Fractionation—The ethanol extract was partitioned between water (1 liter) and chloroform (1 liter) to give fractions of 4.3 (2.2%) and 6.0 (3.0%) g, respectively. The chloroform fraction was then partitioned between petroleum ether (bp 60–90°) (200 ml) and methanol–water (9:1) (200 ml) to yield fractions of 3.5 (18%) and 2.0 (1.0%) g, respectively.

Chromatography—The aqueous methanol fraction was chromatographed on silicic acid[4] (75 g, 2 × 50 cm) in chloroform.

Isolation of II—Elution with 1% methanol–chloroform afforded a residue (233 mg), which was dissolved in chloroform (25 ml) and partitioned with 1% hydrochloric acid (3 × 25 ml). The combined acid layers (75 ml) were made basic to pH 8 with concentrated ammonium hydroxide and partitioned with chloroform (3 × 75 ml). The combined chloroform layers were dried over anhydrous sodium sulfate and evaporated *in vacuo* at 40° to yield a yellowish residue (15 mg).

Repeated attempts at crystallizing quindoline failed, so data were obtained on the amorphous residue; UV: λ_{max} (methanol) 227 (log ϵ 4.30), 269 sh (4.45), 274 (4.46), 330 sh (3.82), and 345 (4.03) nm; λ_{max} (0.01 *N* ethanolic hydrochloric acid) 224 (log ϵ 4.27), 242 sh (3.94), 273 (4.36), 280 (4.38), 350 sh (4.03), and 368 (4.26) nm; IR: ν_{max} (potassium bromide) 1632, 1608, 1487, 1457, 1396, 1370, 1333, 1222, 1150, 1140, 1122, 1105, 1000, 875, 865, 845, 837, 813, 754, 745, 738, 710, and 604 cm^{-1}; mass spectrum (M^+): *m/e* 218 (100%), 217 (8), 190 (8), 109 (12), 95.5 (4), 90 (3), and 89 (5). The alkaloid was found to be identical with an authentic sample of quindoline[5] by direct comparison (UV, IR, and mass spectra and TLC).

Isolation of III—Elution with 2% methanol–chloroform afforded a residue (327 mg), which, when crystallized from methanol, gave III (105 mg), mp > 300°. The structural elucidation of this substance is currently in progress.

Isolation of I—Elution with 30% methanol–chloroform afforded I (300 mg), mp 167–168° dec. [lit. (1) mp 166–169°], hydrochloride mp 263–264° dec. [lit. (1) mp 263–265°], hydroiodide mp 285–287° dec. [lit. (4) mp 284–286°], *methiodide* mp 284–285° dec. [lit. (1) mp 285–288°]; UV: λ_{max} (methanol) 224 (log ϵ 4.11), 246 (3.87), 275 (4.41), 283 (4.43), 355 sh (4.02), 370 (4.33), 4.10 (3.28), and 433 (3.29) nm; λ_{max} (0.01 *N* ethanolic potassium hydroxide) 214 (log ϵ 4.40), 230 sh (4.01), 297 sh (4.38), 307 (4.48), 368 (3.50), and 386 (4.08) nm; IR: ν_{max} (potassium bromide) 1631, 1611, 1585, 1505, 1492, 1460, 1400, 1366, 1357, 1330, 1310, 1300, 1275, 1250, 1160, 1150, 1130, 1040, 900, 887, 875, 850, and 750 cm^{-1}; mass spectrum (M^+): *m/e* 232 (100%), 231 (12), 217 (26), 190 (10), 116 (14), and 89 (15); NMR (trifluoroacetic acid): δ 5.08 (s, 3H, NCH_3), 7.55–8.62 (m, 8H, aromatic), and 8.95 (s, 1H, aromatic) ppm.

[4] Mallinckrodt, 100 mesh.

[5] Reference sample provided by Professor Emery Gellert, Department of Chemistry, University of Wollongong, Wollongong, Australia.

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New Spectrophotofluorometric Assay for Probenecid

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Received January 24, 1977, from the *Department of Medicine, Clinical Pharmacology Program, Emory University School of Medicine, Atlanta, GA 30322.* Accepted for publication June 15, 1977.

Abstract □ A new spectrophotofluorometric assay for probenecid is presented based on conversion of the drug to a fluorescent anthranilic acid derivative. The assay is especially applicable with "clean" biological fluids such as cerebrospinal fluid and offers severalfold greater sensitivity than the commonly used UV method.

Keyphrases □ Probenecid—spectrophotofluorometric analysis in biological fluids □ Spectrophotofluorometry—analysis, probenecid in biological fluids □ Uricosuric agents—probenecid, spectrophotofluorometric analysis in biological fluids

Recently, several GLC procedures (1–4) and a new radioimmunoassay were developed (5) to measure low probenecid levels in biological fluids. Interest in such assays was stimulated by studies of the accumulation of cyclic adenosine monophosphate (6, 7) and metabolites of biogenic amines (8–11) in cerebrospinal fluid caused by probenecid and the well-known inhibitory effect of this drug on active transport systems in the brain (12).

Based on observations with a fluorescent amino derivative of probenecid (13), it was decided that a fluorometric assay would offer definite advantages over existing methodologies—greater sensitivity than the UV procedure (14) and less cost than GLC. In the present study, reaction conditions for quantitative conversion of probenecid to a fluorescent species were investigated and the fluorescent product was characterized. A method for quantitating probenecid in cerebrospinal fluid and plasma is proposed and evaluated.

EXPERIMENTAL

Probenecid[1], ring-labeled ^{14}C-probenecid[1] (specific activity of 0.8 mCi/mmole), and 2′-nitroprobenecid[1] were used as received. The side-chain metabolites were prepared as previously described (15). Radioactivity was measured in a liquid scintillation spectrometer[2] in a counting fluid prepared as described previously (5). Relative fluorescence intensity was measured in 1.0-cm cells in a spectrophotofluorometer[3].

TLC was carried out on silica gel G glass plates[4] (5 × 20 cm, 250 μm)

[1] Courtesy of Dr. John E. Baer, Merck Sharp and Dohme, West Point, Pa.

[2] Beckman LS-255; efficiency for ^{14}C = 90%.

[3] Aminco-Bowman, equipped with an EMI 9558 QC phototube, a Schoeffel microphotometer model 600 at 1200 v, and a magnetic arc stabilizer accessory; slits were set at 3 mm.

[4] Analabs, New England Nuclear, North Haven, Conn.

Table I—Spectrophotofluorometric Method for Probenecid

Probenecid Reacted, μg	Relative Fluorescence Intensity Units per Microgram[a] Standards	Water (1.0 ml)	Dog Cerebrospinal Fluid[b] (1.0 ml)	Dog Plasma[b] (0.1 ml)
4	120	111	113	117
7	106		116	109
10	95	111		133
15	106	106	111	104
30	110	102	105	118
40	112		102	102
80	101	101	101	102
115	93		102	103
150	94	95	97	93

[a] Corrected for ethylene dichloride aliquots with blanks subtracted. Values represent averages of duplicates (duplicates within averages ±4%). Blanks for water, dog cerebrospinal fluid (1 ml), and dog plasma (0.1 ml) were 111, 121, and 450 relative fluorescence intensity units, respectively. [b] Cerebrospinal fluid samples from two other dogs and plasma samples from two human subjects gave blank values of 310 and 115 for cerebrospinal fluid and 370 and 600 for plasma. When probenecid (100 μg/test) was added to these samples and assayed, the relative fluorescence intensity units per microgram values for probenecid were 76 and 81 for cerebrospinal fluid and 95 and 101 for plasma.

using three solvent systems: A, methanol–ethyl acetate–acetic acid (10:10:1 v/v); B, methanol–ethyl acetate–28% ammonium hydroxide (10:10:1); and C, 95% ethanol–acetone–28% ammonium hydroxide (10:10:1). For radiochromatography, 0.5-cm zones of gel were scraped using a thin-layer plate scraper[5] and transferred into counting vials. Radiochemical purity (>95%) was established by TLC as already described.

Two dogs (mongrel male, 14 kg) were anesthetized with pentobarbital sodium (30 mg/kg ip) and administered ^{14}C-probenecid (140 mg, 20 μCi ip). Blood samples were drawn prior to dosing (5 min) and at 1, 2, 3, and 4 hr. Cerebrospinal fluid was collected at 4 hr by cisternal puncture using a 19-gauge spinal needle. Aliquots of plasma (0.1 ml) or cerebrospinal fluid (1.0 ml) were analyzed for probenecid by the spectrophotofluorometric method. As a check on the method, additional aliquots were analyzed by direct counting and by counting ethylene dichloride extracts (evaporated to dryness prior to mixing with counting fluid).

For the spectrophotofluorometric assay, human and dog plasma (0.1 ml diluted to 1 ml with water) or dog cerebrospinal fluid (1 ml) was acidified with 0.5 ml of 3 *N* HCl and shaken for 30 min with 10 ml of ethylene dichloride. After centrifugation (500×*g*, 10 min; all subsequent centrifugation steps were carried out in the same manner), 8 ml of the organic layer was placed in a 40-ml conical centrifuge tube and evaporated to dryness with an air stream at room temperature. Care was taken to transfer only the organic phase and to dry completely.

To the residue, 1 ml of a mixture of concentrated sulfuric acid–70% nitric acid (100:1 v/v) was added. The tube was sealed with a polyethylene stopper to prevent steam uptake during subsequent heating. A hypodermic needle (18 gauge) was inserted through the stopper and left in place throughout the procedure to allow for vapor expansion. After mixing[6] to dissolve the residue, the solution was heated (boiling water bath, 30–35 min) and then diluted with 2 ml of ice-cold water. Stannous chloride (0.2 ml, 2 *M* in 1 *N* HCl) was added, and the heating was resumed for 20–25 min. Upon cooling to room temperature, charcoal[7] (0.5 ml of a 10% water slurry) was added; the mixture was then shaken for 2 min and centrifuged.

The charcoal residue was resuspended in 1 ml of water, briefly stirred, and recentrifuged. Upon repetition of this step with additional water, the final charcoal residue was suspended in 2 ml of 0.2 *N* NaOH and heated (boiling water bath, 20–25 min). After cooling to room temperature, 2 ml of 95% ethanol was added; the mixture was briefly stirred and then centrifuged. Just prior to fluorescence measurement, 3 ml of the supernate was mixed with 0.2 ml of acetic acid.

Relative fluorescence intensity was measured within 5 min of the addition of acetic acid, after which time the sample became turbid and required recentrifugation. The procedure could be stopped for at least 2 days at any point following the addition of stannous chloride and prior to the addition of acetic acid. Excitation and emission wavelengths were 340 and 415 nm, respectively. The spectrophotofluorometer was standardized with anthranilic acid (1 μg/ml in 95% ethanol, 1200 relative fluorescence intensity units).

[5] Autozonal scraper, New England Nuclear, North Haven, Conn.
[6] Vortex mixer.
[7] Norit A, Matheson, Coleman and Bell.

Table II—Comparison of Spectrophotofluorometric and Radiochemical Assays

Dog[a]	Sample and Time, hr	Probenecid, μg/ml Fluorometric	Radiochemical
1	Plasma 1	28	31.9
	Plasma 2	24	27.2
	Plasma 3	25	25.7
	Plasma 4	18	21.6
	Cerebrospinal fluid 4	1.2	1.5
2	Plasma 1	47	38.5
	Plasma 2	37	40.5
	Plasma 3	35	38.1
	Plasma 4	27	34.3
	Cerebrospinal fluid 4	2.6	2.1

[a] Dose of 10 mg/kg ip.

The relationship between fluorescence and concentration of probenecid was determined by reacting known amounts of the drug (obtained by evaporating aliquots of ethanol stock solution of probenecid). Similar determinations were made with solutions of probenecid (1–150 μg/ml) in water, human and dog plasma, and dog cerebrospinal fluid (obtained from pentobarbital-anesthetized animals). Water, plasma, and cerebrospinal fluid control blanks were analyzed simultaneously.

RESULTS

Various reaction conditions were investigated to maximize fluorescence. Of the several nitrating reagents tested (nitronium tetrafluoroborate, cupric nitrate–acetic anhydride, *etc.*), a mixture of concentrated sulfuric acid and 70% nitric acid (100:1 v/v) was the most effective. Virtually complete nitration was obtained by heating at 100° for 20 min. Increasing the ratio of nitric acid resulted in a more rapid reaction but required a concomitant increase in the amount of reducing agent.

A large quantity of stannous chloride (0.4 mmole/test) was needed for complete conversion of the nitro compound to the fluorescent amine. Dilution with water was necessary to keep salts in solution. Charcoal, 50 mg/test, adsorbed 98–100% of radioactivity in samples containing ^{14}C-probenecid when reacted as described. Two 1-ml water washes of the charcoal removed only negligible amounts (<2%) of carbon-14 but eliminated residual mineral acids and salts. Elution of ^{14}C-labeled fluorescent product from the charcoal with 0.2 *N* NaOH (2 ml heated at 90–100° for 20 min) resulted in greater than 90% recovery of carbon-14. Fluorescence was increased about twofold by the addition of an equal volume of 95% ethanol. A pH range of 3.3–3.9 was optimal for maximum fluorescence of the product in an aqueous solution; the addition of 0.1–0.3 ml of acetic acid/3 ml of the final alkaline–alcoholic supernate resulted in maximum fluorescence.

The interference by metabolites of probenecid in the method was evaluated by adding these compounds at the nitration step of the procedure. The metabolites gave the same relative fluorescence as probenecid on a molar basis. Interference in the method, however, would be minimal since the metabolites of probenecid are poorly extracted by ethylene dichloride (16). Pentobarbital and salicylate (20 μg/test) did not contribute detectable fluorescence.

Typical results are summarized in Table I. A linear relationship was obtained between the relative fluorescence intensity and the amount of probenecid (≤ 100 μg/test). When 10 μg of probenecid was reacted on four separate occasions, 940, 910, 975, and 990 relative fluorescence intensity units were obtained (blanks subtracted). Recoveries from water, cerebrospinal fluid (1.0 ml/test), or plasma (0.1 ml/test) were 80–125%. The water or cerebrospinal fluid blank (1 ml) was 1.1–3.4 μg of probenecid equivalent, while plasma blank (0.1 ml) was 3.6–6.4 μg of probenecid equivalent.

Plasma and cerebrospinal fluid samples from two dogs administered ^{14}C-probenecid were assayed by the fluorescence procedure as well as by ^{14}C-measurement. Results obtained by the two methods are compared in Table II. The ratios of cerebrospinal fluid to plasma concentrations of probenecid were in agreement with results obtained in this laboratory using radioimmunoassay for measurement of probenecid in dogs (17).

After carrying ^{14}C-probenecid through the method, the final product was studied by TLC in Solvent Systems A–C. Radioactivity migrated as a single spot with R_f values identical to those of *p*-sulfoanthranilic acid in each system (R_f 0.67, 0.21, and 0.47 in A–C, respectively). The fluorescent product of probenecid and *p*-sulfoanthranilic acid exhibited the

same maximum activation and emission wavelengths as described for 2′-aminoprobenecid (13).

DISCUSSION

The basis of the fluorescence method is conversion of probenecid to an anthranilic acid derivative. Blanchard *et al.* (13) reduced 2′-nitroprobenecid to 2′-aminoprobenecid and described the fluorescent properties of the latter. This observation was applied to the assay of probenecid by first nitrating the drug and then reducing the nitro group. However, the resulting fluorescent product was not 2′-aminoprobenecid, a relatively nonpolar compound that could be extracted into organic solvents, but the more polar *p*-sulfoanthranilic acid. Consequently, interfering substances such as salts and degradation products of biological origin (blank) were only poorly separated from the fluorescent probenecid derivative. The present method achieves a partial separation of interfering substances by means of a charcoal adsorption–elution step. A more effective separation to maximize fluorescence, such as TLC or high-pressure liquid chromatography, seems indicated.

While the fluorometric method for probenecid has only severalfold greater sensitivity than the spectrophotometric method for assay in cerebrospinal fluid and less sensitivity for plasma, there is no significant interference by drugs such as salicylate, unlike the interference by this compound in the UV assay (14). Since metabolites of probenecid are poorly extracted by ethylene dichloride (16), the method is generally specific for probenecid.

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Determination of Fluoxymesterone, Norethandrolone, Prednisolone, and Prednisone in Tablets by Differential Pulse Polarography

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Received February 1, 1977, from the *Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada, M5S 1A1.* Accepted for publication June 22, 1977.

Abstract □ A differential pulse polarographic method for the determination of fluoxymesterone, norethandrolone, prednisolone, and prednisone in tablets is described. This method is more sensitive than dc polarography, and the measurement of diffusion current is greatly simplified. Sørensen phosphate buffer, pH 5.6, was used as the supporting electrolyte. No apparent interference was observed from tablet excipients; the method is rapid, simple, and relatively precise.

Keyphrases □ Fluoxymesterone—differential pulse polarographic analysis in tablets □ Norethandrolone—differential pulse polarographic analysis in tablets □ Prednisolone—differential pulse polarographic analysis in tablets □ Prednisone—differential pulse polarographic analysis in tablets □ Differential pulse polarography—analyses, fluoxymesterone, norethandrolone, prednisolone, and prednisone in tablets □ Androgens—fluoxymesterone and norethandrolone, differential pulse polarographic analysis in tablets □ Glucocorticoids—prednisolone and prednisone, differential pulse polarographic analysis in tablets □ Steroids—fluoxymesterone, norethandrolone, prednisolone, and prednisone, differential pulse polarographic analyses in tablets

The differential pulse polarographic assay of several Δ^4-3-ketosteroids in parenteral formulations (1) and oral contraceptive tablets (2) recently was reported. In addition, the polarographic reduction of certain other Δ^4-3-ketosteroids is well documented (3–7), and a number of workers reported the dc polarographic assay of steroids having this same α,β-unsaturated keto group in pharmaceutical preparations (8–10). Several reports concerned the application of colorimetric (11, 12), spectrophotometric (13, 14), and titrimetric (15) procedures for the estimation of prednisone, prednisolone, and other corticosteroids in pharmaceutical dosage forms.

This paper reports a simple and rapid differential pulse polarographic method for the analysis of fluoxymesterone (I), norethandrolone (II), prednisolone (III), and prednisone (IV) in commercial tablets.

EXPERIMENTAL

Polarographic Analysis—Assays were performed using a polarographic analyzer[1] equipped with a drop timer[2] in the differential pulse

[1] Princeton Applied Research model 174A.
[2] Princeton Applied Research model 172A.