oral dose theophylline bioavailability studies in human adults and pediatric patients.

REFERENCES

(1) J. A. Schack and S. H. Waxler, *J. Pharmacol. Exp. Ther.*, **97**, 283 (1949).

(2) J. W. Jenne, E. Wyze, B. S. Rood, and F. M. MacDonald, *Clin. Pharmacol. Ther.*, **14**, 349 (1972).

(3) R. C. Gupta and G. D. Lundberg, *Anal. Chem.*, **45**, 2403 (1973).

(4) V. P. Shah and S. Riegelman, *J. Pharm. Sci.*, **63**, 283 (1974).

(5) B. Wesley-Hadzija, *Br. J. Clin. Pharmacol.*, **1**, 516 (1974).

(6) A. Arbin and P. O. Edlund, *Acta Pharm. Suec.*, **11**, 249 (1974).

(7) J. D. Lowry, L. J. Williamson, and V. A. Raisys, *J. Chromatogr.*, **143**, 83 (1977).

(8) G. F. Johnson, W. A. Dechtiaruk, and H. M. Solomon, *Clin. Chem.*, **21**, 144 (1974).

(9) B. Wesley-Hadzija and A. M. Mattocks, *J. Chromatogr.*, **115**, 501 (1975).

(10) R. D. Thompson, H. T. Nagasawa, and J. W. Jenne, *J. Lab. Clin. Med.*, **84**, 584 (1974).

(11) D. S. Sitar, K. M. Piafsky, and R. E. Rangao, *Clin. Res.*, **22**, 726A (1974).

(12) G. W. Peng, V. Smith, A. Peng, and W. L. Chiou, *Res. Commun. Chem. Pathol. Pharmacol.*, **15**, 341 (1976).

(13) R. E. Hill, *J. Chromatogr.*, **135**, 419 (1977).

(14) J. J. Orcutt, P. P. Kozak, Jr., S. A. Gillman, and L. H. Cummins, *Clin. Chem.*, **23**, 599 (1977).

(15) A. Arbin and P. Edlund, *Acta Pharm. Suec.*, **12**, 119 (1975).

(16) G. Schmidt and R. Schoyere, *Dtsch. Z. Gesamte Gerichtl. Med.*, **57**, 402 (1966).

(17) A. W. Burg, *Drug Metab. Rev.*, **4**, 199 (1975).

(18) K. K. Midha, S. Sved, R. D. Hossie, and I. J. McGilveray, *Biomed. Mass Spectrom.*, **4**, 172 (1977).

(19) A. Goldstein, "Biostatistics: An Introductory Test," Macmillan, New York, N.Y., 1967.

ACKNOWLEDGMENTS

The author thanks M. Leveque for technical assistance and Dr. Mario Gonzalez of Purdue University and Dr. S. Borodkin for helpful discussions.

Sensitive GLC Assay for Pemoline in Biological Fluids Using Nitrogen-Specific Detection

DANIEL J. HOFFMAN

Received May 31, 1978, from the *Pharmaceutical Products Division, Abbott Laboratories, North Chicago, IL 60064.* Accepted for publication September 9, 1978.

Abstract □ Extractive alkylation was used to determine intact pemoline in serum and urine. Pemoline was extracted into methylene chloride as an ion-pair with tetrapentylammonium hydroxide under alkaline conditions. Evaporation of the solvent at 70° in the presence of methyl iodide yielded the *N,N*-dimethylpemoline derivative. GLC analysis was performed on a 5% FFAP column with nitrogen-specific detection. Sensitivity was 0.05 μg/ml with 1 ml of urine or serum. Calibration curves were linear to at least 4 μg/ml with serum and 15 μg/ml with urine. Precision was excellent with a pooled relative standard deviation of ±7.5% for serum samples in a 0.1–4-μg/ml range.

Keyphrases □ Pemoline—GLC assay using nitrogen-specific detection, biological fluids □ GLC—analysis, pemoline in biological fluids □ Stimulants—pemoline, GLC assay in biological fluids

Several methods to determine pemoline (2-amino-5-phenyl-2-oxazolin-4-one) in biological fluids have been published. In one method (1), pemoline is hydrolyzed to mandelic acid with subsequent oxidation to benzaldehyde. The benzaldehyde is then determined spectrophotometrically. Benzaldehyde generated from pemoline has also been determined by GLC (2). Large sample volumes are required, and the method is tedious and nonspecific. High-pressure liquid chromatography has been applied to urine samples and pharmaceutical preparations (3). However, this method and a TLC method (4) lack the sensitivity for plasma samples.

A GLC method (5) using flame-ionization detection involves hydrolysis of pemoline to 5-phenyl-2,4-oxazolidinedione with subsequent methylation using diazomethane. Extensive cleanup procedures are needed with blood samples, and 60% of the final extract is injected into the chromatograph to achieve sensitivity. A similar approach was recently taken by Libeer and Schepens (6).

A recent GLC method using electron-capture detection determined 5-phenyl-2,4-oxazolidinedione without methylation (7). The method is sensitive but requires careful column preparation and preservation to minimize tailing and to maximize resolution from coextractives.

This paper describes the application of extractive alkylation (8, 9) and nitrogen-specific detection for the analysis of pemoline in biological fluids.

Table I—Precision of Serum and Urine Pemoline Assay

Pemoline Concentration, μg/ml	Peak Area Ratio[a]	*RSD*, %
	Serum	
0	0	—
0.05	0.015 ± 0.0006	4.0
0.10	0.029 ± 0.0040	13.8
0.25	0.097 ± 0.0038	3.9
0.50	0.17 ± 0.023	13.5
1.0	0.33 ± 0.011	3.3
2.0	0.68 ± 0.013	1.9
3.0	1.0 ± 0.025	2.5
4.0	1.4 ± 0.076	5.4
	Urine	
0	0	—
1.0	0.25 ± 0.0050	2.0
5.0	1.4 ± 0.047	3.4
15.0	4.4 ± 0.095	2.2

[a] Mean ± *SD* of triplicate standards.

0022-3549/79/0400-0445$01.00/0

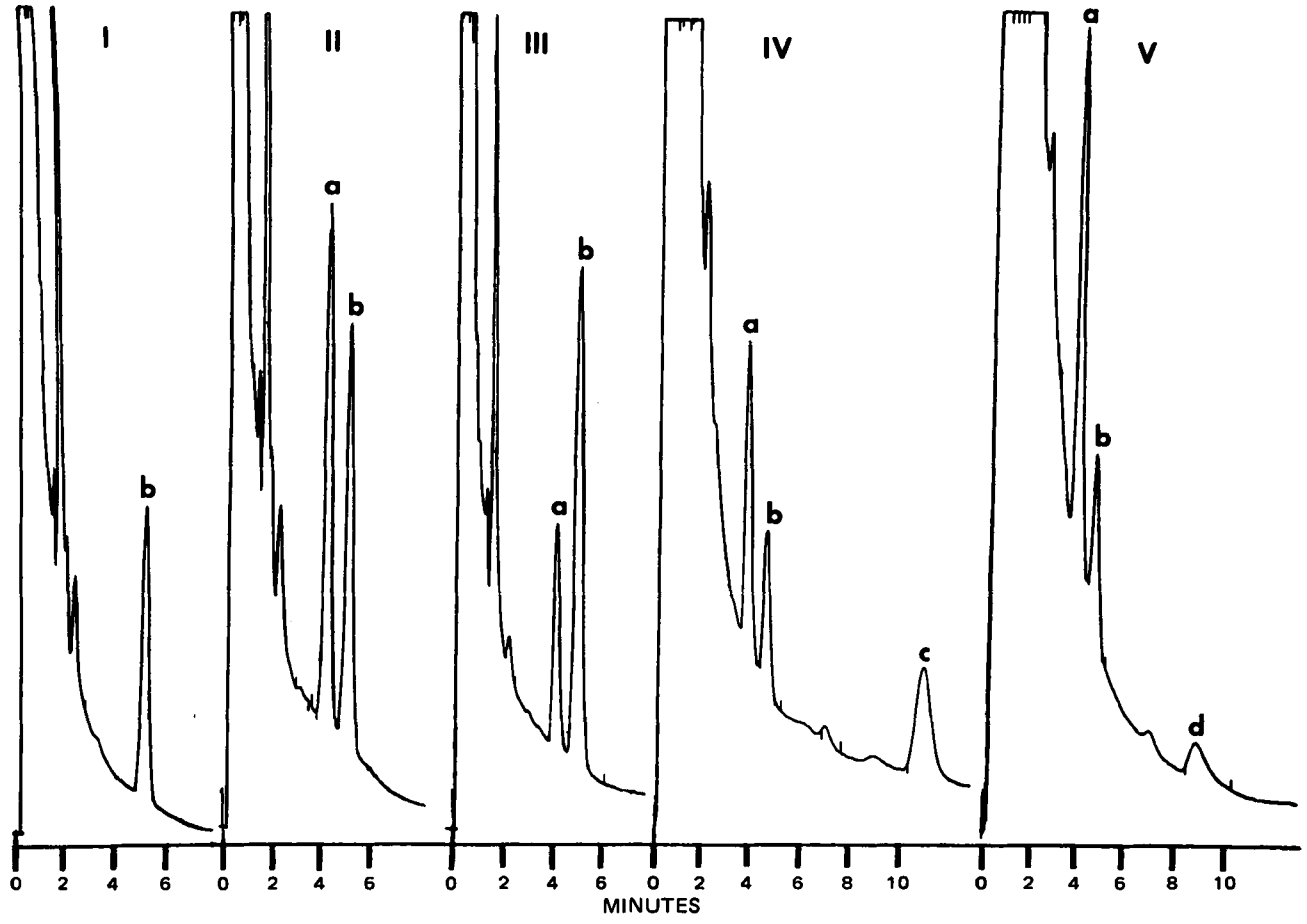


Figure 1—*GLC tracings of serum and urine samples containing pemoline. Key: I, serum blank with internal standard; II, serum standard containing 3 μg of pemoline/ml; III, human serum sample 8 hr after 50-mg oral dose of pemoline; IV, urine standard containing 4 μg of pemoline/ml and 8 μg of* p*-methoxypemoline/ml; V, human urine sample 8 hr after 50-mg oral dose of pemoline; a, pemoline; b, internal standard; c, 5-(*p*-hydroxyphenyl)-2-amino-2-oxazolin-4-one (possible metabolite, although not observed in humans); and d, 5-phenyl-2,4-oxazolidinedione (metabolite).*

EXPERIMENTAL

Reagents—All solvents were analytical reagent grade[1]. Tetrapentylammonium hydroxide[2], pemoline, 2-(*N,N*-dimethylamino)-5-phenyl-2-oxazolin-4-one, 5-phenyl-2,4-oxazolidinedione, and the internal standard 2-amino-5-(2′-methylphenyl)-2-oxazolin-4-one were used as supplied[3].

Instrumentation—A gas chromatograph[4] equipped with a dual nitrogen–phosphorus flame-ionization detector, an automatic sampler[5], and a computing integrator was used. The chromatograph was fitted with a 1.2-m × 2-mm i.d. coiled glass column packed with 5% FFAP[6] on Chromosorb W-HP[7] (80–100 mesh). The column was conditioned overnight at 255° with a helium gas flow rate of 35 ml/min.

Normal operating temperatures were 250, 245, and 280° for the injection port, oven, and detector, respectively. The helium carrier gas flow rate was 38 ml/min. The hydrogen and air flow rates were 3.0 and 50 ml/min, respectively. An attenuation of 2^3 or 2^4 was used.

Preparation of Standards—Standard stock solutions of 1 mg/ml of pemoline and the internal standard were prepared in methanol. A working internal standard solution at 0.5 μg/ml was prepared by diluting 0.5 ml of the 1-mg/ml solution to 1 liter with distilled water. A 4-μg/ml pemoline serum standard was prepared by adding exactly 400 μl of the 1-mg/ml pemoline stock solution to a 100-ml volumetric flask and adjusting to volume with drug-free serum.

Other serum standards were prepared by serial dilutions with serum. Urine standards were prepared in a similar manner using fresh urine. All standards were kept in a freezer until used.

[1] Mallinckrodt.
[2] Eastman.
[3] Abbott Laboratories.
[4] Hewlett-Packard model 5830A.
[5] Hewlett-Packard model 7671A.
[6] Varian Aerograph.
[7] Applied Science Laboratories.

Table II—Accuracy of Serum and Urine Pemoline Assay

Pemoline Concentration, μg/ml		Percent
Actual	Calculated	Difference
	Serum	
1.0	0.97	−3.0
0.24	0.28	+16.7
3.8	3.9	+2.6
2.6	2.5	−3.8
0.12	0.10	−16.7
0.84	0.77	−8.3
1.9	1.9	0
2.5	2.5	0
4.1	4.0	−2.4
3.3	3.1	−6.1
3.4	3.6	+5.9
	Urine	
4.5	4.3	−4.4
10.7	10.8	+0.9
15.2	15.5	+2.0
5.6	5.5	−1.8
1.8	1.6	−11.1
7.7	8.0	+3.9

Extraction Procedure—Serum and urine sample volumes were adjusted, if necessary, to the 0.1–4-μg of pemoline/sample range.

To a 15-ml screw-capped polytef (lined) conical test tube containing 6 ml of 0.5 *M* methyl iodide in methylene chloride were added exactly 1 ml of the internal standard solution and 1 ml of serum or urine sample. Then 0.5 ml of 0.5 *N* NaOH and 100 μl of 0.05 *M* tetrapentylammonium hydroxide were added, and the tube was capped, shaken[8] on a reciprocal

[8] Eberbach Corp.

shaker at about 150 cpm for 10 min, and centrifuged at 20° for 10 min at 3000 rpm.

The upper aqueous phase was discarded, and 5 ml of the organic phase was transferred to a 15-ml conical screw-capped test tube. This separation could also be accomplished using phase separation paper[9]. The extract was evaporated to dryness at 70° plus an additional 10 min after reaching dryness with a stream of filtered air or nitrogen. The residue was reconstituted with 0.3 ml of benzene and mixed for 5 sec. Samples were centrifuged for 5 min at 2500 rpm and then stood at room temperature at least 1 hr before 2 μl was injected into the chromatograph.

Total urinary pemoline levels were determined by incubating 1 ml of an adjusted urine sample[10] with 1000 units of a glucuronidase–sulfatase[11] preparation in 0.2 ml of 0.2 *M* acetate buffer (pH 4.5) for 20 hr at 37°. After incubation, 1 ml of the internal standard solution, 0.6 ml of 0.5 *N* NaOH, 100 μl of 0.05 *M* tetraphenylammonium hydroxide, and 6 ml of 0.5 *M* methyl iodide in dichloromethane were added. Extractive methylation was performed as described for serum samples.

Calculations—A linear calibration curve was constructed by plotting peak area ratios (pemoline/internal standard) against the serum or urine pemoline concentration of pemoline spiked standards. Unknowns were calculated using a computing integrator programmed with the slope of the calibration curve. The slope was obtained by a least-squares linear regression analysis of the pemoline standards.

RESULTS AND DISCUSSION

Under the conditions of alkylative extraction, pemoline was methylated with methyl iodide to 2-(*N,N*-dimethylamino)-5-phenyl-2-oxazolin-4-one

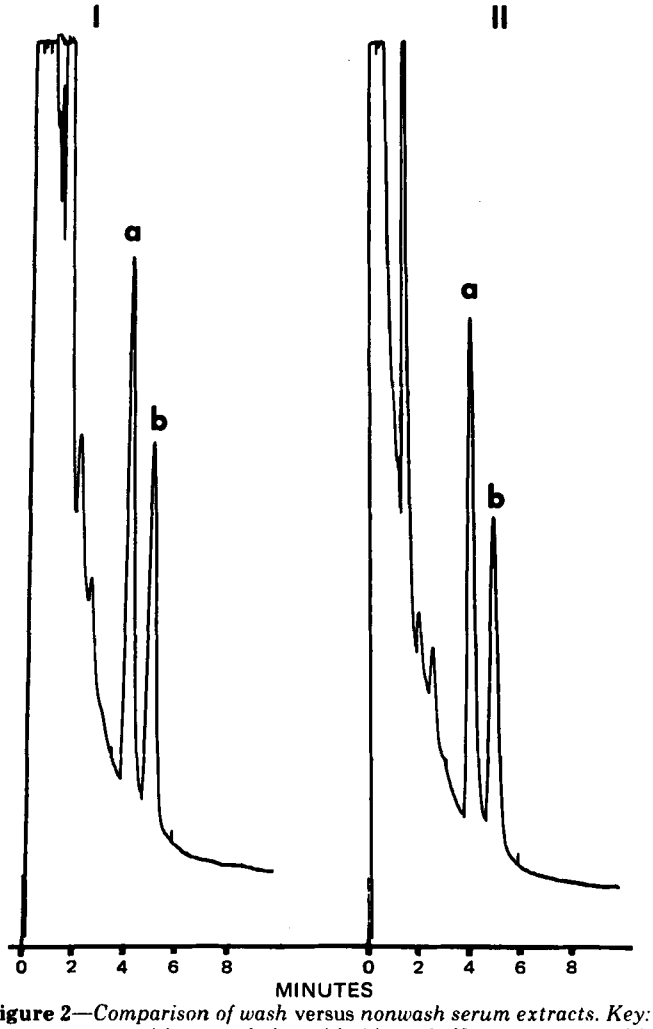
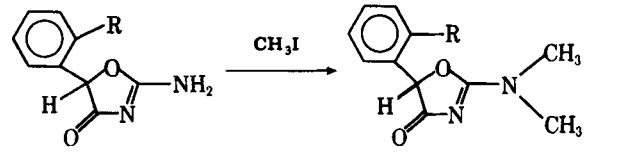


Figure 2—*Comparison of wash* versus *nonwash serum extracts. Key: I, serum extract without methylene chloride wash; II, serum extract with methylene chloride wash; a, pemoline; and b, internal standard.*



pemoline: R = H 2-*N,N*-dimethylamino derivative
internal standard: R = CH_3

Scheme I

(Scheme I). Identification was made by comparison of the GLC–mass spectral properties with those of an authentic sample of 2-(*N,N*-dimethylamino)-5-phenyl-2-oxazolin-4-one. Pemoline (R_T 4.2 min) and the internal standard (R_T 5.0 min) peak shapes were symmetrical and well resolved from coextractives (Fig. 1). The 5-phenyl-2,4-oxazolidinedione metabolite had a retention time of 9.4 min (Fig. 1).

The choice of tetrapentylammonium hydroxide was based on preliminary experiments with radioactive pemoline[3] (1-^{14}C-pemoline). Recoveries from serum (0.5 μg/ml) averaged 63 ± 1.2% using the tetrapentylammonium counterion. Recoveries from serum using tetramethyl-, tetrahexyl-, and tetraheptylammonium counterions were 1.7, 57, and 27%, respectively.

The assay precision was good for both serum and urine samples (Table I). Relative standard deviations ranged from ±1.9 to ±13.8% with a pooled value of ±7.5% for serum samples. Relative standard deviations for urine samples ranged from ±2.0 to ±3.4% with a pooled value of ±2.5%. The assay sensitivity was less than 0.05 μg/sample. Calibration curves were linear over the range of 0.05–4 μg of pemoline/sample. Correlation coefficients were >0.99 with *y*-intercepts not significantly different from zero.

Accuracy was evaluated by analyzing serum and urine samples spiked with pemoline. As shown in Table II, the accuracy was excellent, especially for samples containing more than 0.5 μg of pemoline.

Inclusion of a methylene chloride wash step prior to the addition of tetrapentylammonium hydroxide reduced the amount of coextractives in the solvent front (Fig. 2) with only a slight improvement in precision. Triplicate serum standards at 4 μg/ml were assayed with and without the methylene chloride wash. The relative standard deviation with the methylene chloride wash was ±1.7%; without the wash (described procedure), it was ±2.5%. The additional step may be necessary with highly contaminated serum, plasma, or urine samples.

The efficiency of the FFAP column is maintained at a high level by repacking the first 10 cm of the column every 100–150 sample injections.

Since pemoline is assayed without prior hydrolysis, this method is useful for the analysis of pemoline metabolites. 5-Phenyl-2,4-oxazolidinedione, which is a metabolite in rabbits[12], can be determined, as can the possible metabolite 5-(*p*-hydroxyphenyl)-2-amino-2-oxazoline-4-one (R_T 10.5 min in Fig. 2). The degree of pemoline conjugation in urine also can be determined from the difference between enzymatically hydrolyzed and nonhydrolyzed samples.

Its sensitivity, precision, and specificity make this method desirable for bioavailability and pharmacokinetic studies. Ease of performance allows for the rapid screening of biological samples for pemoline in drug abuse cases.

REFERENCES

(1) L. M. Cummins and J. E. Perry, *J. Pharm. Sci.*, **58**, 762 (1969).
(2) F. DeSio, C. Marena, and E. Bertol, *Med. Sport*, **26**, 23 (1975).
(3) G. P. Cartoni and F. Natalizia, *J. Chromatogr.*, **123**, 474 (1976).
(4) H. Eberhardt and M. Debackere, *Arzneim.-Forsch.*, **15**, 929 (1965).
(5) M. Van Boven and P. Daenens, *J. Chromatogr.*, **134**, 415 (1977).
(6) J. Libeer and P. Schepens, *J. Pharm. Sci.*, **67**, 419 (1978).
(7) S.-Y. Chu and L. T. Sennello, *J. Chromatogr.*, **137**, 343 (1977).
(8) B. Lindström, M. Molander, and M. Groschinsky, *ibid.*, **114**, 459 (1975).
(9) H. Ehrsson, *Anal. Chem.*, **46**, 922 (1974).

ACKNOWLEDGMENTS

The author is grateful to Dr. S. Sun and Dr. S.-Y. Chu for helpful discussions and to Mr. G. Thomas and Ms. M. Leveque for technical assistance.

[9] Whatman 1 PS.
[10] Urine samples from a fasted subject administered a single 75-mg oral dose of pemoline.
[11] Glusulase, Endo Laboratories.
[12] R. Sonders, Abbott Laboratories, North Chicago, Ill., personal communication.