Journal of Chromatography, 137 (1977) 343–350

CHROM. 10,031

GAS CHROMATOGRAPHIC DETERMINATION OF PEMOLINE IN BIOLOGICAL FLUIDS USING ELECTRON CAPTURE DETECTION

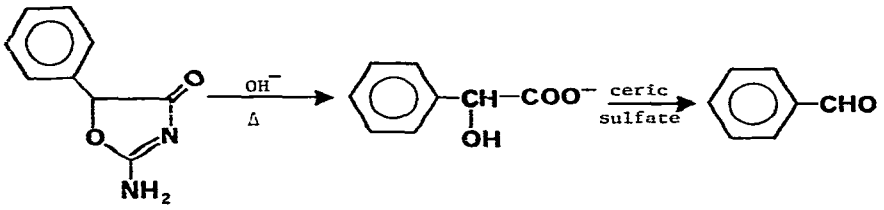
SOU-YIE CHU and LAWRENCE T. SENNELLO
Drug Metabolism Department, Abbott Laboratories, North Chicago, Ill. 60044 (U.S.A.)
(First received December 13th, 1976; revised manuscript received February 28th, 1977)

SUMMARY

A simple and sensitive gas chromatographic (GC) method for the determination of pemoline in biological fluids, utilizing electron capture detection is described. Plasma samples with a pemoline analog added as internal standard were deproteinized with sulfosalicylic acid, and the supernatants were heated at 80°. The pemoline-dione formed was extracted with benzene, and the extract was analyzed on a gas chromatograph equipped with a tritium foil electron capture detector. Poly A-103 (3%) on Gas-Chrom Q (100–120 mesh) packed in a 3-ft. silanized glass column was used as the stationary phase, with nitrogen serving as carrier gas. Under the same GC conditions, benzene extracts of pemoline-dione from acid-hydrolyzed urine samples were analyzed. Using 1 ml of plasma or urine, the lower limit for the assay was about 0.1 μg/ml. The method is accurate and reproducible, with a relative standard deviation within $\pm 4\%$. Mandelic acid (a metabolite of pemoline) does not interfere with the assay.

INTRODUCTION

Pemoline (2-amino-5-phenyl-2-oxazolin-4-one) is a mild central nervous system stimulant which is clinically useful in the treatment of hyperkinesis. The drug is currently marketed as Cylert® by Abbott Laboratories (North Chicago, Ill., U.S.A.). A spectrophotometric method involving the conversion of pemoline to benzaldehyde (as summarized in Fig. 1) has been described for the determination of pemoline concentration in serum and urine[1]. The method suffers from a lack of sensitivity, and requires relatively large serum samples (4–7 ml). It is tedious for the analysis of urine since it includes a time consuming clean-up step utilizing a cation-exchange resin. The specificity is poor because mandelic acid, which has been found at Abbott Laboratories to be a metabolite of pemoline[2], interferes in the assay. Recently, a high-pressure liquid chromatographic (HPLC) procedure has been proposed for the determination of pemoline in pharmaceutical preparations and in urine[3]. The HPLC method should give better specificity, yet no similar procedure has been described for the assay of pemoline in plasma. Our experience in the HPLC analysis of pemoline indicated that the HPLC technique using UV-photometric detection could not

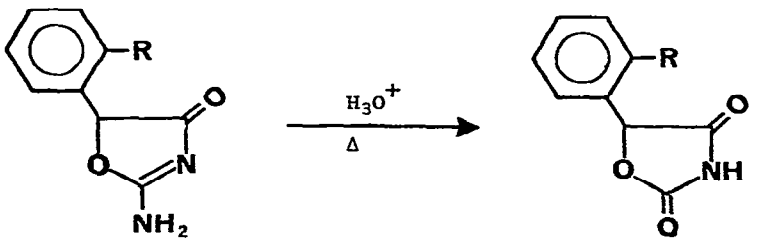


Pemoline — Mandelate — Benzaldehyde Analyzed Spectrophotometrically at 410 nm

Fig. 1. Spectrophotometric assay of pemoline *via* benzaldehyde.

provide the sensitivity needed to monitor low plasma concentrations of that drug at therepeutic dose levels.

It was found in the present study that 5-phenyl-2,4-oxazolidindione (pemoline-dione), an acid-hydrolysis product of pemoline (as illustrated in Fig. 2), evokes a large electron capture response when analyzed by gas chromatography (GC). This discovery, together with the fact that conversion of pemoline to pemoline-dione can be quantitative under moderately acidic conditions[4], led to the development of a new electron capture GC procedure for the determination of pemoline in biological fluids. The method is considerably more sensitive, and less tedious than the existing ones. It offers improved specificity over the spectrophotometric procedure because mandelic acid does not interfere with the assay.



R = H, pemoline — R = H, pemoline-dione

R = CH_3, internal standard — R = CH_3, dione of the internal standard

Fig. 2. Acid hydrolysis of pemoline and its analog.

EXPERIMENTAL

Reagents

Unless otherwise stated, all chemicals were analytical reagent grade from Mallinckrodt (St. Louis, Mo., U.S.A.). 5-Sulfosalicylic acid dihydrate (98%) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). A 50% solution was prepared by dissolving 50 g of the acid in 50 ml of water.

Pemoline, pemoline-dione, and internal standard [2-amino-5-(2-methylphenyl)-2-oxazolin-4-one] were available from Abbott Laboratories stock of chemicals.

Standard solutions of pemoline were prepared at 100 and 10 μg/ml concentrations in methanol. A stock solution of the internal standard was prepared in methanol at 1 mg/ml. A standard solution of the internal standard at 100 μg/ml was prepared by diluting the stock solution 1:10 with distilled water. Additional solutions of the internal standard at 10 and 2 μg/ml were obtained through serial dilutions of the standard solution with 10% aqueous methanol. All solutions of pemoline and internal standard were refrigerated until used.

Chromatographic system and conditions

A Varian Aerograph Series 1400 gas chromatograph with a tritium foil electron capture detector (ECD) was used. A 3-ft. coiled glass column (2 mm I.D.) was silanized by treating with a 10% solution of dimethyldichlorosilane in toluene for 30 min. The column was then packed with 3% Poly A-103 on Gas-Chrom Q (100–120 mesh) (Applied Science Lab., State College, Pa., U.S.A.). The packed column was conditioned overnight at 260° with nitrogen gas flowing. The temperatures in the injection port, column oven, and detector were all 230°. The nitrogen flow-rate was about 45 ml/min. Poly A-103 packing was found to decompose slowly at this operating temperature, resulting in peak tailing and loss of resolution. Columns were replaced after about one week of use.

Assay of plasma samples

For samples containing 2–5 μg/ml of pemoline, 0.3-ml aliquots of each sample were pipetted into 15-ml screw-cap conical centrifuge tubes. To each tube were added 0.3 ml of internal standard solution (10 μg/ml), and 0.1 ml of 50% sulfosalicylic acid. The mixtures were stirred briefly on a Vortex mixer, centrifuged at *ca.* 1500 *g* for 10 min, and the supernatants were transferred to clean conical centrifuge tubes. The tubes were sealed with Teflon®-lined screw caps, and the solutions were heated in a water bath at 80° for 1.5 h. After the contents were allowed to cool to room temperature, 1.0 ml of benzene was added to each tube. The mixtures were shaken for 10 min and then centrifuged at *ca.* 1000 *g* for 3 min. The organic layers were transferred to clean conical centrifuge tubes (each containing about 50 mg of anhydrous sodium sulfate), and after a brief drying, a 5–8-μl aliquot of each solution was injected into the gas chromatograph and analyzed.

A set of samples prepared from control plasma spiked with known amounts of pemoline was analyzed along with the unknown samples. Typically, plasma samples to which pemoline had been added at 0, 1, 2, 5, 10, and 20 μg/ml levels were prepared. Aliquots (30, 60, 150, 300, and 600 μl) of pemoline standard solution (10 μg/ml) were transferred to conical centrifuge tubes, and the organic solvent was evaporated at 40° under a stream of air. To each tube plus a blank, were added 0.3 ml of control plasma and 0.3 ml of an internal standard solution (10 μg/ml). These spiked plasma standards were then carried through the analytical procedure along with the unknown samples.

The results from analysis of spiked plasma standards were used to construct a calibration curve from a plot of peak height ratio of pemoline to internal standard *vs.* concentration of pemoline. Alternatively, a calibration curve was plotted with

weight ratio of pemoline to internal standard *vs.* the corresponding peak height ratio. The concentrations of the unknown were derived from the calibration curve.

The same procedure was followed for plasma samples containing 0.5–4 μg/ml of pemoline, except that 0.5 ml of plasma, 0.5 ml of internal standard solution (2 μg/ml), and 0.2 ml of 50% sulfosalicylic acid were used. For plasma samples containing 0.1–2 μg/ml of pemoline, 1.0 ml of plasma, 0.5 ml of internal standard solution (2 μg/ml), and 0.3 ml of 50% sulfosalicylic acid solution were used.

Assay of urine samples

For urine samples containing 10–250 μg/ml of pemoline, 0.3 ml of each sample were transferred to a separate 15-ml screw-cap conical centrifuge tube containing 0.3 ml of internal standard (100 μg/ml). After addition of 0.1 ml of 4 *N* aqueous hydrochloric acid, and a brief stirring on a Vortex mixer, each tube was capped and heated in a water bath at 80° for 1.5 h, and then allowed to cool. Benzene (2.0 ml) was added to each tube, and the mixtures were shaken for 10 min and centrifuged at *ca.* 1000 *g* for 3 min. The organic layers were transferred to clean 15-ml screw-cap conical tubes containing about 100 mg of anhydrous sodium sulfate. After a brief drying, a 2–10 μl aliquot of each of the solutions was injected into the gas chromatograph and analyzed.

A set of samples prepared from control urine spiked with known amounts of pemoline was prepared and analyzed along with the unknown samples. The pemoline levels added to these control urine samples may vary in order to suit the concentration ranges of the intended analysis. A typical set is presented here. A 0.3- or 1.0-ml aliquot of standard pemoline solution (100 and 10 μg/ml) was transferred to each of four conical centrifuge tubes. The contents of each tube were evaporated to dryness at 40° under a stream of air, and 0.3 ml of control urine was added to each of these tubes plus a blank. To each of these urine samples, to which pemoline had been added to levels of 0, 10, 33.3, 100, and 333.3 μg/ml, were added 0.3 ml of internal standard solution (100 μg/ml), and 0.1 ml of 4 *N* HCl. These urine standards were carried through the analytical procedure along with unknown samples. From these data, a standard curve was constructed from peak height ratios of pemoline to internal standard *vs.* the corresponding weight ratios of pemoline to internal standard.

RESULTS AND DISCUSSION

The key to development of this assay was the discovery of the electron capturing capability of pemoline-dione and its analogs. The ECD response to these compounds is quite large. Data from analysis of pemoline-dione solutions in benzene indicated that 0.5 ng of the compound injected on-column can readily be detected using the proposed GC procedure.

Fig. 3 shows gas chromatograms of samples prepared from blank plasma, a plasma blank spiked with pemoline to a level of 0.328 μg/ml and internal standard to a level of 2.36 μg/ml, blank urine, and blank urine spiked with pemoline at 4.26 μg/ml and internal standard at 7.08 μg/ml. No interfering peaks were observed in samples from either blank plasma or urine. A small peak with a slightly shorter retention time, t_R, than that of pemoline dione appeared in both plasma and urine blanks. This peak (t_R = 5.51 min in Fig. 3) could be well resolved from those of the internal

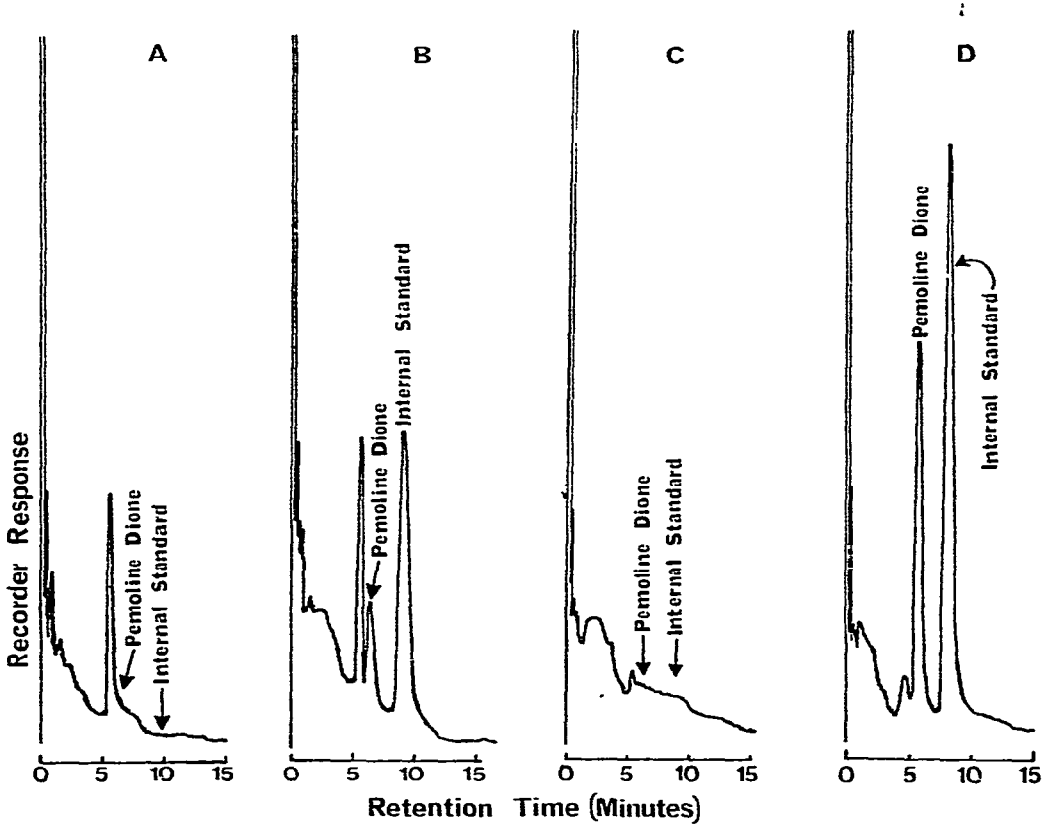


Fig. 3. Electron capture gas chromatograms of samples prepared from A, blank dog plasma; B, blank dog plasma spiked with pemoline at 0.382 μg/ml and internal standard at 2.36 μg/ml; C, blank dog urine spiked with pemoline at 4.26 μg/ml and internal standard at 7.08 μg/ml.

standard and pemoline in the chromatograms when the column was packed and conditioned properly. Silanization of glass columns was found to be important, since silanized columns gave sharper peaks and greatly improved resolution. A newly packed column should be conditioned at 260° for approximately 16 h with nitrogen flowing. Once the column is installed and conditioned, the oven temperature should be reduced to about 70° when the column is not in use in order to increase the serviceable life of the packing.

Pemoline-dione was shown to be chromatographed without chemical alteration on a 6-ft. Poly A-103 column under the same conditions used in this study. When the eluted material was subjected to combined GC–mass spectrometry (MS), spectral data were consistent with the pemoline-dione structure. In the same manner, the peak corresponding to the internal standard was found to have the expected dione structure, based upon GC–MS data.

In the procedure for assay of plasma samples, sulfosalicylic acid served both as a protein precipitating agent and as an acid catalyst for the hydrolysis of pemoline and internal standard. Under the conditions specified for plasma samples, the effect of reaction time on the formation of pemoline dione was studied. The results (shown

in Fig. 4A) indicate that the hydrolysis is complete in 1.5 h at 80°, and that further heating up to 4 h does not increase the amount of product. Fig. 4B shows the results of the hydrolysis of pemoline in urine at 80° under the conditions described for assay of urine samples. The reaction was essentially complete in 40 min, with no change being observed after further heating up to 2 h.

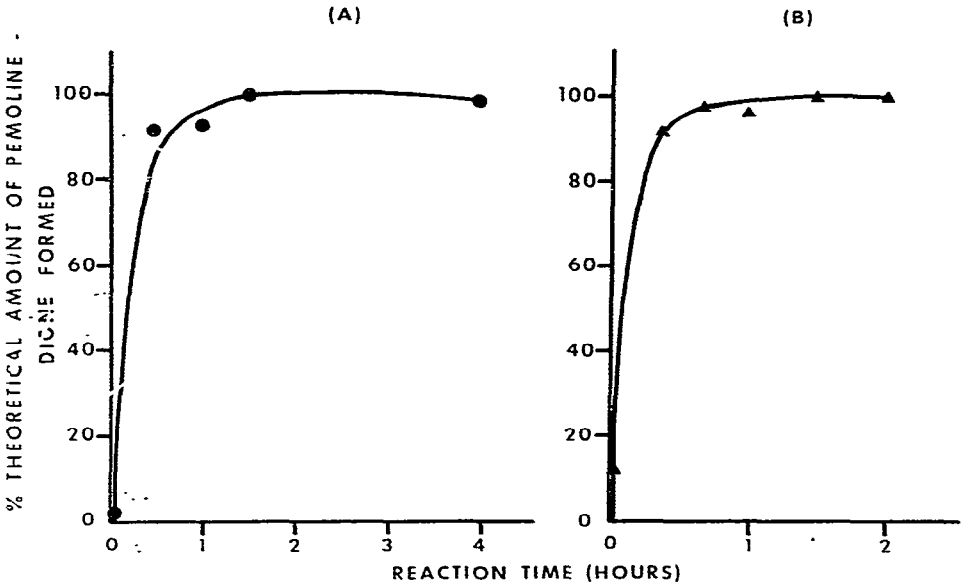


Fig. 4. Formation of pemoline-dione from acid hydrolysis of A, pemoline-spiked plasma sample and B, pemoline-spiked urine. Samples were hydrolyzed by heating in water bath at 80°.

Preliminary studies indicated that the recovery of pemoline-dione from samples decreased gradually when the benzene extract was evaporated at 50° under a gentle stream of air. Thus, concentrating the benzene solution by evaporation could not be used in this procedure. However, this did not create a serious problem for the assay, since the corresponding diones could readily be extracted into a relatively small amount of benzene, and an adequate concentration could be achieved in this extraction step. Ether was not used since it was found that ethereal extracts gave a broad solvent peak that interfered with the assay.

In order to assess the accuracy and precision of the method, the following experiment was performed. Five samples of approximately 10 mg of pemoline were each accurately weighed and individually dissolved in 10 ml of methanol to form five stock solutions. The stock solutions were serially diluted (1:10) twice to give five standard solutions at a concentration of about 10 μg pemoline per ml of methanol. A 50-, 100-, or 500-μl aliquot of each of the standard solutions was transferred to a 15-ml conical centrifuge tube, and the solvent was removed by evaporation at 40° under a stream of air. Plasma standards were prepared by pipetting 0.5 ml of control plasma into each tube and shaking the contents briefly on a Vortex mixer. An aliquot (1.0 ml) of the internal standard solution (3.38 μg/ml) was added to each sample.

A blank was also prepared from control plasma. The plasma standards and the plasma blank were carried through the analytical procedure as described above. The results, as shown in Table I, indicated the method to be accurate and reproducible, with a relative standard deviation within ±2%. A least squares best fit was calculated for peak height ratios, y, *vs.* the corresponding plasma pemoline concentrations, x. The equation of the resulting line was $y = 0.1321x + 0.0038$, where the y intercept

TABLE I

DATA FOR THE DETERMINATION OF PRECISION AND LINEARITY OF THE GC–ECD ANALYSIS OF PEMOLINE IN PLASMA

Theoretical plasma pemoline conc. (μg/ml)	*Weight ratio of pemoline to internal standard*	*Relative peak height of pemoline to internal standard*	*Recalculated plasma pemoline concentration (μg/ml)*	*Percent of theory*
0.96	0.1422	0.1275	0.94	97.9
1.22	0.1807	0.1618	1.20	98.4
1.27	0.1881	0.1667	1.23	96.9
1.48	0.2191	0.2036	1.51	102.0
1.59	0.2355	0.2195	1.63	102.5
1.92	0.2844	0.2611	1.95	101.6
2.44	0.3614	0.3253	2.43	99.6
2.54	0.3762	0.3378	2.53	99.6
2.96	0.4384	0.3804	2.85	96.3
3.18	0.4709	0.4217	3.16	99.4
9.60	1.4218	1.3095	9.88	102.9
12.2	1.8068	1.6233	12.3	100.8
12.7	1.8809	1.6659	12.6	99.2
14.8	2.1919	1.9651	14.9	100.7
15.9	2.3549	2.0854	15.8	99.4
		Mean		99.8
		Standard deviation		±1.96
		Relative Standard Deviation		±2.0%

TABLE II

ANALYSIS OF PEMOLINE IN SPIKED URINE SAMPLES

Sample no.	*Pemoline concentration (μg/ml)*					
	4.26		*70.2*		*175.5*	
	Found (μg/ml)	*% of theory*	*Found (μg/ml)*	*% of theory*	*Found (μg/ml)*	*% of theory*
1	4.27	100.2	71.5	101.9	171.4	97.7
2	4.46	104.7	69.6	99.2	177.0	100.9
3	4.40	103.3	73.0	103.9	176.8	100.8
4	4.35	102.1	66.6	94.8	171.4	97.7
5	4.36	102.3	68.4	97.4	177.7	101.2
6			70.1	99.9		
Mean ± R.S.D.	4.37	102.5 ± 1.6%	69.9	99.5 ± 3.2%	174.9	99.6 ± 1.8%

of 0.0038 was not significantly different from zero. The correlation coefficient was 0.9998, clearly establishing the linearity of the data. A similar linear relationship was obtained between weight ratios and relative peak heights from these data which had weight ratios ranging from 0.14 to 2.4.

Table II shows precision and recovery data for the assay when applied to urine samples. Five to six separately spiked urine samples were prepared at each concentration using the same standard solution of pemoline for each set. The data indicated that recovery was quantitative (95–105%) at various levels, and that the method had a relative standard deviation of about $\pm 3.2\%$.

The method has been used for the assay of plasma and urine samples collected from animals that had been orally dosed with pemoline. Using 1 ml of plasma or urine, pemoline concentrations of about 0.1 μg/ml can be measured. Since mandelic acid (a known metabolite of pemoline), added at a level of 1.06 mg/ml did not interfere with the assay, the method is more specific than the previously published assay involving conversion to benzaldehyde.

REFERENCES

1 L. M. Cummins and J. E. Perry, *J. Pharm. Sci.*, 58 (1969) 762.
2 *Drug Monograph on Cylert®*, Abbott Laboratories, North Chicago, Ill., 1975, p. 9.
3 G. P. Cartoni and F. Natalizia, *J. Chromatogr.*, 123 (1976) 474.
4 J. W. Clark-Lewis, *Chem. Rev.*, 58 (1958) 63.