetone and 10 μl of this solution were applied to the thin-layer plate as a spot by hand using a microsyringe. A series of extracts from plasma spiked with measured amounts of caffeine and a standard of caffeine in acetone were also applied to the same plate. The plate was developed in the solvent system [4] chloroform—carbon tetrachloride—methanol (8:5:1) in a saturated tank allowing the solvent to migrate about 8 cm.

Gas—liquid chromatography

Materials and methods

A Pye Unicam 104 gas chromatograph equipped with an alkaline flame ionisation detector (AFID) was used for this study. Two columns were found suitable: (1) 1.5 m × 4 mm I.D. borosilicate glass packed with 10% Apiezon M (Supelco, Bellefonte, Pa., U.S.A.) on Supasorb, 60—80 mesh (BDH, Poole, Great Britain); (2) 2.4 m × 4 mm I.D. borosilicate packed with 3% Poly S-179 (Supelco) on Gas-Chrom Q (DCMS-treated), 60—80 mesh (Supelco).

Extraction and chromatographic procedures

To 0.5 ml plasma in a ground-glass stoppered tube were added 20 μl of the internal standard solution of phenacetin (100 μg/ml in methanol), 0.1 ml 0.1 *N* NaOH and 3 ml ethyl acetate. The mixture was vortex mixed for 20 sec and centrifuged at 750 *g* for 5 min. The supernatant organic phase was transferred to a conical tube and evaporated to dryness under a stream of nitrogen in a water-bath at 75°. The residue was dissolved in 20 μl methanol and 1—3 μl were injected into the gas chromatograph. The chromatograph operating conditions for both columns were: column oven 225°; detector 330°; carrier

gas (nitrogen) flow-rate 75 ml/min; hydrogen flow-rate 37 ml/min; and air flow-rate 480 ml/min.

RESULTS AND DISCUSSION

A typical recording of the scanning of the thin-layer plate in the direction of solvent flow is shown in Fig. 1. In the system used to develop these plates the R_F of caffeine is 0.5. Calibration curves relating concentration of caffeine to peak height were linear over the range investigated (0–50 μg/ml). The minimum level of detection of the TLC assay was 0.1 μg/ml. Reproducibility was assessed by replicate analyses of spiked plasma samples. The coefficient of

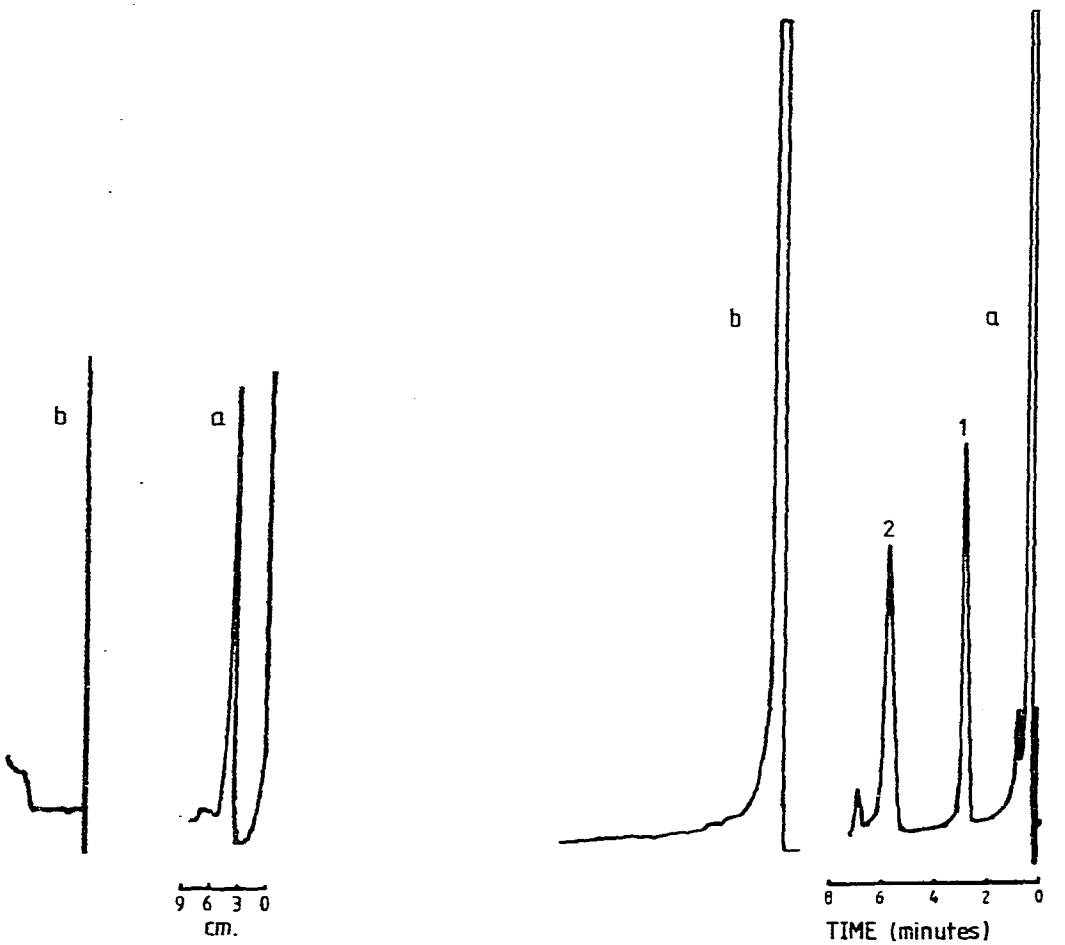

Fig. 1. Scan of thin-layer chromatogram along the plate in the direction of solvent flow. (a) Plasma containing caffeine (10 μg/ml); (b) control plasma.

Fig. 2. Gas chromatograms on Apiezon M system of human plasma. (a) At 5 h following oral administration of 300 mg caffeine base; peaks: 1, phenacetin (internal standard), 2, caffeine (3.2 μg/ml). (b) Control caffeine-free plasma.

variation between assays was 7.18 at 10 μg/ml (n = 15) and 7.25% at 2 μg/ml (n = 5). There were no interfering substances in blank plasma or urine.

Typical chromatograms for the GLC assay are shown in Fig. 2. The retention times on Apiezon M were phenacetin 2.5 min and caffeine 5.4 min, and on Poly S-179 were phenacetin 1.5 min and caffeine 3 min. The peak shape for both substances was symmetrical allowing quantitation by the peak-height ratio method. The minimum level of detection was 0.05 μg/ml, and the calibration curve was linear over the range 0—50 μg/ml. The coefficients of variation between assays were 1.67% at 10 μg/ml (n = 6) and 6.5% at 1 μg/ml (n = 6). There were no interfering endogenous substances in blank plasma, urine or saliva.

Duplicate samples of plasma were analysed by both TLC and GLC methods. The data were correlated by linear regression analysis which yielded a correlation coefficient of 0.91 for 76 observations. A paired Student's t-test of these values gave a value of 2.12, which is significant at the 5% level and indicates a systematic trend for the TLC assay to give a lower estimation than the GLC assay. This is demonstrated by the plot of the difference of the two assays vs. the mean of the assays (Fig. 3). Both assays yield data that are a measure of the caffeine level in plasma, and in a study of the pharmacokinetics of orally administered caffeine in man (to be published shortly) both gave identical estimates of the plasma $t_{½}$.

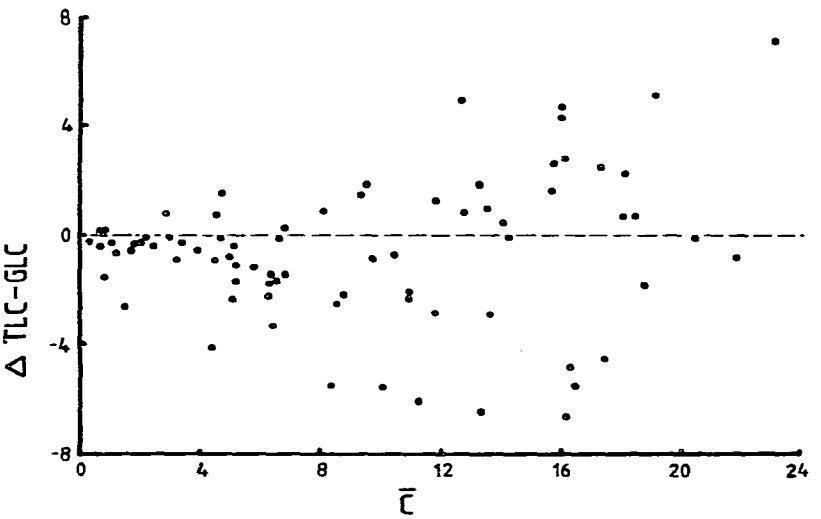

Fig. 3. Plot of difference between plasma levels estimated by TLC and GLC methods (ordinate, μg/ml) vs. the mean of the assays (C, = μg/ml) for 76 paired observations.

The TLC technique is simple, rapid and allows processing of a large number of samples. It is, however, less sensitive than GLC or HPLC methods. Although the use of the AFID allows a relatively simple extraction procedure, the GLC method requires more complex sample preparation but has greater sensitivity than the TLC method or that claimed for HPLC [2]. It is suitable for estimates of caffeine in saliva and can be used for very small volumes of blood. The

presently described TLC and GLC methods are considerably more sensitive than the previously described GLC assay [8] which required a 5-ml blood sample to achieve a reported sensitivity of 0.25 μg/ml. Both techniques are therefore suitable for use in studies of caffeine pharmacokinetics.

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