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GLC Determination of Caffeine in Plasma Using Alkali Flame Detection

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Abstract □ A rapid, specific, and sensitive GLC assay for caffeine in plasma was developed utilizing alkali flame-ionization detection. The procedure involves the addition of mepivacaine as an internal standard, alkalinization of the sample, and extraction with chloroform. Peak height ratio measurements produced linear standard curves in the 0.25–10.0-μg/ml range. Absolute sensitivity from a 1.0-ml plasma sample was 0.1 μg/ml. The relative standard deviation of a 2.0-μg/ml pooled plasma standard run repeatedly over several months was 5.2%. The method is applicable to time–concentration studies in human and animal plasma following typical oral doses of caffeine.

Keyphrases □ Caffeine—GLC analysis in plasma □ GLC—analysis, caffeine in plasma □ Stimulants, central—caffeine, GLC analysis in plasma

The widespread occurrence of caffeine in popular beverages and over-the-counter medications makes it the drug most consumed by many individuals. The average amount of caffeine in a cup of coffee is about 80–100 mg; however, depending on the manner of preparation, strong coffee can contain as much as 300 mg/cup (1). The amount of caffeine in tea varies considerably, mainly as a function of water temperature and time of contact, with typical averages of 25–50 mg/cup (1). However, caffeine concentrations in tea approach those of coffee when packaged instructions for typical products are followed. Cola and cocoa beverages contain smaller, but substantial, amounts of caffeine.

BACKGROUND

Although caffeine has long been known for its diuretic and central nervous system stimulating activities, many other pharmacological and some toxic effects have been reported, particularly in individuals consuming large quantities. Gilbert (2) reviewed a large number of epidemiologic studies suggesting that ulcers of the stomach and duodenum, carcinomas of the kidney and urinary tract, and certain types of heart disease occur more frequently in individuals ingesting more than 600 mg of caffeine/day. In addition, a large number of symptoms associated with anxiety occur more frequently in this group.

These reports prompted investigations of the relationships among caffeine consumption, adverse effects, and body fluid concentrations. Rather surprisingly, there is a scarcity of published methods suitable for the routine determination of caffeine. The spectrophotometric method of Axelrod and Reichenthal (3) is widely used, as is a modified procedure (4), but these procedures have distinct sensitivity and specificity limitations and are relatively tedious. A GLC method (5) was reported with improved sensitivity, but it suffered from an inadequate internal standard which was added just prior to injection and hampered the reproducibility.

Although several liquid chromatographic methods were reported for theophylline (6, 7), none has been reported specifically for caffeine. Caffeine elutes slowly and broadly in these systems. A radioimmunoassay with a high level of sensitivity recently was reported (8). The inherent specificity of GLC, coupled with the increased sensitivity and selectivity of an alkali flame detector for nitrogen-containing compounds, led to the development of the present assay for caffeine in plasma of animals and humans. Data also are presented that support the application of this method for obtaining plasma time–concentration data on caffeine after ingestion of typical doses.

EXPERIMENTAL

Reagents—Caffeine[1] (purity >99%) was obtained commercially and used directly. A standard stock solution containing 200 μg/ml was prepared in methanol, protected from light, and stored at 4°.

Mepivacaine, the internal standard, was prepared by extraction of the alkalinized commercial dosage form[2] with dichloromethane. The solvent was removed, and the solid was recrystallized from ether, mp 150–152°. A standard stock solution containing 650 μg/ml was prepared in methanol and stored in the same manner as the caffeine stock solution.

Chloroform[3] and methanol[3] were spectral grade. All other reagents were analytical grade and were used as received. Other drugs were commercially available dosage forms.

Standard Curve—Appropriate aliquots of the stock caffeine standard in methanol were added to conical centrifuge tubes, the solvent was evaporated, and 1.0 ml of pooled human plasma was added to produce final concentrations of 0.25, 0.5, 1.0, 5.0, and 10.0 μg/ml. Identical concentrations were used to prepare aqueous standard curves. The tubes were vortexed to dissolve the drug, and the assay was carried out as described later. Standard curves were constructed from peak height ratio measurements of caffeine to internal standard.

Analytical Procedure—To 1.0 ml of plasma or aqueous sample in a 15-ml centrifuge tube were added 25 μl of mepivacaine standard in methanol and 30 μl of 12 *N* NaOH. This solution was vortexed, 10.0 ml of chloroform was added, and the tube was shaken for 15 min. The tube was then centrifuged at 2500 rpm for 10 min, and the aqueous (top) layer was aspirated off. The organic layer was filtered[4] into a 15-ml centrifuge tube and evaporated using a stream of air in a 40° water bath. The residue

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Carbocaine Hydrochloride, Winthrop Laboratories, New York, N.Y.
[3] Burdick and Jackson Laboratories, Muskegon, Mich.
[4] Whatman No. 1 filter paper.

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Table I—Reproducibility of Analysis of Caffeine Added to Plasma[a]

Caffeine Concentration Added, μg/ml	n	Mean Peak Height Ratio (Caffeine to Mepivacaine)	RSD, %
0.25	5	0.210	9.2
0.50	8	0.310	7.5
1.00	8	0.462	7.9
2.00	20	0.837	5.2
5.00	7	1.532	6.9
8.00	5	2.214	5.5
10.00	10	2.905	5.1

[a] Pooled plasma blanks typically demonstrated a caffeine concentration of 0.20 μg/ml.

was reconstituted in 25 μl of methanol and vortexed for 30 sec, and 1–5 μl was injected into the gas chromatograph.

Chromatographic Conditions—Chromatography was performed on a dual-column instrument[5] equipped with an alkali flame-ionization detector. The U-shaped glass column, 182 × 0.25 cm i.d., was packed with 3% OV-17 on 60–80-mesh Chromosorb P[6]. The column oven temperature was 210° (isothermal), and the injector and detector were maintained at 250°. Nitrogen carrier gas, air, and hydrogen flows were 25, 250, and 38 ml/min, respectively. Caffeine eluted at 3.8 min and mepivacaine eluted at 6.5 min under these conditions.

Plasma Time–Concentration Studies—Venous blood samples drawn into heparinized vacuum tubes[7] were collected periodically from normal volunteers following ingestion of varying amounts of coffee containing 350 μg of caffeine/ml (~70 mg/cup). As a part of physiological and histological studies, colonies of mice drank various standard brews of coffee. They were then sacrificed, and the plasma from several animals was pooled for caffeine analysis. Samples were analyzed directly or the separated plasma was frozen until analysis.

RESULTS AND DISCUSSION

Typical chromatograms from the analysis of a human plasma sample and a pooled plasma blank are shown in Fig. 1. The relatively broad peak in the blank eluting after 9.0 min generally was present in all patient samples assayed but did not interfere with the quantitation of caffeine. The small peak eluting at about 3.8 min in the pooled plasma blank was present in all pools and most individual patient blanks and was probably residual caffeine. This peak was not present in mouse plasma blanks or human samples from individuals who had abstained from all caffeine ingestion for 48 hr.

Standard curves from water and pooled plasma were linear up to 10 μg/ml. Based on the unextracted standard curve, the overall efficiency of the extraction was estimated to be 85%. The minimum detectable amount of caffeine over baseline was about 10 ng injected, which provided a practical limit of sensitivity of 0.1 μg/ml for a 1.0-ml plasma sample. This assay is considerably more sensitive than the literature procedure (5) in which a 5.0-ml blood sample was required to produce a reported sensitivity of 0.25 μg/ml using regular flame-ionization detection. This improvement is due mainly to a decrease in background noise seen with the alkali flame detector.

Data from replicate determinations of caffeine added to plasma to produce a working standard curve from 0.25 to 10.0 μg/ml are presented in Table I. Replicate analyses of a spiked 2.0-μg/ml plasma standard over several months produced a relative standard deviation of 5.2%.

Since caffeine is essentially nonionized throughout the physiological pH range, preliminary extractions to allow sample cleanup were not feasible. The relatively high pH used here (>12) was selected primarily to minimize emulsion formation during extraction. Because of the simplicity of the extraction, possible interference, particularly from basic and neutral drugs, was investigated even though no interfering peaks were seen in either pooled or individual plasma blanks.

Lidocaine, procainamide, quinidine, isoniazid, glutethimide, salicylamide, phenylbutazone, and acetaminophen did not interfere at a spiked plasma concentration of 15 μg/ml. As expected, theophylline, theobromine, and the major human caffeine metabolites, methyluric acid, 1-methylxanthine, and paraxanthine (2, 9), also did not interfere under these analytical conditions. Because of the relatively high pH, no other acidic species are anticipated to interfere.

[5] Model 2100, Varian Instruments, Palo Alto, Calif.
[6] Applied Science Laboratories, State College, Pa.
[7] Vacutainers, Becton-Dickinson Co., Rutherford, N.J.

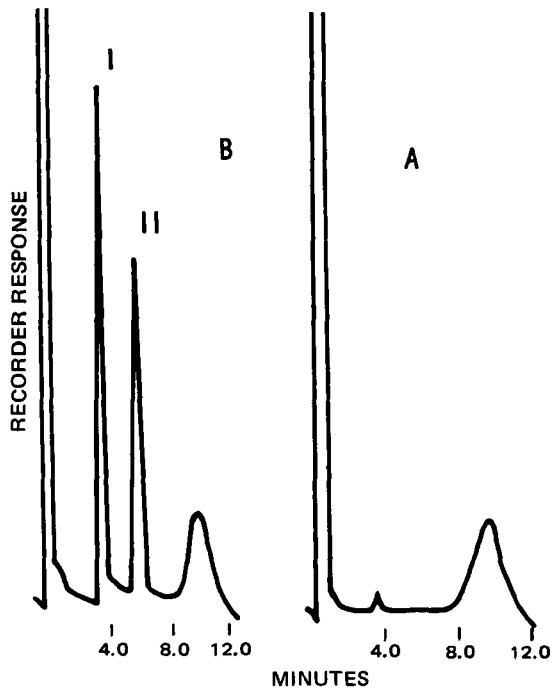

Figure 1—*Gas chromatograms of human plasma samples. Key: A, pooled plasma blank; and B, plasma sample from a normal volunteer containing 5 μg of caffeine/ml (I) and 12 μg of mepivacaine/ml (II).*

The procedure was developed to allow the investigation of plasma time–concentration patterns in humans and mice following various caffeine ingestion patterns. Figure 2 illustrates these profiles for four normal volunteers who consumed varying amounts of coffee through a working day. Peak plasma levels generally occurred 1–2 hr following ingestion, and levels were maintained between 1 and 5 μg/ml for individuals consuming less than 5 cups of coffee per day. This result agrees with previously reported data (5).

The disappearance rate of caffeine from the blood following the absorptive phase varied considerably. In eight subjects, the range in observed half-lives was from 2.1 to 6.5 hr. Literature reports of average terminal phase elimination half-lives of caffeine in humans range from 2.5 (3) to 4.5 (8, 9) hr. Fasting mice appear to absorb and eliminate caf-

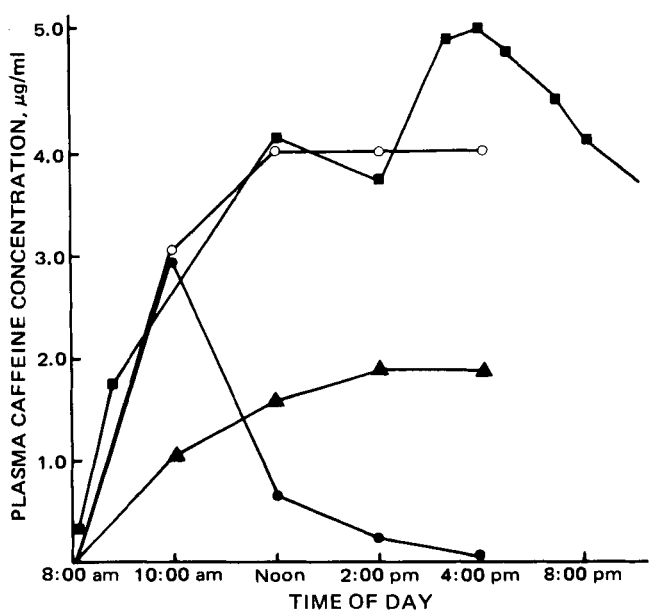

Figure 2—*Caffeine plasma time–concentration curves for four normal volunteers ingesting various amounts of coffee containing 350 μg of caffeine/ml. Key: ●, P-1, 2 cups at 8:00 am; ▲, P-2, approximately 3 cups* ad libitum; *○, P-3, approximately 5 cups* ad libitum; *and ■, P-4, 240 ml at 8:00 am, 11:30 am, and 2:30 pm.*

Table II—Plasma Concentrations in Colonies of Fasted Mice following Ingestion of Caffeine

Minutes	Plasma Caffeine Concentration, μg/ml
0	<0.1
15	5.9
30	1.9
60	1.2

feine more rapidly than humans. Table II indicates some typical plasma level data for a colony of mice fasted overnight and then given approximately 1 mg of caffeine as a single dose in the morning. This dose is equivalent to about 10 cups of coffee for a 70-kg human. Peak absorption occurred within 15 min, and concentrations declined rapidly thereafter. In colonies of nonfasted mice allowed to drink standard brews of coffee (350 μg/ml) *ad libitum*, typical plasma concentrations between drinks were 0.3–1.5 μg/ml throughout the day.

In general, the assay appears to be well suited to the rapid and routine measurement of human or animal plasma caffeine concentrations following administration of caffeine-containing beverages or medications. It is sensitive and highly selective and has permitted a variety of human and animal studies aimed at quantitating plasma concentration–time profiles following varying amounts of caffeine ingestion and correlating them to the possible incidence of adverse effects. The high sensitivity attainable also allows this method to be useful in assessing plasma concentration of caffeine in toxic situations from small volumes of blood.

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GLC Determination of Ticrynafen and Its Metabolites in Urine, Serum, and Plasma of Humans and Animals

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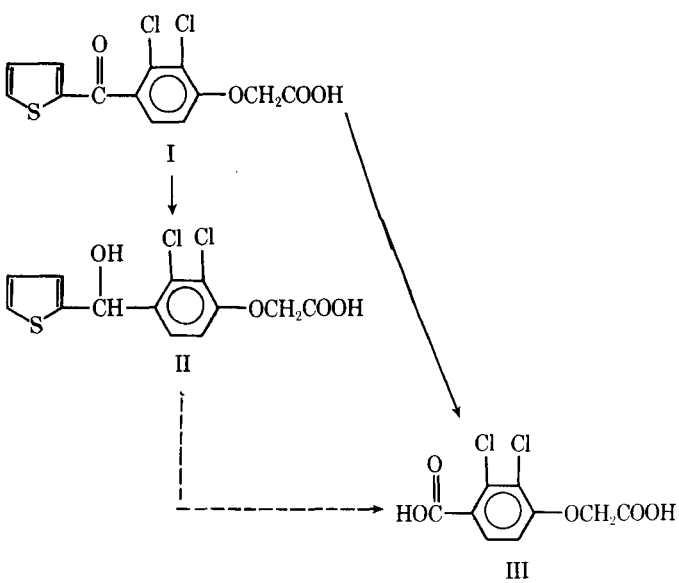
Received June 2, 1977, from the *Research and Development Division, Smith Kline and French Laboratories, Philadelphia, PA 19101.* Accepted for publication November 17, 1977.

Abstract □ A sensitive GLC assay for ticrynafen, a diuretic agent with uricosuric properties, and its two metabolites in urine, serum, and plasma is described. The method employs methylation of carboxylic acid groups and trimethylsilylation of the hydroxyl group on one metabolite that cannot otherwise be separated readily from ticrynafen as a simple methyl ester. Urinary output and serum or plasma levels of ticrynafen and its two metabolites were measured in specimens from human volunteers receiving one 250-mg tablet.

Keyphrases □ Ticrynafen—and metabolites, GLC analyses in biological fluids □ GLC—analysis, ticrynafen and metabolites in biological fluids □ Diuretics—ticrynafen and metabolites, GLC analyses in biological fluids

Ticrynafen [tienilic acid[1] or [2,3-dichloro-4-(2-thienylcarbonyl)phenoxy]acetic acid] (I) is a new potent diuretic agent with uricosuric properties (1–4). A pharmacokinetic study with the ^{14}C-labeled drug in animals (5) and a GLC method (6) for the drug were reported recently. However, the reported GLC method does not distinguish the parent drug from one of its metabolites, [2,3-dichloro-4-(α-hydroxy-2-thienyl)phenoxy]acetic acid (II). The drug is metabolized by two pathways (5): reduction of the ketone function to the corresponding alcohol (II) and oxidation of the thienylcarbonyl moiety to form [2,3-dichloro-4-carboxyphenoxy]acetic acid (III) as shown in Scheme I.

This report describes a procedure capable of measuring



Scheme I

[1] WHO approved name.