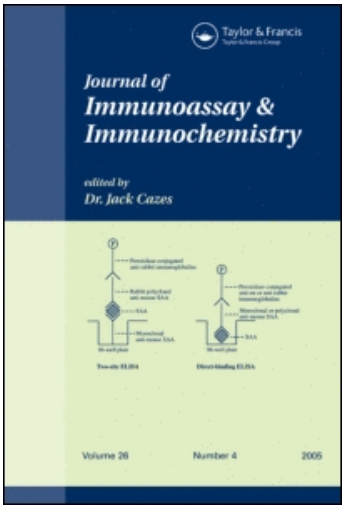

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Development of Fluoroimmunoassays for the Determination of Individual or Combined Levels of Procainamide and N-Acetylprocainamide in Serum

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DEVELOPMENT OF FLUOROIMMUNOASSAYS FOR THE DETERMINATION OF INDIVIDUAL OR COMBINED LEVELS OF PROCAINAMIDE AND N-ACETYLPROCAINAMIDE IN SERUM

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ABSTRACT

A fluoroimmunoassay has been developed for the simultaneous determination of serum levels of procainamide and its active metabolite N-acetylprocainamide. It employs procainamide linked through its aromatic amino group to fluorescein isothiocyanate as tracer and an antiserum raised against procainamide conjugated to human thyroglobulin through the same position. Separation is rapidly and simply achieved by covalently linking the antiserum to magnetisable microparticles and use of a magnet.

Specific magnetisable particle fluoroimmunoassays were also developed for procainamide and for N-acetylprocainamide by the use of suitable immunogens and fluorescein-labelled tracers. That for procainamide uses an antiserum raised to a procainamide-enzyme conjugate and fluorescein-labelled p-aminobenzoic acid while the fluoroimmunoassay for N-acetylprocainamide employs an antiserum against a N-acetylprocainamide-enzyme conjugate and fluorescein-labelled p-acetamidobenzoic acid.

INTRODUCTION

Procainamide has been used for many years to treat cardiac arrhythmias (1,2). However, it is difficult to base such treatment on dose alone because of wide individual variations in the drug's rates of absorption, distribution and elimination. Fortunately, circulating levels of procainamide correlate with clinical effectiveness, the normal therapeutic range being 4 to 8 mg/l while adverse effects are common when serum levels exceed 12 mg/l (3). However, the main metabolite of procainamide, N-acetylprocainamide, is also pharmacologically active (4,5). Thus it may be necessary to determine both the drug and its metabolite for effective therapeutic monitoring (6,7,8,9) and to maintain their combined serum levels in the range 5 to 30 mg/l (6,10).

Gas chromatography (5) and high performance liquid chromatography (10,11) can determine both procainamide and N-acetylprocainamide levels at the same time on the same serum sample. This is not possible with a single immunoassay although the availability of specific enzymoimmunoassays for procainamide (12) and its active metabolite (13) can provide similar information by two separate determinations on each sample (14). An alternative, when the drug and metabolite have similar therapeutic efficacy and toxicity, is to employ an immunoassay in which both cross-react equally and to determine their combined serum level. Such an approach has been described previously for amitriptyline and its active metabolite nortriptyline (15).

N-Acetylprocainamide differs from procainamide only by acetylation of the aromatic amino group (Figure 1). In this study, fluoroimmunoassays have been designed for the determination of the drug and its active metabolite in serum either alone or together by the use of appropriate tracers and immunogens.

MATERIALS AND METHODS

Fluorescein isothiocyanate isomer I (FITC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide and digoxin were from Sigma (Poole, Dorset, U.K.); Triton X-100, sodium azide, starch-iodide test papers and all organic solvents (Analar grade) from BDH (Poole, Dorset, U.K.); procaine, p-aminobenzoic acid and p-acetamidobenzoic acid from Aldrich (Gillingham, Dorset, U.K.); silica gel thin-layer chromatography sheets DC-Alufolien Kieselgel 60 F_{254} from Merck (Darmstadt, F.R.G.); Sephadex G-25 fine grade from Pharmacia (Hounslow, Middlesex, U.K.); Freund's complete and incomplete adjuvants from Difco (West Molesey, Surrey, U.K.); and procainamide and N-acetylprocainamide enzyme conjugates from the appropriate EMIT enzymoimmunoassay kits (Syva, Palo Alto, California, U.S.A.). Other materials were gifts including procainamide hydrochloride and N-acetylprocainamide hydrochloride from Squibb Europe (Twickenham, Middlesex, U.K.); amiodarone hydrochloride from S.A. Labaz (Brussels, Belgium); disopyramide phosphate from Searle (Chicago, Illinois, U.S.A.); phenytoin from Parke-Davis (Pontypool, Gwent, U.K.); quinidine sulphate from Lewis Laboratories (Leeds, West

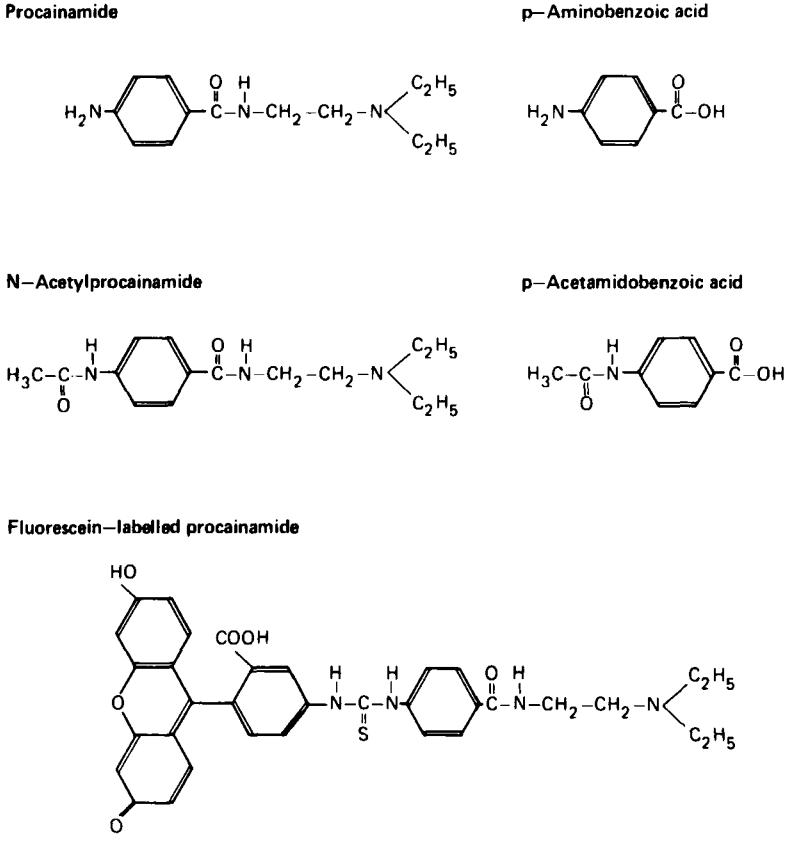

Figure 1. Structures of procainamide, N-acetylprocainamide and some compounds used in the development of combined or specific fluoroimmunoassays.

Yorkshire, U.K.); and human thyroglobulin from Dr. L. Nye (Department of Immunology, Middlesex Hospital Medical School, London W1, U.K.).

Sodium phosphate buffer (100 mmol/l, pH 7.5) containing 1 ml/l Triton X-100 and 1 g/l sodium azide was used as diluent throughout.

Procainamide and N-acetylprocainamide standards were prepared by addition of their solid hydrochlorides to pooled normal human serum and stored at -20°C.

Assays were performed in duplicate at room temperature, using disposable polystyrene test tubes (55 x 12 mm, No. 55.484, from Sarstedt, Leicester, Leics., U.K.).

Combined Procainamide/N-Acetylprocainamide Fluoroimmunoassay

Tracer: Fluorescein-labelled procainamide was prepared by dissolving 2.5 mg (9.2 μmol) of procainamide hydrochloride in 500 μl of distilled water and adding 3.6 mg (9.2 μmol) of FITC dissolved in 1 ml of methanol containing 10 ml/l triethylamine. After 3 h at room temperature, the reaction mixture was applied to silica gel sheets (200 μl per sheet) and developed with methanol/chloroform (90:10 v/v). The single major band, with R_f 0.43 (FITC had R_f 0.88 in this system), was scraped from each plate, eluted into 1 ml of methanol and stored at 4°C in the dark. The concentration of the product was estimated spectrophotometrically, assuming an extinction coefficient for the fluorescein group of 8.78×10^4 l mol^{-1} cm^{-1} at 492 nm in sodium bicarbonate buffer (50 mmol/l, pH 9.0) (16).

Antiserum: A procainamide-human thyroglobulin conjugate, prepared as recommended by Pinckard and Weir (17), was employed as the immunogen. Procainamide hydrochloride (43.2 mg) was dissolved in 1 ml of HCl (100 mmol/l) at 4°C and aqueous $NaNO_2$ (10 g/l) added dropwise with constant stirring. After each addition, the reaction mixture was tested for the presence of free nitrous acid using starch-iodide paper, and diazotisation was considered complete

when a positive test was obtained 15 min after the last addition (270 µl was required). The product was added slowly, with constant stirring, to thyroglobulin (300 mg) dissolved in 4 ml of sodium borate buffer (25 mmol/l, pH 9.5) at room temperature. When the pH of the reaction mixture (monitored by pH paper) fell below 7.0 it was adjusted to 9.5 by addition of NaOH (100 mmol/l). The reaction mixture (which had a deep wine-red colour) was then adjusted to pH 9.0 and maintained at this pH while being stirred for 2 h. After standing overnight at 4°C, the reaction mixture was dialysed for three days against daily changes of distilled water (4-5 l) and the dialysate freeze-dried to obtain 280 mg of the immunogen, which was stored at 4°C.

When dissolved to 700 mg/l in sodium borate buffer (25 mmol/l, pH 9.5) the immunogen displayed an absorption peak at 370 nm with an optical density (1 cm) of 0.46 which was attributed to the diazo-coupled hapten. The hapten/carrier ratio was estimated to be 37:1, taking the molecular weight of human thyroglobulin to be 660,000 (18).

Three female New Zealand White rabbits (R001-R003) and six female Border Leicester cross sheep (S305-S310) were each immunised at several intramuscular and subcutaneous sites with 2 mg of the immunogen in 2 ml of an emulsion of 1:3 parts by volume of saline and complete Freund's adjuvant. Six weeks later, and at fortnightly intervals thereafter, the animals were reimmunised in the same way with 1 mg of the immunogen, using complete adjuvant for the rabbits and incomplete adjuvant for the sheep. The

animals were bled ten days after the first and then each alternate booster immunisation.

Each antiserum (2 ml) was covalently coupled by the cyanogen bromide method (19) to 1 g of magnetisable cellulose/iron oxide particles (20). The products were suspended in 20 ml of sodium phosphate buffer (100 mmol/l, pH 7.5) and stored at 4°C where they remained stable for at least one year.

Antibody Dilution Curves: Aliquots (100 µl) of doubling dilutions of each solid-phase antiserum were incubated with 100 µl of tracer (200 nmol/l) for 1 h on a mechanical shaker, 1 ml of diluent buffer added, the particles sedimented on a magnet (multi-polar ferrite block from Magnet Applications, City Road, London EC1, U.K.) for 2 to 5 min and the supernates (containing the free fraction and endogenous fluorophores and other interfering factors) aspirated to waste. Then 1.5 ml of elution reagent (methanol/water, 90:10 v/v) was added to elute the antibody-bound fraction, the tubes vortex mixed and the particles again sedimented. The tubes were finally placed directly in a Perkin-Elmer Model 1000 fluorimeter (Perkin-Elmer, Beaconsfield, Bucks., U.K.) equipped with filters and an adapter for disposable test tubes as described previously (21). The antibody dilution curves were constructed by plotting fluorescence signal (in arbitrary units) against the amount of magnetisable solid-phase per tube.

Standard Curves: To 10 µl of serum sample or standard was added 100 µl of fluorescein-labelled procainamide (200 nmol/l) followed by 50 µl of the appropriate solid-phase antiserum (25 g/l). After

incubation for 30 min on a mechanical shaker, the particles were washed and the bound fraction eluted and its fluorescence determined as above. Standard curves were constructed by plotting total fluorescence signal (in arbitrary units) against serum standard concentration.

Specific Fluoroimmunoassay For Procainamide

Tracer: Fluorescein-labelled p-aminobenzoic acid, which is similar in part of its structure to procainamide (Figure 1), was prepared by dissolving 2 mg (14.6 μmol) of p-aminobenzoic acid in 500 μl of dimethylformamide (DMF) and adding 16.8 mg (146 μmol) of N-hydroxysuccinimide and 28 mg (146 μmol) of EDC dissolved in 500 μl of DMF/water (80:20 v/v). The reaction mixture was stirred at room temperature for 1 h then 6.6 mg (14.6 μmol) of fluoresceinthiocarbamyl ethylenediamine, prepared as described previously (16), dissolved in 500 μl of DMF was added slowly. After 2 h at room temperature, the reaction mixture was applied to silica gel sheets (150 μl per sheet) and developed with methanol/chloroform/ammonia (40:40:1 v/v). The single major band, with R_f 0.34 (fluoresceinthiocarbamyl ethylenediamine had R_f 0.08 in this system), was scraped from each plate, eluted into 1 ml of methanol and its concentration estimated spectrophotometrically as described earlier.

Antiserum: The reconstituted enzyme-labelled reagent (2.5 ml) from an EMIT procainamide kit was applied to a column (1.2 x 20 cm) of Sephadex G-25 equilibrated with 10 mmol/l phosphate-buffered saline (pH 7.9) containing 1 ml/l Tween 20, and eluted with the

same buffer. Fractions (500 µl) were collected and their absorbance measured at 280 nm to detect protein. The entire peak was pooled (4 ml) and emulsified with an equal volume of complete Freund's adjuvant. Three female New Zealand White rabbits (R1-R3) each received 1 ml of the emulsion and were reimmunised and bled as described above.

Assay Procedures: Each antiserum (2 ml) was coupled to magnetisable particles (1 g) and used to construct antibody dilution curves with 100 µl of fluorescein-labelled p-aminobenzoic acid (150 nmol/l) per tube. Standard curves were constructed using 10 µl of serum sample or standard, 100 µl of the tracer and 100 µl of the solid-phase antiserum (25 g/l), with a 1 h incubation period.

Specific Fluoroimmunoassay For N-Acetylprocainamide

Tracer: Fluorescein-labelled p-acetamidobenzoic acid, which is similar in part of its structure to N-acetylprocainamide (Figure 1), was prepared by dissolving 2 mg (11.2 µmol) of p-acetamidobenzoic acid in 500 µl of DMF and adding 14 mg (122 µmol) of N-hydroxy-succinimide and 28 mg (146 µmol) of EDC dissolved in 500 µl of DMF/water (80:20 v/v). The reaction mixture was stirred at room temperature for 1 h then 5 mg (11.2 µmol) of fluoresceinthiocarbamyl ethylenediamine dissolved in 500 µl of DMF was added slowly. After 2 h at room temperature the reaction mixture was applied to silica gel sheets (150 µl per sheet) and developed with methanol/chloroform/ammonia (40:40:1 v/v) to give a single major band with R_f 0.33. This was eluted with 1 ml of methanol and its concentration estimated spectrophotometrically as described earlier.

Antiserum: The reconstituted enzyme-labelled reagent from an EMIT N-acetylprocainamide kit was chromatographed, emulsified with complete Freund's adjuvant and used to immunise three female New Zealand White rabbits (R4-R6) as described above.

Assay Procedures: Antisera were coupled to magnetisable particles and used to construct antibody dilution curves and standard curves as described for the specific procainamide assay, with 100 µl of fluorescein-labelled p-acetamidobenzoic acid (150 nmol/l) per tube.

RESULTS

Combined Procainamide/N-Acetylprocainamide Fluoroimmunoassay

Fluorescein-labelled procainamide was 98% bound by an excess of solid phase and the antisera obtained from all three rabbits and six sheep had a titre suitable for use in a separation fluoroimmunoassay. Standard curves, using procainamide or N-acetylprocainamide serum standards, showed that the drug was less immunoreactive than its metabolite in every case. Thus procainamide cross-reacted between 50% and 80% relative to N-acetylprocainamide with the three rabbit antisera and only between 8% and 10% with the sheep antisera, as calculated by the method of Abraham (22). The second bleed from rabbit R001 was used as the antiserum for all further experiments. The curves obtained using procainamide and using N-acetylprocainamide standards are shown in Figure 2, and cover the clinically important range for the combined levels of the two.

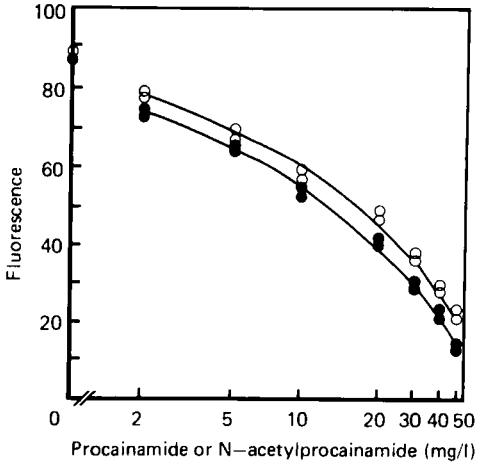

Figure 2. Typical standard curves for the combined fluoroimmunoassay using procainamide (○) or N-acetylprocainamide (●) serum standards.

TABLE 1

CROSS-REACTIVITIES IN THE COMBINED AND THE SPECIFIC PROCAINAMIDE AND N-ACETYLPROCAINAMIDE FLUOROIMMUNOASSAYS

SUBSTANCE	CROSS-REACTIVITY (%) COMBINED ASSAY	SPECIFIC PROCAINAMIDE ASSAY	SPECIFIC N-ACETYL-PROCAINAMIDE ASSAY
Procainamide	80	100	12
N-Acetylprocainamide	100	8	100
Procaine	0.02	2.1	0.02
p-Aminobenzoic acid	0.05	0.6	0.03

Procainamide cross-reacted 80% relative to its metabolite (Table 1), while the cardioactive drugs amiodarone, digoxin, disopyramide, phenytoin and quinidine showed no detectable effect at serum concentrations of 1 g/l. The recovery of procainamide added to pooled normal human serum at concentrations of 2, 10 and 35 mg/l was satisfactory as was the within- and between-assay precision (Table 2). Twenty serum samples from patients receiving procainamide were assayed by an established, specific gas chromatography technique (at the Clinical Chemistry Department, Mount Sinai Hospital, Toronto, Canada) and by the fluoroimmunoassay. The results (Table 3) showed a reasonable correlation ($r = 0.89$) but those by the immunoassay were some two to three times higher.

Specific Fluoroimmunoassay For Procainamide

The fluorescein-labelled p-aminobenzoic acid was 85% bound by an excess of solid phase and the antisera obtained from all three rabbits had a titre suitable for use in a separation fluoroimmunoassay. The third bleed from rabbit R2 was used as the antiserum in further experiments. Standard curves obtained using procainamide and N-acetylprocainamide serum standards are shown in Figure 3a. The metabolite cross-reacted only 8% and the curve for procainamide covered the clinically important range. Procaine and p-aminobenzoic acid cross-reacted 2.1 and 0.6%, respectively, relative to the drug (Table 1), while none of the other cardioactive compounds studied had any effect at a serum level of 1 g/l.

The recovery of procainamide added to pooled normal human serum at concentrations of 2, 8 and 16 mg/l was satisfactory as

TABLE 2

PRECISION AND ACCURACY OF THE COMBINED AND THE SPECIFIC PROCAINAMIDE AND N-ACETYLPROCAINAMIDE FLUOROIMMUNOASSAYS

ASSAY	AMOUNT OF DRUG (mg/l)	RECOVERY (%) (MEAN ± SD, n = 10)	WITHIN-ASSAY CV (%) (n = 20)	BETWEEN-ASSAY CV (%) (n = 20)
Combined assay	2	105 ± 5	5.8	6.2
	10	102 ± 5	6.8	7.2
	35	100 ± 11	9.5	10.6
Specific procainamide assay	2	102 ± 9	4.6	5.8
	8	98 ± 11	6.5	7.2
	16	106 ± 13	9.4	11.5
Specific N-acetyl-procainamide assay	2	105 ± 7	6.3	7.5
	8	109 ± 12	8.6	9.8
	16	113 ± 6	10.5	12.2

was the within- and between-assay precision (Table 2). When the results for the 20 serum samples determined by gas chromatography were compared with those by this fluoroimmunoassay (Table 3) they correlated with an r value of 0.997. A regression line (Fluoroimmunoassay) = 0.97(Gas chromatography) + 0.6 was calculated on the assumption that the two methods have equal precision characteristics (23).

TABLE 3

PROCAINAMIDE LEVELS (mg/l) IN TWENTY PATIENT SAMPLES MEASURED BY GAS CHROMATOGRAPHY AND BY THE COMBINED AND THE SPECIFIC PROCAINAMIDE FLUOROIMMUNOASSAYS

SAMPLE NUMBER	GAS CHROMATOGRAPHY	SPECIFIC FLUOROIMMUNOASSAY	COMBINED FLUOROIMMUNOASSAY
1	4.1	4.5	27
2	4.0	4.6	25
3	4.7	5.2	13.5
4	2.2	3.1	11.2
5	6.6	7.1	15.8
6	3.3	4.0	6.4
7	17.9	17.0	45
8	9.3	10.0	28.9
9	6.1	6.4	14.5
10	1.6	1.5	7.1
11	1.7	2.1	6.5
12	14.1	15.0	28
13	16.1	16.9	34
14	15.8	15.2	26
15	9.6	10.3	18.2
16	2.3	3.1	10.5
17	1.3	1.6	7.2
18	19.8	19.2	60
19	16.1	15.8	42
20	16.3	17.5	51

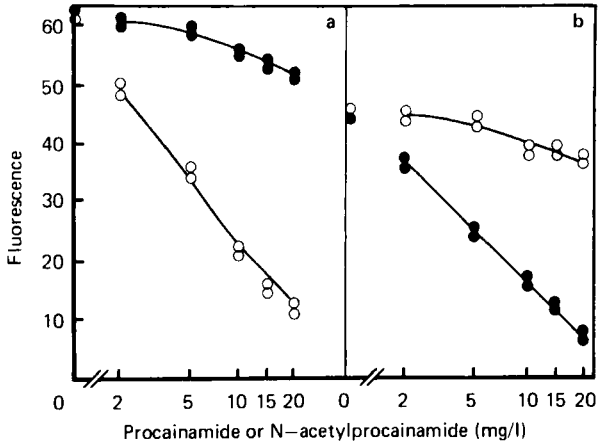

Figure 3. Standard curves obtained using procainamide (○) or N-acetylprocainamide (●) serum standards. Figure 3a shows the results employing the specific fluoroimmunoassay for procainamide and Figure 3b that for N-acetylprocainamide.

Specific Fluoroimmunoassay For N-Acetylprocainamide

The fluorescein-labelled p-acetamidobenzoic acid was 65% bound by an excess of antisera and those obtained from all three rabbits had a titre suitable for use in a separation fluoroimmunoassay. The third bleed from rabbit R6 was used as the antiserum in further experiments. Standard curves obtained using N-acetylprocainamide and procainamide serum standards are shown in Figure 3b. The parent drug cross-reacted only 12% and the curve for its active metabolite covered the clinically important range. Procaine and p-aminobenzoic acid cross-reacted only 0.02 and 0.03%, respectively, relative to N-acetylprocainamide (Table 1), while none of the other cardioactive compounds studied had any effect at a serum level of 1 g/l.

The mean analytical recovery of the metabolite added to pooled normal serum at concentrations of 2, 8 and 16 mg/l was satisfactory as was the within- and between-assay precision (Table 2).

DISCUSSION

The procainamide/N-acetylprocainamide system is one of a number in which a major metabolite has similar pharmacological (and toxic) activity to that of the parent drug. In such situations use of an immunoassay in which both cross-react equally permits the measurement of their combined level and is suitable for therapeutic drug monitoring provided the pharmacological effects of the drug and its metabolite are similar. When individual levels of both are required, for example in the discrimination between genetically fast and slow acetylators, chromatographic techniques capable of measuring both simultaneously are often preferred. Alternatively, two separate immunoassays can be employed provided one is specific for the drug and the other for its metabilite.

The present work was based on the use of fluorophore-labelled tracers and antisera covalently linked to magnetisable particles, an approach which has been successfully applied to other drugs (21,24). The simple separation step also enabled removal of endogenous fluorophores and other interfering factors present in serum, with measurement of the fluorescence of the bound fraction after its elution from the magnetisable particles. The fluoroimmunoassays were performed in disposable polystyrene tubes,

which also served as the fluorimeter cuvettes, and were relatively simple, quick and precise.

One fluoroimmunoassay (the combined assay) determined serum levels of both procainamide and N-acetylprocainamide and covered the clinically important range for their combined values. Despite using fluorescein-labelled procainamide and a rabbit antiserum raised against a procainamide-thyroglobulin conjugate the parent drug showed only 80% of the immunoreactivity of its metabolite. This probably reflects a similarity in structure between the bridge and the N-acetylprocainamide molecule (Figure 1). Variation of the assay conditions, such as of pH which had a significant effect on cross-reactivity in an amitriptyline/nortriptyline immunoassay (15), did not alter the relative immunoreactivity of procainamide and N-acetylprocainamide.

It has been found previously (25) that readily available drug-enzyme conjugates, normally used as the tracers in enzymoimmunoassay kits, provide excellent immunogens. Specific antisera against procainamide and N-acetylprocainamide were raised in rabbits by immunisation with appropriate enzyme conjugates. Fluorescein-labelled p-aminobenzoic acid and fluorescein-labelled p-acetamidobenzoic acid were synthesised for the purpose of obtaining specific fluoroimmunoassays for procainamide and for N-acetylprocainamide respectively. The assays covered the clinically important ranges and the results obtained with the specific assay for procainamide correlated closely with those by a specific gas chromatographic technique.

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