Table I—Urine Levels (Percent of Dose)

Method	0–12 hr	12–25 hr
GLC	2.9	0.31
UV	2.4	0.25

used in this procedure is 0.5 μg/ml. A similar GLC assay designed to measure both I and II was reported recently (4).

Interference of Aspirin and Salicylic Acid—Since tolmetin often could be used with large doses of aspirin, the interference of aspirin or salicylic acid in the spectrophotometric and GLC assay for tolmetin was evaluated. Plasma was seeded with 500 μg/ml[6] of aspirin or 500 μg/ml of salicylic acid and assayed by both procedures.

No interference was observed from either aspirin or salicylic acid with the GLC assay, but significant interference was observed in the spectrophotometric assay. For 500 μg/ml of aspirin, the spectrophotometric assay gave an absorption equivalent to ~10 μg/ml of tolmetin. For 500 μg/ml of salicylic acid, the assay gave absorption equivalent to 50 μg/ml of tolmetin. Since the levels of tolmetin at therapeutic doses (400–600 mg) are between 10 and 30 μg/ml, the spectrophotometric assay would not be suitable for the determination of plasma levels of tolmetin in patients who are simultaneously receiving aspirin products.

With the spectrophotometric assay, one can easily assay 36 samples/day. With the GLC assay, using a dual-column gas chromatograph and manual injection, analytical productivity is reduced by approximately half. Therefore, the spectrophotometric assay offers significant economic advantages over the GLC assay in controlled situations, *e.g.*, bioavailability studies (6). Because of the greater specificity of the GLC procedure, it offers greater utility in the assay of plasma levels of tolmetin in clinical practice (6).

[6] The 500-μg/ml level of aspirin or salicylic acid is at least two to three times the level expected from a 4.5-g dose of aspirin per day (5).

Comparison of the Two Analytical Procedures—To evaluate the applicability of the assay procedures, plasma and urine samples from a fasted subject dosed with 600 mg of tolmetin as an oral solution were assayed using both methods. The results are shown in Fig. 5. Studies with ^{14}C-tolmetin (3) showed that 100% of the administered dose of radioactivity is excreted in the urine within 24 hr. These radioactive studies also demonstrated that only 2–3% of the administered dose is excreted in the urine as intact drug. Therefore, the urine assay (Table I) provides a severe test of both assay procedures. The agreement of the urine and plasma assays by both methods, as well as the agreement of the results with data from other studies using radioactive and nonradioactive tolmetin (3, 4), validates the utility of both assay procedures.

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GLC Determination of Procainamide in Biological Fluids

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Abstract □ A GLC method for the determination of procainamide in biological fluids is presented. By using a dipropyl analog of procainamide as an internal standard, both compounds can be chromatographed directly, yielding linear calibration curves and a sensitivity that allows quantitative determination of concentrations as low as 0.1 μg/ml. The extraction procedure was carefully modified to avoid hydrolysis of *N*-acetylprocainamide, a major metabolite of procainamide. The usefulness of the procedure is demonstrated by following the disappearance of procainamide from the plasma and urine of human subjects treated with the drug.

Keyphrases □ Procainamide—GLC analysis, biological fluids, therapeutic range □ GLC—analysis, procainamide in biological fluids

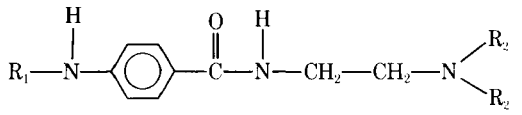
The effectiveness of procainamide[1] (I) in the prevention or treatment of cardiac arrhythmias has been firmly established (1, 2). Careful clinical studies demonstrated that plasma levels of procainamide may be correlated with pharmacological responses. The range of therapeutic plasma concentrations is 4–8 μg/ml (2–4).

Due to individual differences in absorption and elimination of procainamide, dosages necessary for therapeutic effects may vary widely (2). Several methods are presently available for the determination of procainamide plasma concentrations within the therapeutic range.

The most commonly used method of determining procainamide in biological fluids is a colorimetric method (1) where, following complexation with *N*-(1-naphthyl)ethylenediamine, absorbance is read at 550 nm. This method has been used by many investigators (2, 4–11) and has been shown to have satisfactory precision above 2–3 μg. However, its specificity recently has been questioned. Gibson *et al.* (12) reported that *N*-acetylprocainamide, the major metabolite in humans (5), is hydrolyzed to procainamide under the conditions of the procedure of Mark *et al.* (1).

Spectrophotofluorometric detection is also possible

[1] Pronestyl, E. R. Squibb & Sons. The chemical name is *p*-amino-*N*-[2-(diethylamino)ethyl]benzamide hydrochloride.



I: procainamide, $R_1 = H$, $R_2 = -CH_2CH_3$
II: internal standard, $R_1 = H$, $R_2 = -CH_2CH_2CH_3$
III: *N*-acetylprocainamide, $R_1 = CH_3C(=O)-$, $R_2 = -CH_2CH_3$

since procainamide fluoresces at 385 nm following excitation at 295 nm at pH 11 (13). This method has been useful (14–17) and successfully compared to the colorimetric method (2), but it retains the limitations of sensitivity and specificity.

A GC method for procainamide determinations was developed (18) but shows nonlinearity in the calibration curve at low concentrations as a result of adsorption to active sites on the column and/or packing material. Karlsson (14) used GC for the determination of procainamide in urine, but the method has not been published.

Sterling *et al.* (19) developed a GC method using polyamide incorporated onto the packing to reduce adsorption. They also developed a fluorometric method which involves coupling with fluorescamine to enhance the fluorescence of procainamide. Both methods were tested with pooled human plasma spiked with procainamide to concentrations as low as 2 μg/ml and with plasma from patients chronically maintained on procainamide.

However, to conduct safe, low-dose pharmacokinetic studies with procainamide in normal subjects (where many blood samples have to be drawn), it is necessary to have an assay that requires 1 or at most 2 ml of plasma and is sensitive at levels below 1 μg/ml. A GLC method was developed in this laboratory in which adsorption is minimized to yield linearity and sensitivity to levels as low as 0.1 μg/ml. Furthermore, the extraction procedure is modified to eliminate the hydrolysis of *N*-acetylprocainamide.

EXPERIMENTAL

Reagents—Procainamide and the internal standard, a dipropyl analog of procainamide, *p*-amino-*N*-[2-(dipropylamino)ethyl]-benzamide hydrochloride (II), mp 188–190°, were used[2]. Stock solutions of procainamide contained 2, 5, 10, 15, and 20 μg/ml for plasma and 10, 25, 50, 75, and 100 μg/ml for urine in water. Stock solutions of the internal standard contained 5, 10, 25, and 50 μg/ml in water. Solutions of phthalate buffer (pH 3) (20) and 2 *N* Na_2CO_3 buffer (pH 10.8) also were used.

All solvents were reagent grade, except for chloroform which was spectral grade. *N*-Acetylprocainamide (III), mp 189–190°, was synthesized according to the method of Dreyfuss *et al.* (6). GC and colorimetric analysis showed it to be 95% pure with 5% unchanged procainamide.

Extraction of Plasma Standards and Specimens from Dosed Subjects—*Control Plasma Standards*—The extraction procedure is a modification of the approach proposed by Mather and Tucker (21) for basic amine drugs. To 1 ml of pooled control plasma samples in 15-ml Pyrex culture tubes, fitted with Teflon-lined screw caps, were added 0.5 ml of aqueous procainamide solution, 0.5 ml of the appropriate internal standard solution, and 0.5 ml of carbonate buffer (pH 10.8).

The samples were extracted with 8 ml of ether by shaking at high speed on a reciprocating shaker for 5 min. Then sufficient sodium chloride was added to saturate the aqueous layer completely. The samples were shaken again at a low speed for 30 sec and at high speed for 5 min, followed by centrifugation at 2000 rpm for 20 min. This shaking procedure minimizes emulsion formation from the plasma protein–ether mixture.

The samples were then frozen in an acetone–carbon dioxide bath, the ether layers were poured into 15-ml test tubes, and the aqueous layer was discarded. After the addition of 0.2 ml of phthalate buffer (pH 3), each sample was mixed[3] for 60 sec and centrifuged as before for 5 min. The samples were frozen again, and the ether layers were discarded. Then the samples were heated in a water bath at 50° for 10 min to thaw the aqueous layer and evaporate remaining traces of ether.

The aqueous layers were poured from the test tubes into 1-ml conical centrifuge vials[4]. To each vial were added 0.1 ml of carbonate buffer (pH 10.8) and sufficient sodium chloride to saturate the aqueous layer; then the vials were mixed[3] for 30 sec. Samples were extracted with 10 μl of water-saturated chloroform by high speed shaking for 20 min, followed by centrifugation at 2000 rpm for 20 min. From the chloroform bead formed in the conical tip of the vial, 1–3 μl was injected directly into the gas chromatograph.

Plasma Samples from Dosed Subjects—Appropriate aliquots (0.5–2 ml) of plasma were pipetted into 15-ml tubes. Following addition of the internal standard, the samples were extracted by the same procedure as was used for the plasma standards.

Extraction of Urine Standards and Specimens from Dosed Subjects—*Control Urine Samples*—Procainamide concentrations in urine are about 10 times that found in plasma, and significant protein is not usually present. Therefore, the plasma assay was simplified for urine samples by omitting the ether extraction. Calibration curves were prepared by adding 0.1-ml samples of both procainamide and internal standard stock solutions and 0.1 ml of carbonate buffer (pH 10.8) directly into the 1-ml vials[4]. Sufficient sodium chloride was added to saturate the aqueous layer, followed by mixing[3] for 30 sec. Samples were then extracted with 10 μl of water-saturated chloroform by high speed shaking for 20 min, followed by centrifugation at 2000 rpm for 20 min. From the chloroform bead formed in the conical tip of the vial, 1–3 μl was injected directly into the gas chromatograph.

Urine Samples from Dosed Subjects—A 0.1-ml sample of urine, suitably diluted to contain between 1 and 10 μg of procainamide, was pipetted directly into the 1-ml vials[4]. To each sample was added 0.1 ml of internal standard solution and then the same extraction procedure was used as for the urine standards.

GLC—GLC was carried out on a gas chromatograph[5] equipped with a flame-ionization detector. Glass columns (coiled 0.91 m × 0.63 cm, 2 mm i.d.), packed with 10% OV-7-coated Gas Chrom Q[6] (60–80 mesh), were maintained for isothermal chromatography at 245° with the injector at 280° and the detector at 300°. Helium, the carrier gas, was maintained at 40 ml/min, with hydrogen and air at 30 and 450 ml/min, respectively. Under these conditions, procainamide, the internal standard, and *N*-acetylprocainamide have retention times of ~3, ~4, and ~9 min, respectively (Fig. 1).

To prolong column life, temperatures were reduced at night. Prior to raising temperatures at the beginning of a run and before igniting, the columns were conditioned with 30 μl of a trimethylsilyl mixture[7]. With these precautions, columns were used for up to 8 months.

The first sample each day was injected two or three times to ensure reproducibility and column stability. Where possible, samples were usually injected in order of decreasing drug concentration, and larger volumes were injected into the gas chromatograph as drug concentrations decreased.

Calculations—Peak height ratios were calculated by dividing the height of the procainamide peak by the height of the internal standard peak. Calibration curves were constructed daily from the

[2] Provided by Dr. T. Q. Spitzer, E. R. Squibb and Sons, New Brunswick, N.J.

[3] Vortex mixer, Scientific Products, American Hospital Supply Corp., Evanston, Ill.

[4] Solu-vials, Chemical Research Services, Addison, IL 60101

[5] Aerograph 200, Varian Aerograph, Walnut Creek, CA 94598. It was equipped with a Speedomax W recorder, Leeds and Northrup, Philadelphia Pa.

[6] Applied Science Labs, State College, PA 16801

[7] Silyl 8, Pierce Chemical Co., Rockford, IL 61105

Table I—Calibration Curve and Precision Data for Procainamide Determination from Spiked Plasma Samples

Procainamide Added, μg[a]	Peak Height Ratio[b]	Procainamide Found, μg[b]
1.0	0.225 ± 0.022	0.98 ± 0.09
2.5	0.572 ± 0.021	2.50 ± 0.09
5.0	1.137 ± 0.054	4.97 ± 0.23
7.5	1.717 ± 0.112	7.50 ± 0.49
10.0	2.282 ± 0.132	9.96 ± 0.58

[a] $n = 6$. [b] Mean ± SD.

results of spiked pooled control plasma samples by plotting peak height ratios against concentration of procainamide (micrograms per milliliter).

GLC–Mass Spectrometry—A gas chromatograph–mass spectrometer[8], fitted with a Gohlke separator, was used to check the identity and purity of the procainamide, internal standard, and *N*-acetylprocainamide peaks in a control aqueous solution and in plasma and urine samples. The ionizing energy was 70 ev.

RESULTS AND DISCUSSION

GLC—Figure 1 shows typical chromatograms obtained after addition of 1 and 10 μg of procainamide to pooled plasma samples and a chromatogram of a patient urine sample showing procainamide, internal standard, and *N*-acetylprocainamide peaks. Procainamide and the internal standard gave well-resolved, symmetrical peaks with no tailing. The *N*-acetylprocainamide peak, although well resolved, was broad and tailing, which makes quantification more difficult under these chromatographic conditions.

The identity and purity of the peaks were confirmed by mass spectrometry. The spectra and fragmentation patterns were comparable to those reported previously (17, 18).

The linearity of the system was established by analyzing the results of six calibration curves assayed simultaneously (Table I). The calibration curve constructed from the mean values had the linear regression equation $y = 0.229x - 0.002$ ($r = 1.000$). The precision of this method was determined from these data; for amounts ranging from 1 to 10 μg, coefficients of variation ranged from 3.6 to 9.5%. The standard deviation of the slope of nine routine calibration curves over 8 months was 4.0%.

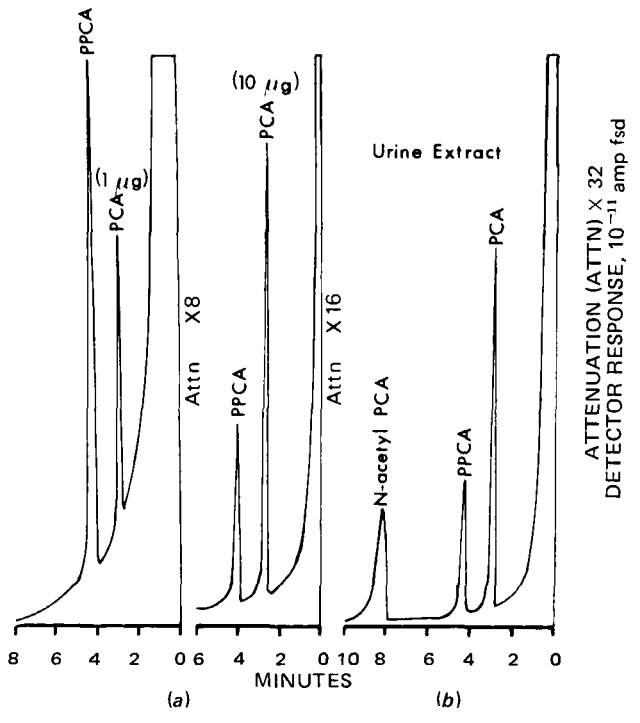


Figure 1—(a) *Gas chromatograms of procainamide (PCA) and internal standard (PPCA), showing 1 and 10 μg of drug extracted from spiked human plasma samples.* (b) *Chromatogram of a urine sample from a patient given 3.5 mg/kg iv showing procainamide (PCA), internal standard (PPCA), and* N*-acetylprocainamide* (N*-acetyl PCA*).

[8] Finnigan model 3000.

Table II—Recovery of Procainamide from Spiked Plasma and Urine Samples

	Plasma (Acid/Base Wash)		Urine (Single Extraction)	
Procainamide Added, μg/ml[a]	Procainamide Found, μg/ml	Recovery, %	Procainamide Found, μg/ml	Recovery, %
1.0	0.35	34.67	0.86	85.67
2.5	0.98	38.68	2.29	91.72
5.0	1.82	36.34	3.74	74.80
7.5	2.80	37.29	6.55	87.33
10.0	3.95	39.47	7.49	74.93
		37.29 ± 1.90		82.89 ± 7.65

[a] $n = 2$.

The standard deviation of the peak height ratio of the 1-μg sample of the same nine calibration curves over 8 months was 7.2%. The absolute peak height of a 1-μg sample from 1 ml of plasma was as high as 90 mm. Levels as low as 0.1 μg/ml, using 2-ml aliquots, could be measured. This sensitivity was made possible by using a small volume (10 μl) of chloroform in the final extraction. Although amounts of 0.2–0.5 μg were not included in the daily calibration curve, experience with duplicate determinations showed a variability of less than 10%. Duplicate calibration curves using 1 and 2 ml of plasma had the same slope, demonstrating that the extraction efficiency was not affected by increasing the plasma volume to 2 ml.

The recovery of procainamide from spiked plasma samples was determined to be 37.3%, and the recovery from urine samples was 82.8% (Table II). The low recovery in the double-extraction procedure is due to several factors: (*a*) the unfavorable solvent ratio of 8 ml of ether to 0.2 ml of phthalate buffer (pH 3), (*b*) the transfer of this small volume from test tubes to the vials, and (*c*) the necessity of two extractions at pH 10.8 [procainamide pKa 9.23 (2)] to prevent hydrolysis of *N*-acetylprocainamide.

However, in spite of the low absolute recovery, the overall precision of the method was satisfactory and comparable to that obtained by other GLC methods (18, 19). The variability of recovery shown in Table II is larger than that suggested by Table I, because in this instance the internal standard was not added until the final extraction step was completed and the extraction steps were not made quantitative.

Chromatographic Adsorption—The problem of procainamide adsorption onto the column and/or support during GLC is adequately documented (18, 19). Two approaches generally are used to prevent drug adsorption: (*a*) formation of a derivative of the drug, by which means the active sites on the drug are protected; and (*b*) use, if possible, of a combination of column supports and stationary phases that eliminates active sites for adsorption.

In the present work, it was possible to form derivatives of both procainamide and the internal standard with dimethylformamide dimethyl acetal[9], but the resulting derivatives still showed adsorption characteristics. Benzoyl derivatives, synthesized and chromatographed, also revealed that the adsorption problem was not resolved. Attempts were made to use base-washed column supports and amine packings, including 10% Apiezon L plus 2% KOH[10] and 28% Penwalt 223 plus 4% KOH[6]. When using these stationary phases, it was not possible to develop narrow peaks even at maximum stationary phase temperatures.

The approach of Meijer (22) was then used in which the percent of the stationary phase (of optimum polarity) is increased, the par-

[9] Methyl-8, Pierce Chemical Co., Rockford, IL 61105
[10] Supelco, Inc., Bellefonte, PA 16823

Table III—Hydrolysis of *N*-Acetylprocainamide

N-Acetylprocainamide Added, μg/ml	Condition	Hours	Procainamide Found, μg/ml	Hydrolysis, %
	At 50°[a]			
80	1 *N* HCl	8	60.29	77.48
80	pH 1.3	10	11.75	11.03
80	pH 2.8	32	0.0	0.0
80	pH 4.95	40	0.0	0.0
80	pH 7.0	40	0.0	0.0
80	pH 9.0	32	0.0	0.0
80	pH 9.98	20	0.0	0.0
80	1 *N* NaOH	8	64.31	82.97
Procainamide Added, μg/ml				
80	1 *N* HCl	8	79.26	0.92
80	1 *N* NaOH	8	80.31	0.0
N-Acetylprocainamide Added, μg/ml	At 25°[a]			
5	1 *N* HCl	18	1.44	26.49
10	1 *N* HCl	18	2.90	26.66
20	1 *N* HCl	18	5.62	25.70
				26.28 ± 0.51

[a] Determined by measuring the appearance of procainamide using this GLC method for samples at 50° and the colorimetric method (4) for samples at 25°.

ticle size of the support is also increased, thereby decreasing the surface area, and the column length is shortened. The percent of OV-17[6] was increased from 3 to 10% on 80–100-mesh support, yielding improved peaks and a reduced solvent front but little effect on adsorption. Ten percent OV-17[6] on 60–80 mesh also gave little improvement. Increasing the percent of this phase to 20% on 60–80-mesh[6] support yielded no improvement over the 10%, and the column temperature had to be raised to obtain usable peaks.

A switch to an OV-1[6] phase allowed the use of lower temperatures, and adsorption was decreased when column length was reduced to 0.91 m. A 10% OV-7[6] phase showed optimum polarity and reduced adsorption to the extent that the calibration curves became linear with negligible intercepts (less than 0.1 μg) (Table I).

Specificity—Elson *et al.* (23) found that patients maintained on procainamide therapy often have plasma levels of *N*-acetylprocainamide several times higher than those of procainamide. In addition, Gibson *et al.* (12) reported on the potential of hydrolysis of *N*-acetylprocainamide during the colorimetric analysis of procainamide using the method of Mark *et al.* (1). Therefore, an assessment of the specificity of any procedure is dependent on an understanding of the conditions of hydrolysis of *N*-acetylprocainamide.

A study of the hydrolysis of *N*-acetylprocainamide in 1 *N* HCl revealed 77.5% hydrolysis in 8 hr at 50° and 26.3% hydrolysis in 18 hr at 25°. In 1 *N* NaOH, 83.0% hydrolysis of *N*-acetylprocainamide was found in 8 hr at 50°. Control studies on procainamide showed no hydrolysis in either 1 *N* HCl or 1 *N* NaOH in 8 hr at 50°. There was, however, no hydrolysis of *N*-acetylprocainamide between pH 3 and 10 for up to 40 hr at 50° (Table III). Therefore, buffers at pH 3 (phthalate buffer) and pH 10.8 (2 *N* Na_2CO_3) were substituted for the 2 *N* HCl and 2 *N* NaOH solutions, respectively, used in the procedure of Mather and Tucker (21). Analysis of solutions of *N*-acetylprocainamide by the proposed method showed no more procainamide present than control solutions of *N*-acetylprocainamide injected directly into the gas chromatograph without extraction.

Application—This method was used to follow the disappearance of procainamide from the plasma and urine of seven normal human subjects given 4 mg/kg iv. The plasma concentration *versus* time curves showed that all plasma procainamide concentrations following the first 0.5 hr postadministration were less than 1 μg/ml and that values fell to below 0.1 μg/ml in less than 6 hr after the dose.

CONCLUSIONS

This GLC method for the determination of procainamide in biological fluids minimizes the adsorption problem so that concentrations as low as 0.1 μg/ml can be quantitatively determined. Also, it has the added advantage of specificity, thus eliminating the interference of *N*-acetylprocainamide. With respect to monitoring procainamide concentrations in the therapeutic range, this procedure is slightly more tedious than other currently available GLC methods. However, with its added sensitivity, it is useful when only small volumes of plasma are available.

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