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High-Performance Liquid Chromatographic Determination of Tracazolate and Its Major Metabolite in Plasma

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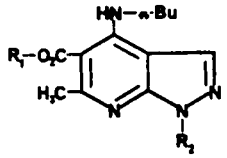
Abstract □ A high-performance liquid chromatographic (HPLC) method is reported that provides for either separate or simultaneous plasma determination of tracazolate and its major metabolite, the free carboxylic acid. Tracazolate is extracted from plasma with hexane and is quantitated by reverse-phase HPLC using an internal standard. The metabolite is extracted in an additional step, methylated, and either quantitated using the same HPLC conditions in a separate determination or combined with the hexane extract for the simultaneous HPLC determination of drug and metabolite. The plasma concentrations of metabolite were as much as 60 times greater than free drug in some animal species, therefore requiring the use of separate determinations for the two compounds. The analyses have limits of reproducible quantitation of 20 ng/mL for tracazolate and 50 ng/mL for the metabolite in plasma. The simultaneous analysis for tracazolate and the metabolite was used to evaluate plasma levels of both compounds *versus* time and dose. Data generated in conjunction with a toxicology study are reported to demonstrate the applicability of the procedure.

Keyphrases □ Tracazolate—free carboxylic acid metabolite, individual and simultaneous assay in plasma, HPLC □ HPLC—tracazolate and its free carboxylic acid metabolite, individual and simultaneous assay in plasma

Tracazolate (I) (ICI 136,753; 4-(butylamino)-1-ethyl-6-methyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylic acid ethyl ester), a nonbenzodiazepine agent with potential clinical utility as a nonsedative anxiolytic drug (1, 2), is extensively metabolized by a first-pass effect in the rat and dog. The major metabolite (II) is the acid resulting from deesterification of I (3). Although II is inactive in pharmacological screening tests, it is of interest since the circulating levels can be 3- to 60-fold greater than I depending on the species and dosage. Metabolite (II) may be the only drug-related compound in sufficient concentration to measure in plasma, therefore, the quantitation of II may provide an indication of absorption of I at low dosages should plasma levels of I fall below the limits of detection.

The analytical procedures described herein allow for either the separate determination of I and II or for the simultaneous determination of both in plasma. The internal standard used was the 1-butyl analogue of tracazolate (III). Compound IV is the methyl ester of II.



I: $R_1 = R_2 = -CH_2CH_3$ (mol. wt. 304)
II: $R_1 = H$; $R_2 = -CH_2CH_3$ (mol. wt. 276)
III: $R_1 = -CH_2CH_3$; $R_2 = -(CH_2)_3CH_3$ (mol. wt. 332)
IV: $R_1 = -CH_3$; $R_2 = -CH_2CH_3$ (mol. wt. 290)

EXPERIMENTAL

Chemicals and Reagents—All organic solvents used were HPLC grade[1]. The water was purified by ion exchange, activated charcoal filtration, and distillation. Ammonium hydroxide was high purity[2], and 10% aqueous trichloroacetic acid was made from reagent-grade trichloroacetic acid[3]. The methylation of II was carried out with diazomethane[4] (4).

Instrumentation—A liquid chromatograph fitted with an automatic sampler[5] was used with a variable-wavelength UV detector[6] set at 240 nm. The columns used were: a 30 cm × 3.9 mm i.d. stainless steel precolumn (prior to the injector) hand packed with 30–38-μm pellicular packing[7], a 3-cm guard cartridge (between the injector and the analytical column) packed with 5-μm octadecylsilane-bonded packing (ODS)[8], and a 25 cm × 4.6-mm i.d. stainless-steel analytical column packed with 7-μm spherical ODS packing[9]. The mobile phase of methanol–water–20% NH_3OH (90:10:0.05 v/v/v) was pumped at ~2 mL/min and produced ~3000 psi back-pressure. A strip-chart recorder[10] provided a real time record of the chromatography; however, the quantitation was done by computer[11]. The detector signal for the computer was tapped prior to attenuation and had an output of 1 V/AU.

Standard Solutions—Stock solutions of I, II, IV, and the internal standard (III) (1.0 mg/mL) were prepared in methanol. Aliquots of the above stock solutions were further diluted in methanol as spiking solutions. Standard solutions of I, III, and IV were brought to volume with mobile phase solution and were chromatographed directly. Ester IV was previously synthesized in bulk and was used directly to make a stock solution. Matrix standards were prepared with control plasma, 200 μL in a 15-mL centrifuge tube, and spiked with methanol solutions of I and III, for the analysis of I only; II only for the separate analysis of II; or I, II, and III for the dual analysis of I and II. Matrix blanks were not spiked. The internal standard (III) was added to samples obtained from dosed animals for either the single or dual analysis where I was being determined. A 20-μL aliquot of a 1.0-μg/mL internal standard solution in methanol was added to the 200-μL plasma aliquots. The resulting concentration of internal standard was 110 ng/mL.

Sample Preparation—For the extraction of I, 2.5 mL of *n*-hexane was added to each plasma sample in a 15-mL centrifuge tube, vortexed for 30 s, and centrifuged. The hexane layer containing I and III was removed with a Pasteur pipet, and if I was to be measured it was transferred into a labeled test tube. If II was to be extracted, a 10% trichloroacetic acid solution (0.04 mL) and 2.5 mL of methyl *tert*-butyl ether were added to the aqueous residues, which were then vortexed and centrifuged. The methyl *tert*-butyl ether layer containing II was removed with a Pasteur pipet and transferred into a test tube. If both I and II were to be determined simultaneously the methyl *tert*-butyl ether layer was added to the *n*-hexane layer from the previous extraction. The

[1] Burdick and Jackson; methanol, *n*-hexane, methyl *tert*-butyl ether, and ether; USI Chemicals: 200 proof ethanol.
[2] Baker Chemicals; "Ultrex" grade 20% ammonium hydroxide.
[3] Baker Chemicals; trichloroacetic acid.
[4] Aldrich Chemical Co., diazald (*N*-methyl-*N*-nitroso-*p*-toluene); Baker Chemical Co., glacial acetic acid (diluted 25% in water); Fisher Chemical Co., potassium hydroxide (0.7 M in 10% v/v water–ethanol).
[5] Model 1081B HPLC; Hewlett-Packard, Palo Alto, Calif.
[6] Spectromonitor III (model 1204); Laboratory Data Control, Riviera Beach, Fla.
[7] 30–38-μm HC Pellosil; Whatman, Clifton N.J.
[8] Guard Holder and 5 μm C18 cartridges; Brownlee, Santa Clara, Calif.
[9] Zorbax ODS packing and columns; DuPont, Wilmington, Del.
[10] Model 391 recorder; Linear Instruments, Irvine, Calif.
[11] Model 3356 Laboratory Automation System; Hewlett-Packard, Palo Alto, Calif.

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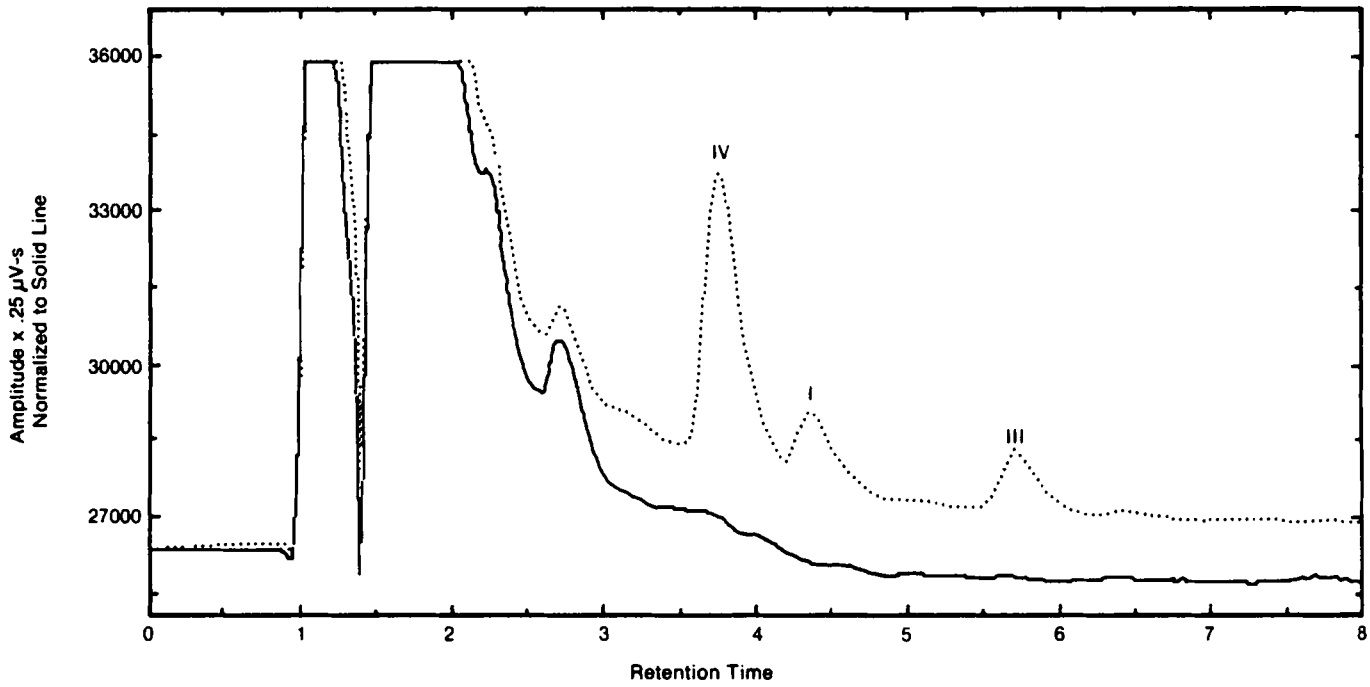

Figure 1—*Tracing of a computer reconstructed chromatogram of a 50-μL injection of a matrix standard (. . .) with 500 ng/mL of IV, 100 ng/mL of I, and 100 ng/mL of III from canine plasma superimposed on the chromatogram of a matrix blank (—). Compound II is not retained on the column, so its methyl ester IV is the species chromatographed and quantitated.*

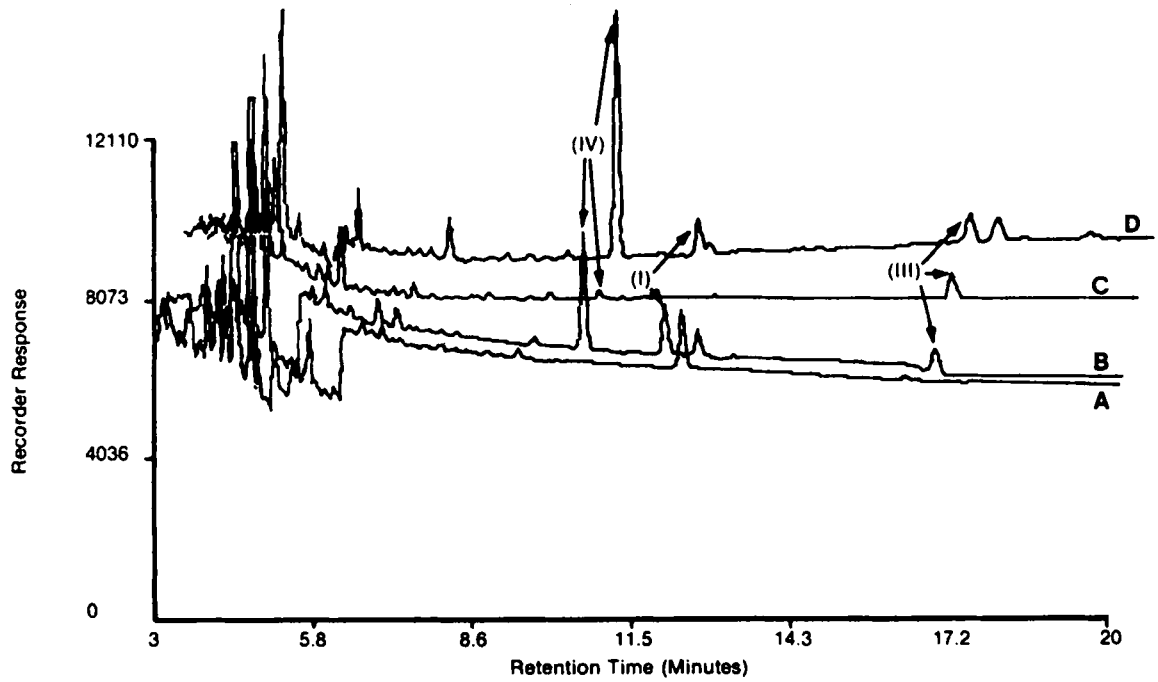

Figure 2—*Tracing of the computer reconstruction of capillary gas chromatograms of a plasma blank (A), a spiked plasma standard (B), and samples at 1 (C) and 6 h (D) postdose from a dog dosed with 80 mg/kg of I.*

organic extracts containing II were evaporated under a gentle stream of dry nitrogen at room temperature to ~0.1 mL. The diazomethane-ether solution (~0.2 mL) was added to each tube, and the tubes were carefully agitated, loosely covered, and let stand for 30 min at room temperature[12]. The extracts were evaporated under an efficient hood with a gentle stream of dry nitrogen at room temperature and removed as soon as they were dry. The dried residues were either tightly capped and stored at −10°C for up to 72 h or diluted with mobile phase solution which remained stable overnight at room temperature.

[12] Mild conditions were required for derivatization because of the potential for the decarboxylation of II at elevated temperatures.

Liquid Chromatographic Procedure—At least four matrix standards were prepared for each sequence of samples. The matrix standard solutions were positioned between the standard solutions and the samples and again after the samples as a reproducibility check. Compound concentration ranges were the same for the standard solutions and matrix standards (see Table I). Peak heights (PH) of I, IV, and III were used as the measure of detector response. Results for the dual determination of I and II or the separate determination of I were calculated by the internal standard method using the average of matrix standard response factors in a multipoint calibration. The concentration of II in the separate determination was calculated using the external standard method, since the internal standard (III) was removed in the initial *n*-hexane extraction.

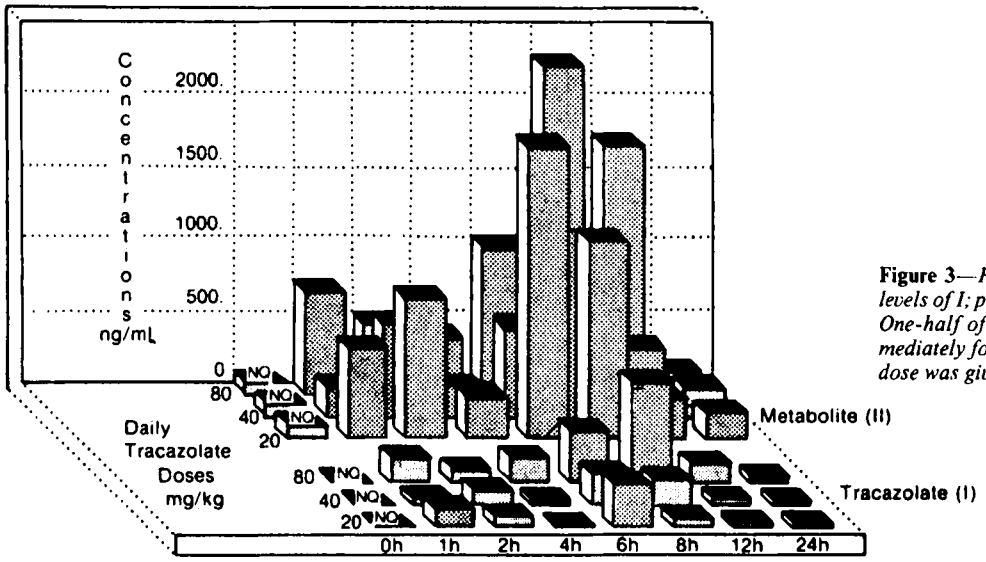

Figure 3—*Pharmacokinetic data from dogs at 3 dosing levels of I; plasma levels of I and II are reported in ng/mL. One-half of the daily dose of I was given by gavage immediately following the predose sample, and the remaining dose was given after the 4-h sample.*

RESULTS

A representative chromatogram, shown in Fig. 1, was from a study for which the dual analysis of I and II was used.

Recovery—Control of plasma pH was found not to be critical for the efficient extraction of I or III. However both pH control and efficient protein precipitation were found to be critical for the extraction of II from plasma. The plasma sample was buffered to pH 1.6 and protein was precipitated (5) when 10% trichloroacetic acid was added at a 1:5 volume ratio to the samples. Methanol spiking solutions were not found to affect the recoveries of I and II when added to plasma at levels of <1% up to 20% (v/v). External standard response factors were calculated and averaged for the matrix standards and the standard solutions. The ratio of the average standard solution response factor and the average matrix standard response factor was used to evaluate the percent recovery of desired compound[13]. Average recoveries of I and II, when extracted from spiked plasma, were 102 and 96%, respectively, during the production of the data listed in Table II. The coefficients of variation for recoveries across the concentration ranges listed in Table II were 4.3% for I and 6.8% for II. The diazomethane methylation of II had an efficiency of 94% *versus* standard solutions of IV. Hexane extracts of plasma resulted in a recovery of 86% of ^{14}C-labeled I added at the 1.0-μg/mL level measured by radiochemical techniques. Methyl *tert*-butyl ether extracts of plasma recovered 85% of ^{14}C-labeled II added at the 2 μg/mL level. Recoveries of [^{14}C]I and [^{14}C]IV were complete when injected directly into the HPLC as solutions in mobile phase.

UV detection was done at the maximum absorbance of I, which occurred at 240 nm with $\epsilon > 30{,}000$ and provided high sensitivity. From Table I, the peak height ratios for the matrix standards were reproducible and linear from 20 to 2000 ng/mL for I and from 100 to 5000 ng/mL for II measured as IV. Correlation coefficients were >0.99 for both curves, and y-intercepts were 0.0478 and 0.5834, respectively. Accuracy and precision were measured from routine calibration data which reflected the accuracy and precision expected in practice. The coefficients of variation were <10% and relatively constant over a wide analytical range for both compounds. The limits of quantification, represented in Table II, were selected to ensure reproducibility of the determination of the low standards on a daily basis.

Specificity—Capillary GC of sample extracts from animals dosed with I was used in the evaluation of specificity. The samples were carried through the sample preparation procedure for the dual determination of I and II described above. The dry residue of the sample extractions containing I, IV, and III was reconstituted in isooctane. This mixture was injected onto a 15-m fused silica capillary column coated with SE52 methyl silicone gum[14]. The column was temperature programmed from 80°C to 230°C in a gas chromatograph equipped with an alkali bead nitrogen detector[15]. As demonstrated in Fig. 2, the elution order of IV, I, and III was the same order as in the HPLC method.

Table I—Peak Height Ratios from Matrix Standards: Dual Determination of I and II [a]

Spiked Conc. in Plasma, ng/mL	Peak Height Ratios[b]	
	I	II (Measured as IV)
20	0.296	
	0.292	
50	0.710	
	0.691	
100	1.280	1.534
	1.276	1.384
250		2.850
		2.732
500	6.360	4.682
	6.504	4.572
2000	26.607	
	26.773	
2500		19.715
		20.036
5000		40.438
		41.135

[a] Procedure used for canine plasma samples. [b] Peak height ratios: a sample peak height/III peak height.

No interfering peaks with I or IV were seen under these GC conditions. Specificity was also evaluated by a GC–MS analysis of samples prepared according to the dual procedure from animals dosed with I. Isolated fractions of HPLC eluant of these samples corresponding to the HPLC peaks of I and IV were dried under nitrogen and the residue analyzed by GC–MS[16]. The pure compounds displayed molecular ions of m/z 304 and 290 for I and IV, respectively, a base peak of m/z 215, and several fragments including m/z 99. The mass spectra as well as the GC retention times of the HPLC eluants corresponded to the respective data of the pure compounds.

Stability of Prepared Samples—Plasma samples from animals dosed with I were analyzed several times for both I and II over a period of 5 months and were found to be stable. These plasma samples were stored frozen with intermittant thawing and maintenance at room temperature for several hours during preparation for analysis. Stock and standard solutions of I, II, III, and IV were stable, without measurable alteration, for at least 1 month if refrigerated between uses.

DISCUSSION

The quantitative HPLC procedures for tracazolate and its major metabolite described herein have been used to evaluate circulating levels of these com-

[13] The molecular weight ratio of II and IV was used to properly adjust the percent recovery to a molar basis.

[14] *Fused silica capillary column*; J & W Scientific, Rancho Cordova, Calif.

[15] Hewlett-Packard Model 5730 GC fitted for capillary columns.

[16] Model 4000 GC/MS; Finnigan Instruments, Sunnyvale, Calif., utilizing the same GC capillary conditions described above with the quadrupole MS operating under EI conditions at 70 eV and scanning from m/z 60–350.

Table II—Accuracy and Precision from Matrix Standards: Individual Determinations of I and II [a]

Compound	Value Expected, ng/mL	Mean Found[b], ng/mL	CV[c], %
I	20	23	4.7
	50	50	3.3
	100	100	6.3
	500	503	1.5
	2000	1852	4.5
II	100	100	7.9
	250	250	1.0
	500	515	4.3
	2500	2405	2.0
	5000	5146	3.6
	10,000	9835	3.5

[a] Procedure used for mouse plasma samples. [b] Found calculated from peak height ratio and peak height ratio response factors for I and II, respectively, from four standard curves each run on a different day for each compound. [c] For four determinations.

pounds in pharmacological, pharmacokinetic, and toxicological studies in rats, dogs, mice, monkeys, and rabbits. These procedures had reproducible quantifiable limits of 20 ng/mL for I and 50 ng/mL for II. Separate determinations of I and II were required for animal samples in which ratios of the concentration of II to the concentration of I were large enough to significantly degrade the resolution between the HPLC peaks. These high ratios occurred in plasma samples from dosed rats, mice, and rabbits. The separate determinations were used in these cases; otherwise, the selectivity, sensitivity, and other analytical indicators were the same for the plasma of all species tested.

Results from the dual determination of I and II in plasma from dogs are shown in Fig. 3. Part of these results were generated using the standardization curve data reported in Table I. The animals were dosed orally with one-half of the daily dose of I just after the time zero samples were taken and with the remainder of the daily dose after the 4-h samples. The data were evaluated for peak plasma level times (6–8 h) as well as for relationships between dose and plasma levels (no consistent dose response). The low detection limits for both I and II and the low plasma volume requirements for analysis have made this procedure very useful in multisampling and small animal studies. These points and the versatility of this procedure are amply demonstrated in Fig. 3.

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Collaborative Study of the USP Dissolution Test for Prednisone Tablets with Apparatus 2

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Abstract □ Five lots of prednisone tablets that disintegrate within 5 min were collaboratively studied by 11 laboratories using USP Apparatus 2 under carefully controlled conditions. One lot gave complete dissolution. The reproducibility and repeatability of Apparatus 2 for the four lots still dissolving at the end of the test were 2.6 and 1.6% of label claim, respectively, for the 11 laboratories.

Keyphrases □ USP dissolution test—collaborative study of Apparatus 2, five lots of prednisone tablets, 11 laboratories □ USP Apparatus 2—collaborative study of dissolution of five lots of prednisone tablets, 11 laboratories □ Prednisone tablets—dissolution of five lots with USP Apparatus 2, collaborative study by 11 laboratories

The USP dissolution test for prednisone tablets (1) requires that when 12 tablets are tested, an average of ≥80% of the labeled amount of prednisone must dissolve in 30 min. The tablets are individually tested under experimental conditions which must be carefully controlled if reproducible results are to be obtained.

Four common sources of error associated with Apparatus 2 have been identified: misalignment of equipment (2), nonuniformity of the bottom curvature of vessels (3), excess gases in the dissolution medium (4), and the interaction of the test with slowly disintegrating tablets (4). Equipment, tools, and technique were developed and improved between 1978 and 1980 to control the first three sources of error. Certain products consist of slowly disintegrating tablets that do not always settle at the center of the bottom of the vessels; such variability of tablet position before disintegration can give imprecise results (4). However, rapidly disintegrating tablets gave results of sufficient precision to warrant a collaborative study.

The purpose of this collaborative study, conducted by 11 laboratories in the second half of 1980, was to measure the reproducibility and repeatability of Apparatus 2 under state-of-the-art conditions for prednisone tablets that disintegrate within 5 min. The secondary objectives were to determine whether personnel in many laboratories could correctly adjust Apparatus 2 by following a set of detailed instructions and whether the apparatus would hold the adjustment over an ~2-week test period.

EXPERIMENTAL

Dissolution Test—The instructions[1] to collaborators conformed to the USP conditions for testing prednisone tablets (1) with two exceptions. The collaborators were instructed to drop a tablet down the side of the vessel with the paddle rotating rather than to drop a tablet into the vessel and then start paddle rotation. The collaborators were instructed to position each vessel so that its vertical axis was not more than 1 mm at any point from the axis of the paddle shaft. A 2-mm tolerance is allowed in the USP specifications. If this second

[1] The complete instructions are available from the authors on request.

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