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MICRODETERMINATION OF CAFFEINE IN BLOOD BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY*

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SUMMARY

A micromethod for the quantitative analysis of caffeine present in small quantities (100 µl) of whole blood is described. It is based on the gas chromatographic—mass spectrometric analysis of chloroform extracts of biological samples. The method is relatively simple, rapid, specific and sensitive; as little as 20 ng of caffeine can be measured.

INTRODUCTION

Caffeine, a plant alkaloid, possesses several pharmacological actions of therapeutic significance such as its ability to stimulate the central nervous system, cardiac muscle and skeletal muscle, to relax smooth muscle, and to produce diuresis. For these reasons, it is formulated in many pharmaceutical preparations. Moreover, caffeine is found in several widely consumed beverages including coffee, tea, cocoa, maté and various cola-flavored "soft drinks". Of potential significance are reports that it is mutagenic in bacteria [1, 2] and clastogenic in cultured human lymphocytes [3—5], and that it inhibits cyclic 3′:5′-nucleotide phosphodiesterase [6, 7], post-replication DNA repair [8, 9], and mitosis [10] in various mammalian cells.

A great deal of investigative effort has been directed toward determining the physiological disposition of caffeine [11]. However, these investigations were often handicapped in that a rapid, simple, specific and sensitive method for measuring this drug in biological samples was not available. Of those available prior to 1968 (listed and referenced in ref. 12), the methods of choice were

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based on spectrophotometric analysis. These methods lacked sufficient sensitivity and specificity for estimating pharmacologically reasonable caffeine concentrations in biological material.

A significantly improved method for the determination of caffeine was described by Grab and Reinstein [12]. This method was based on the gas chromatographic separation and quantitation of caffeine extracted from biological samples with chloroform. The minimum amount of caffeine that could be quantitated accurately was about 500 ng. Ingestion of pharmacological amounts of caffeine, ca. 100 mg, produces blood caffeine concentrations that can be accurately monitored for several hours by this method provided that blood samples greater than 1—2 ml can be repeatedly obtained.

The present report describes a gas chromatographic—mass spectrometric (GC—MS) micromethod that is even more rapid, specific and sensitive; as little as 20 ng of caffeine can be measured. Therefore, accurate estimates of caffeine concentrations in 100 µl or less of biological samples can easily be made following the ingestion of pharmacological amounts of caffeine*.

EXPERIMENTAL

Apparatus

A Finnigan Model 3200 gas chromatograph—mass spectrometer (Finnigan, Sunnyvale, Calif., U.S.A.) was used. The column was a silanized U-shaped Pyrex glass tube, 0.25 in. O.D. (2 mm I.D.) and 5 ft. long, packed with 3% Dexsil 300 on 80—100 mesh Chromosorb Q (Supelco, Bellefonte, Pa.,U.S.A.). Prior to its initial use, it was conditioned overnight at 350° with the carrier gas flowing. Helium (14 SCCM) was used as the carrier gas. Operating temperatures were: column, 210°; injection port, 260°; separator, 255°; transfer line, 245°. Electron energy was 68 eV and the emission current was 102 µA. Responses were recorded by a Sargent-Welch Model DSRG two-pen recorder (Sargent-Welch, Skokie, Ill., U.S.A.).

Chemicals

Caffeine, the emulsifying agent mannide monooleate, and glutethimide were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.), Sigma (St. Louis, Mo., U.S.A.), and Ciba-Geigy (Summit, N.J., U.S.A.), respectively. Chloroform (ACS certified) and acetone (ACS certified) were obtained from Fisher (Fair Lawn, N.J., U.S.A.).

A caffeine-containing oil—water emulsion was prepared for subcutaneous injection by sonicating a mixture of caffeine in 0.9% NaCl solution (12.5 ml; 20 mg caffeine/ml), mannide monooleate (5 ml), and light mineral oil (7.5 ml).

Glutethimide was dissolved in chloroform (1.35 µg/ml). It was chosen for use as the internal standard for the GC—MS assay of caffeine because (1) both it and caffeine are readily extracted from aqueous solutions by chloroform,

*Subsequent to the completion of the present investigation but prior to its submission for publication, a report describing the use of GC—MS to quantitate caffeine content was called to our attention [13]. Deuterated caffeine was used as the internal standard in these studies. The lower limit of sensitivity was not reported.

(2) its retention time is very similar to that of caffeine (Fig. 1), and (3) it shows a convenient fragmentation pattern for use in GC—MS.

Preparation of biological samples

Caffeine was subcutaneously injected, 200 mg/kg (20 ml/kg), into the inguinal region of 80—90-g male Holtzman rats. Blood samples, 0.1 ml, were drawn from the tail vein into heparinized pipettes at various times after caffeine injection and analyzed for caffeine content as described below.

Analytical procedure

Blood samples 0.1 ml, or caffeine in 0.9% solution, 0.1 ml, were added to test tubes containing 0.2 ml of 0.9% NaCl solution and 0.5 ml of the glutethimide in chloroform solution. The mixtures were mixed for 60 sec and the phases were separated by low-speed centrifugation. Preliminary experiments established that virtually all (>95%) of the caffeine and glutethimide partitioned into the chloroform phase. The water layer was removed by aspiration and the bulk of the remaining chloroform phase was transferred with a Pasteur pipette to clean test tubes in order to remove it from precipitated blood constituents present at the chloroform—water interface. The samples were then evaporated nearly to dryness, dissolved in 0.5 ml of acetone and assayed for caffeine content. Caffeine concentrations, relative to glutethimide concentrations, were determined by monitoring the molecular ion of caffeine (*m/e* 194) and the *M*—28 ion of glutethimide (*m/e* 189) in a gas chromatograph—mass spectrometer. A standard curve establishing the relationship between peak-height ratios of caffeine/glutethimide vs. caffeine concentrations was constructed (Fig. 2) and was used in estimating caffeine concentrations in biological samples.

Data analysis

Caffeine/glutethimide peak-height ratios were determined in triplicate for each caffeine concentration to establish a standard curve. The equation describing this relationship was determined by weighted linear regression analysis [14]. The reciprocals of the variances were used as the weights for each datum point. All calculations were conducted with the aid of a Control Data Corporation 6400 Series computer.

RESULTS AND DISCUSSION

The major fragment ion of glutethimide was the *M*—28 ion (*m/e* 189); the molecular ion of caffeine (*m/e* 194) was also the base peak (data not presented). These observations are in agreement with those of others [15, 16].

Retention times for caffeine and glutethimide were approximately 5—6 min (Fig. 1), and sharp peaks with minimal tailing were observed for both agents. The relationship between caffeine/glutethimide peak height ratios and caffeine concentrations is shown in Fig. 2. A linear (r = 0.998) and reproducible relationship was observed over a wide range (1 μM to at least 1 mM) of caffeine concentrations. Values for the slope and y intercept of the regression line defining this relationship were 0.093 and —0.027, respectively. In

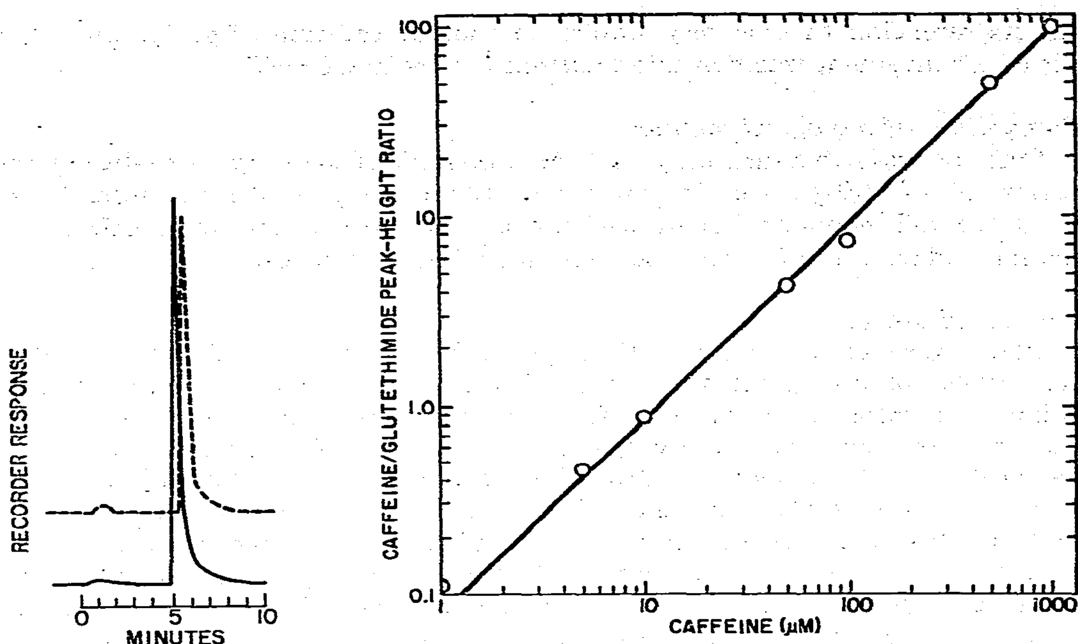

Fig. 1. Gas chromatogram of caffeine (— — —) and glutethimide (————). Recorder responses were obtained by monitoring the appearance of the molecular ion of caffeine (m/e 194) and the M—28 ion of glutethimide (m/e 189) as described in Experimental.

Fig. 2. Standard curve describing the relationship between caffeine/glutethimide peak height ratios and caffeine concentration. Peak height ratios were obtained by GC—MS as described in Experimental.

subsequent experiments, blood caffeine concentrations were estimated from the relationship:

$C = (\text{R} - \text{intercept})/\text{slope} = (R + 0.027)/0.093$

where C represents the caffeine concentration in μmoles/l and R represents the caffeine/glutethimide peak height ratio.

The lowest caffeine concentration that could be measured with accuracy was about 1 μM since the relationship between caffeine/glutethimide peak height ratios and caffeine concentrations deviated from linearity at lower caffeine concentrations in that the amount of caffeine present was overestimated (data not presented); the reason for this phenomenon was not established. The sample volume was 100 μl. Thus, as little as 20 ng of caffeine could be measured with accuracy.

The maximum plasma caffeine concentration after ingestion of coffee containing 100 mg of the drug is about 100 to 200 ng/0.1 ml [12]. This amount of caffeine would easily be measured by the present method but not by previously available methods unless relatively large sample volumes were obtained. Because the increased sensitivity of the present method permits the use of smaller sample sizes, caffeine concentrations could be determined in blood drawn from the finger tip, ear lobe, or umbilical cord and repeated sampling in a single small animal would be possible.

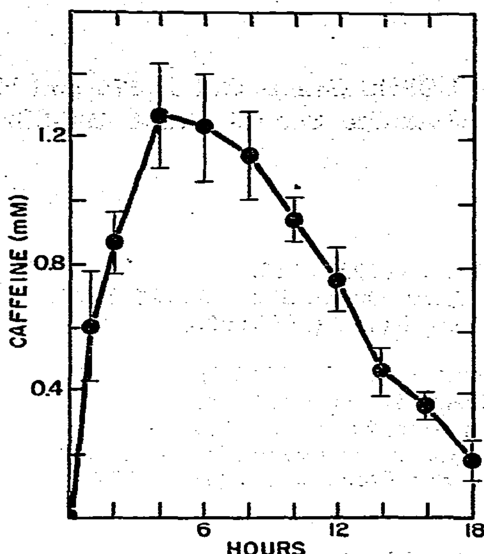

Fig. 3. Blood caffeine concentrations in rats following the subcutaneous injection of caffeine. Three male Holtzman rats were injected with caffeine, 200 mg/kg, and blood caffeine concentrations were determined as described in Experimental. Each point represents the mean ± S.E. of the values obtained.

The present method is also more specific than any method previously employed. Specificity is promoted by (a) differential extraction into chloroform from many potentially troublesome endogenous compounds, (b) differential separation of the extract on the gas chromatographic column, and (c) monitoring of specific mass fragments. Consequently, the probability of an interfering substance or blank error is small.

The method is simple and rapid in that the retention times of caffeine and glutethimide on the gas chromatographic column are only 5–6 min and extraction of caffeine and glutethimide into chloroform is quantitatively and rapidly effected.

Application of the method to a problem of current interest in our laboratory provided data illustrating its utility (Fig. 3). In this experiment, a sustained-release preparation of caffeine was injected subcutaneously into each of three rats and 100-μl blood samples, taken at various times thereafter, were analyzed for caffeine content. Eleven blood samples were taken from each of the three rats. Blood samples taken just prior to caffeine injection showed no interfering peaks. This experiment did not challenge the expected lower limit of sensitivity of the method; the lowest caffeine concentration measured was about 0.2 mM (approximately 4 μg of caffeine). However, the experiment does illustrate that, because of the sensitivity of the method, only small sample volumes are required and therefore multiple blood samples can be taken from small animals, e.g., rats, so that half-lives, etc., could be determined in single animals.

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