(10) N. J. Kerkhof, R. K. Vanderlaan, J. L. White, and S. L. Hem, *J. Pharm. Sci.*, **66**, 1528 (1977).
(11) C. J. Serna, J. L. White, and S. L. Hem, *J. Pharm. Sci.*, **67**, 324 (1978).
(12) J. S. Fordtran, *N. Engl. J. Med.*, **298**, 1082 (1978).
(13) R. W. Welch, *Arch. Intern. Med.*, **138**, 1208 (1978).
(14) "The Merck Manual," 13th ed., Merck and Co., Rahway, N.J., 1977, p. 1132.
(15) S. L. Hem, J. L. White, J. D. Buehler, J. R. Luber, W. M. Grim, and E. A. Lipka, *Am. J. Hosp. Pharm.*, **39**, 1925 (1982).
(16) A. J. Frueh and J. P. Golightly, *Can. Mineral.*, **9**, 51 (1967).
(17) J. A. Gadsden, "Infrared Spectra of Minerals and Related Inorganic Compounds," Butterworth, Reading, Mass., 1975, p. 74.

ACKNOWLEDGMENTS

This study was supported in part by William H. Rorer, Inc. This report is Journal Paper 9493, Purdue University Agricultural Experiment Station, West Lafayette, IN 47907.

Rapid Quantitative Liquid Chromatographic Determination of Caffeine Levels in Plasma after Oral Dosing

S. E. O'CONNELL ˣ **and F. J. ZURZOLA**

Received May 6, 1983, from *Bristol-Myers Products, Hillside, NJ 07207.* Accepted for publication July 11, 1983.

Abstract □ A simple method is described for the rapid, quantitative analysis of caffeine in human plasma. Caffeine levels present in plasma following drug administration were determined by high-performance liquid chromatography with UV detection at 273 nm after plasma protein precipitation. Caffeine was detectable at levels as low as 0.1 μg/mL. Mean recoveries of 98% with a coefficient of variation of 3% were obtained for plasma standards, in which concentrations ranged from 0.1 to 8 μg/mL. Interassay variability of the slope of the standard curve had a coefficient of variation of 3%. Application of this method during human bioavailability studies is described.

Keyphrases □ Caffeine—HPLC, human plasma, bioavailability □ Bioavailability—caffeine, HPLC, human plasma, oral dosing

Caffeine is a commonly ingested drug that is present in foods, beverages, and medicaments. Its widespread use has stimulated an interest in the development of a simple and rapid plasma determination for caffeine suitable for analyzing multiple samples.

Although GC (1-3) and spectrophotometric (4) methods exist for caffeine determination in biological fluids, high-performance liquid chromatographic (HPLC) methods are relatively few, and those available suffer from the disadvantages of inadequate sensitivity (5), time-consuming extraction steps (5-7), dedicated equipment (8), inadequate sample cleanup, inadequate sensitivity for pharmacokinetic studies (9-11), and insufficient testing in human plasma (11).

The method described herein involves a single plasma protein precipitation followed by liquid chromatographic determination of caffeine in the clear supernatant. The plasma proteins were denatured and precipitated with 0.15 M $Ba(OH)_2$ and 5% $ZnSO_4$ solutions, as described previously (12). The use of this combination of reagents results in a supernatant with a neutral pH and a clean chromatographic profile due to efficient coprecipitation of both plasma proteins and excess reagents. The method is capable of detecting at least 0.1 μg/mL of caffeine, and the reproducibility eliminates the need for an internal standard. Furthermore, the ease and rapidity of sample workup make this method ideal for multiple-sample analyses.

This method has been used routinely on long-term human bioavailability studies, resulting in clean predose plasma chromatograms with no interference and no column deterioration after several hundred analyses, and has been found to be precise and accurate.

EXPERIMENTAL SECTION

Reagents—Standard solutions were prepared in distilled water. The 0.15 M $Ba(OH)_2$ and 5% $ZnSO_4$ solutions were obtained commercially[1].

Instrumentation and Operating Conditions—The analysis was performed with a high-performance liquid chromatograph[2] with a variable-wavelength UV detector[3] set at 273 nm, an automated injection system[4] fitted with a 75-μL loop, and a 30 cm × 4-mm i.d. reverse-phase, high-efficiency C_{18} col-

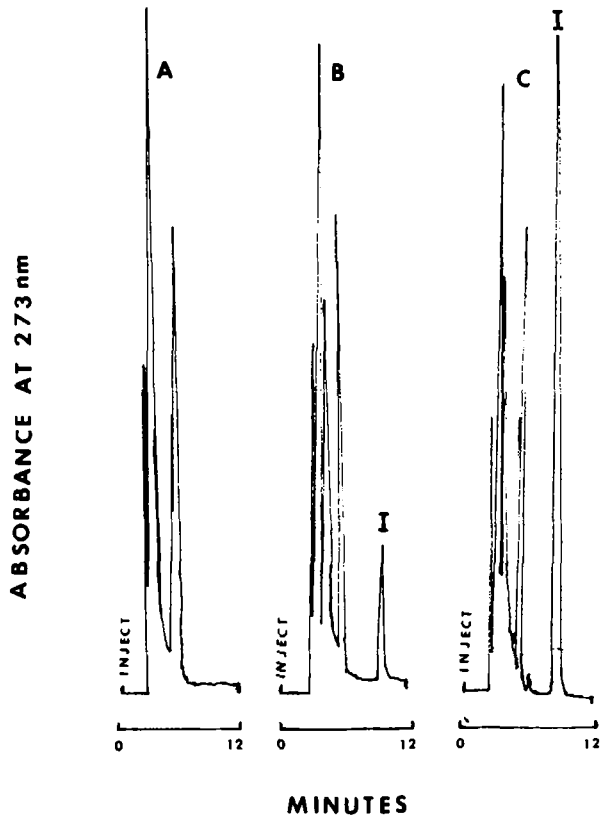

Figure 1—*Chromatograms for a typical subject. Key: (A) taken prior to caffeine (I) administration; (B) taken 10 min after oral administration of two 65-mg tablets; (C) taken 20 min postdose.*

[1] $ZnSO_4$ solution (lot SO-Z-10), $Ba(OH)_2$ solution (lot SO-B-138); Fisher Scientific Co.
[2] Model 6000; Waters Associates, Milford, Mass.
[3] Model 773; Kratos, Ramsey, N.J.
[4] Model 725; Mircomeritics, Norcross, Ga.

0022-3549/84/0700-1009$01.00/0

Table I—Accuracy of HPLC Assay for Caffeine in Human Plasma

Amount Added, μg	Experiment 1 Amount Found, μg	1 Error, %	2 Amount Found, μg	2 Error, %	3 Amount Found, μg	3 Error, %	4 Amount Found, μg	4 Error, %	5 Amount Found, μg	5 Error, %
0	0.01		0.01		0		0		0	
0	0.01		0.01		0		0		0	
0.1	0.10	0.0	0.10	0.0	—		—		—	
0.1	0.11	10.0	0.10	0.0	—		—		—	
0.25	0.25	0.0	0.26	4.0	0.24	4.0	0.26	4.0	0.27	8.0
0.25	0.23	8.0	0.26	4.0	0.24	4.0	0.27	8.0	0.26	4.0
0.50	0.48	4.0	0.50	0.0	0.50	0.0	0.47	6.0	0.51	2.0
0.50	0.51	2.0	0.52	4.0	0.50	0.0	0.50	0.0	0.55	10.0
1.00	0.99	1.0	1.00	0.0	0.96	4.0	0.99	1.0	0.89	1.0
1.00	1.05	5.0	1.05	5.0	1.01	1.0	0.99	1.0	1.01	1.0
2.00	1.93	3.5	1.99	0.5	2.04	2.0	1.92	4.0	1.95	2.5
2.00	2.04	2.0	1.95	2.5	1.99	0.5	2.04	2.0	1.95	2.5
4.00	3.86	3.5	3.84	4.0	3.94	1.5	3.91	2.3	4.01	0.3
4.00	4.08	2.0	3.91	2.3	3.96	1.0	4.10	2.5	4.00	0.0
8.00	7.78	3.0	8.02	0.3	7.92	1.0	7.93	0.8	7.97	0.4
8.00	8.25	3.0	8.11	1.4	8.12	1.3	8.08	1.0	8.08	1.0

umn[5]. The mobile phase was methanol-water (30:70), which was filtered through a 0.45-μm pore-size filter[6] and was deaerated before use. The flow rate was 1.0 mL/min. The column was fitted with a precolumn packed with C_{18} Corasil, 37–50 μm[7]. This precolumn was repacked once a week during panel use, since particulate matter accumulated on the precolumn frit and started to increase the column pressure after a weekly load of 300–400 injections. The detector was set at a sensitivity of 0.02 AUFS.

Plasma Caffeine Study in Humans—Because the described method was so sensitive to low levels of caffeine, it became necessary to insist that panelists abstain from caffeine-containing food, drugs, and beverages for at least 72 h before dosing. The panelists were fasted overnight and then received a 130-mg oral dose of caffeine in the form of two multicomponent tablets[8] taken with 100 mL of water. It had been determined in earlier panels that coadministration of aspirin and acetaminophen do not result in drug or metabolite peaks which interfere with the caffeine peak. Venous blood specimens were withdrawn by syringe and discharged into centrifuge tubes containing heparin as an anticoagulant. Specimens were taken before and at specified times after drug administration through 4 h. Collected specimens were centrifuged immediately for 15 min at 3500 rpm in a bench-top centrifuge. Plasma was separated and analyzed for caffeine by the proposed method.

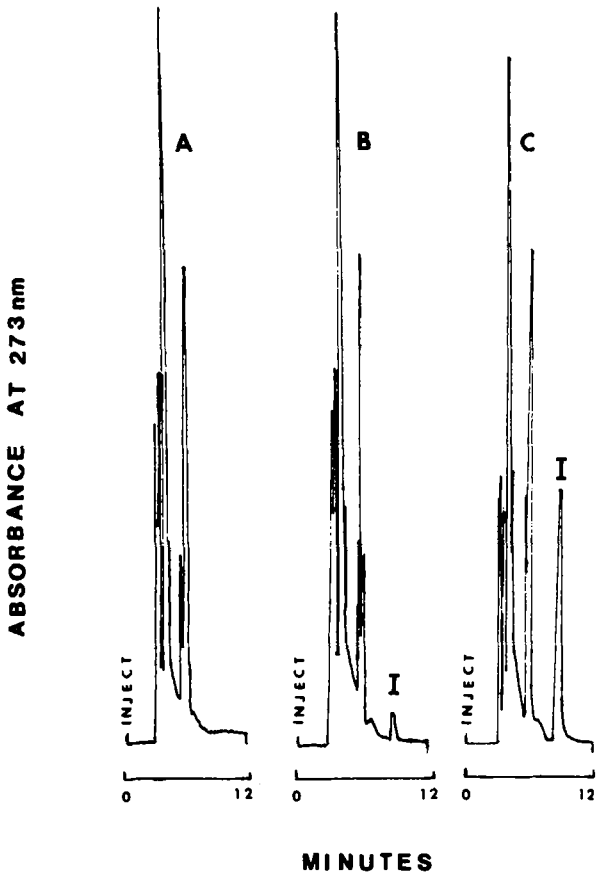

Figure 2—*Chromatograms of a 1-mL human plasma sample processed by the described precipitation method. The mobile phase was 30% methanol at a flow rate of 1.0 mL/min, and the chart speed was 15 cm/h. Key: (A) unspiked plasma; (B) plasma spiked with 0.1 μg of caffeine (I); (C) plasma spiked with 1 μg of I.*

Analysis—One milliliter of freshly drawn plasma was added to a screw-top culture tube (16 × 100 mm). To each tube was added 1 mL of the saturated 0.15 M $Ba(OH)_2$ solution. The tubes were vortexed for 2 min at sufficient speed on a multiple tube vortex mixer[9] to allow thorough mixing and sufficient reaction time. The addition of 1 mL of the 5% $ZnSO_4$ solution was made only after each sample became opaque in appearance. Each tube was then capped and vortexed at high speed for 1 min. The tubes were then centrifuged in a bench-top centrifuge at high speed for 10 min. The resulting clear, water-like supernatant was separated from the precipitate and filtered directly into an autoinjector vial with a Pasteur pipet plugged with glass wool. This ensured a minimal volume loss and a particulate-free sample.

Standard Curves—Calculations were carried out with standard curves constructed by analyzing 1-mL samples of the pooled predose plasma spiked with 0.1–8 μg of caffeine and by plotting the peak height *versus* the corresponding concentration. The standard curve data were subjected to least-squares linear regression analyses, and the resulting equation was utilized for the calculation of the drug concentration in the unknown samples.

RESULTS AND DISCUSSION

Typical chromatograms of a predose plasma sample (Fig. 1A) and the 10- and 20-min postdose plasma samples (Fig. 1B and C, respectively) obtained for a typical subject demonstrate the specificity of this method by the absence of interfering peaks. With 30% methanol in water as the eluant at a flow rate of 1.0 mL/min, the retention time for caffeine was 9 min.

Figure 2 represents the chromatograms of a 1-mL plasma blank, a 1-mL plasma sample spiked with 0.10 μg of caffeine, and a 1-mL plasma sample spiked with 1 μg of caffeine. The standards and samples are chromatographically clean to the extent that caffeine concentrations <0.1 μg/mL can be detected by this procedure. The adequate peak size and clean baseline resolution for a 75-μL sample injection containing 0.10 μg/mL of caffeine indicates the potential for detecting caffeine at concentrations of <0.1 μg/mL *via* an increased injection volume or more sensitive detector setting. Since the lowest caffeine level normally encountered in our therapeutic drug monitoring was ~0.25 μg/mL, the present sensitivity of this method is more than adequate for use in blood level and pharmacokinetic studies.

Recovery—The recovery of caffeine from plasma relative to its recovery from water, as measured by the ratio of the slopes of the standard curves in plasma and water, respectively, was 0.98 ± 0.03, with a coefficient of variation

[5] μ-Bondapak C_{18}, Waters Associates.
[6] Millipore Corp., Bedford, Mass.
[7] Waters Associates.
[8] Excedrin; Bristol-Myers Products, Hillside, N.J.
[9] Kraft Apparatus, Inc., Mineola, N.Y.

Table II—Caffeine Plasma Levels [a]

Subject	Caffeine Levels, μg/mL						
	10 min	20 min	40 min	60 min	120 min	180 min	240 min
Females							
1	0.7578	4.3575	3.9549	3.2918	2.2024	1.4683	1.0657
3	1.7288	4.3338	3.8839	3.5760	2.6524	2.1314	1.6814
5	2.3238	3.9185	3.8729	3.5312	3.0756	2.7566	2.5516
7	0.0000	1.1394	3.5811	4.2594	3.7439	3.3370	2.9843
9	2.9160	3.6563	3.2301	2.9160	2.5572	2.2431	1.9964
11	0.4561	2.6294	4.4807	4.2124	3.7026	3.1392	2.7367
13	1.7465	4.3202	4.0904	4.1134	3.5389	3.2632	3.2402
15	4.5783	5.6424	4.3555	3.8111	3.2172	2.7470	2.3015
17	0.3960	5.3454	5.0732	4.6030	3.8853	3.4646	3.1182
19	0.7061	3.1482	5.7374	5.6491	4.7370	4.0015	3.5601
21	2.0590	4.3696	3.8434	3.2715	2.5623	2.1276	1.7845
23	1.4077	4.7510	4.3771	4.0692	3.5853	3.1014	2.5955
25	4.2452	5.8676	5.0294	4.1912	3.0555	2.4065	1.8928
27	0.7842	6.8141	5.1376	4.5968	3.8126	3.3530	3.1907
29	0.1978	1.6808	2.6201	2.5954	2.3235	2.1011	1.9775
31	2.1887	4.3993	3.3050	2.8453	2.1231	1.5759	1.1819
33	2.3090	5.2018	3.9279	3.5032	2.7336	2.2293	1.9109
35	0.2261	1.2886	3.2555	3.1650	2.7807	2.6677	2.4868
37	4.2461	4.9246	4.3118	3.9178	3.3487	2.9329	2.7140
39	2.2933	4.5110	4.0826	3.7046	2.7974	2.3185	1.8397
Males							
2	0.8973	2.3553	3.4768	2.7815	2.3777	2.0188	1.8394
4	0.0000	1.4353	3.8478	2.4149	2.0276	1.8454	1.6631
6	1.4209	3.1971	2.7945	2.6998	2.3919	2.0603	1.7288
10	3.3641	4.4493	3.0657	2.6587	1.9533	1.5735	1.3022
12	0.6440	2.4148	2.6026	2.1197	1.7440	1.4757	1.3148
14	0.4455	2.0788	2.8955	2.7222	2.5242	2.2768	2.2025
16	1.8513	3.8977	2.6830	2.4952	2.5220	2.3342	2.1464
18	1.3558	3.9065	3.4929	3.3320	2.7575	2.7346	2.4588
20	1.6914	4.0158	3.7510	3.5744	3.1037	2.8389	2.4564
22	0.1352	1.0816	3.3259	3.1096	2.4065	2.0821	1.8387
24	0.6863	2.3792	3.7061	2.9054	2.1275	1.8073	1.6014
26	0.1730	1.2112	2.7437	3.3122	2.1752	1.6067	1.1865
28	0.1977	0.9887	2.5707	2.9415	2.5213	2.1258	1.8539
30	0.0000	0.9458	2.4855	2.1556	1.7157	1.4077	1.2318
34	2.1232	3.0521	2.8398	2.7071	2.4682	2.3355	2.3090
40	1.6416	3.2174	3.3050	2.9548	2.2106	1.9261	1.7729

[a] $n = 36$.

of 3%. Interassay variability of the slope of the standard curves generated during the bioavailability study had a coefficient of variation of <5%. A typical calibration regression line is $y = 44.33x - 0.37$, where x is concentration and y is peak height; this is linear over a concentration range of 0.1–8 μg/mL. The good linearity between the peak height and caffeine concentration in plasma is indicated by the correlation coefficient of >0.999.

Accuracy—Table I shows the actual amounts with which 1-mL blank plasma samples were spiked and the amounts found when the plasma was analyzed by the described method. The experiment was performed in duplicate on each of 5 d. The percent error for each unknown sample was calculated according to (13):

$$\text{percent error} = \left(\frac{\text{amount added} - \text{amount found}}{\text{amount added}}\right) \times 100$$

The average percent error was 3%. In no case did it exceed 10%.

Plasma Levels of Caffeine—A comparison was made of caffeine levels produced by two different caffeine-containing tablet formulations. The total caffeine dose administered was 130 mg. Table II shows the caffeine levels for one of the formulations for 36 subjects in a two-way crossover study with samples taken at 10, 20, 40, 60,120, 180, and 240 min postdose. The 10-min plasma levels indicate a slower absorption by male panelists, with the lowest detectable level of caffeine being slightly less than 0.2 μg/mL. A further inspection of the data reveals that caffeine was rapidly absorbed from the oral dose, reaching a peak plasma level of 3.6 μg/mL in <40 min.

The proposed method is extremely simple, inexpensive, and sensitive for the rapid determination of caffeine in plasma or blood at levels likely to be encountered after a usual 65- or 130-mg total dose. Plasma proteins are denatured by the addition of a saturated $Ba(OH)_2$ solution and then precipitated with a 5% $ZnSO_4$ solution. The resulting colorless clear supernatant is analyzed by HPLC with conventional 3.9-mm i.d. columns. The reproducibility, sensitivity, and selectivity of the method make the use of an internal standard unnecessary. The reproducibility and reliability of this method have been demonstrated in >500 determinations of plasma concentrations of caffeine.

REFERENCES

(1) R. L. Merriman, A. Swanson, M. W. Anders, and N. E. Sladek, *J. Chromatogr.*, **146**, 85 (1978).

(2) F. L. Grab and J. A. Reinstein, *J. Pharm. Sci.*, **57**, 1703 (1968).

(3) J. L. Cohen, C. Cheng, J. P. Henry, and Y. Chan, *J. Pharm. Sci.*, **67**, 1093 (1978).

(4) J. I. Routh, N. A. Shane, E. G. Arredondo, and W. D. Paul, *Clin. Chem.*, **15**, 661 (1969).

(5) C-N. Ou and V. L. Frawley, *Clin. Chem.*, **28**, 1671 (1982).

(6) A. A. Tin, S. Somani, H. Bada, and N. Khanna, *J. Anal. Toxicol.*, **3**, 26 (1979).

(7) D. B. Haughey, *J. Chromatogr.*, **229**, 387 (1982).

(8) Sj.van der Wal, S. J. Bannister, and L. R. Snyder, *J. Chromatogr. Sci.*, **20**, 250 (1982).

(9) J. Blanchard, J. Mohammadi, and K. Conrad, *Clin. Chem.*, **26**, 1351 (1980).

(10) J. Blanchard and J. Mohammadi, *J. Trang, Clin. Chem.*, **27**, 637 (1981).

(11) F. L. S. Tse and D. W. Szeto, *J. Chromatogr.*, **226**, 231 (1981).

(12) S. E. O'Connell and F. J. Zurzola, *J. Pharm. Sci.*, **71**, 1291 (1982).

(13) M. A. Schwartz and J. A. De Silva, in "Principles and Perspectives in Drug Bioavailability," J. Blanchard, R. W. Sawchuck, and B. B. Brodie, Eds., S. Karger, Basel, Switzerland, 1979, p. 92.