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## Note

# Comparison of high-performance liquid chromatography with an enzyme multiplied immunoassay technique for the analysis of serum procainamide and N-acetylprocainamide

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The use of procainamide (PA) for the control of cardiac arrhythmias is well established [1]. The major metabolite, N-acetylprocainamide (NAPA) has been found to be equally effective against cardiac arrhythmias and often exceeds the concentration of the parent compound in serum [2]. Therapeutic drug level monitoring of PA and NAPA in serum is recommended because the therapeutic range is narrow (4—8 mg/l), toxicity above these levels can be serious, and individual differences in absorption, distribution, and elimination make serum drug concentration of PA a better guide for clinical effectiveness than total dose [3].

A homogeneous enzyme immunoassay, the Enzyme Multiplied Immunoassay Technique (EMIT®), for the assay of serum PA and NAPA, has been developed recently by the Syva Corporation (Palo Alto, Calif., U.S.A.) [4, 5]. While analysis of PA and NAPA by EMIT requires a separate assay for each drug, both tests are rapid and require essentially no sample preparation.

High-performance liquid chromatography (HPLC) for the simultaneous determination of serum PA and NAPA is also a relatively recent analytical technique [6—9]. Simultaneous determination by HPLC of serum PA and NAPA involves simple organic extraction and reversed-phase chromatography and has been shown to be sensitive, specific and suitable for routine clinical use.

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A comparison of these two procedures in a clinical setting would be useful to laboratories considering the future use of either of these methods. This communication reports the results of a study in which serum samples, obtained from patients in a coronary intensive care unit who were receiving a number of drugs in addition to PA, were assayed by both EMIT and HPLC for serum PA and NAPA.

## MATERIALS AND METHODS

### *Samples*

A total of 79 blood samples was obtained from patients in the Cardiac Care Unit in the University of California, Davis, Medical Center (Sacramento, Calif., U.S.A.). All patients had cardiac arrhythmias requiring therapy by pharmacological agents. PA was administered in doses ranging from 3.0 to 6.0 g/day orally (p.o.) and from 1 to 5 mg/min intravenously (i.v.). Blood samples were obtained at various times after drug administration to obtain as broad a range of drug concentrations as possible. These included pre-drug (PA) control samples.

A variety of other drugs were used in the clinical management of these critically ill patients; these included digoxin, diazepam, phenobarbital, furosemide, dopamine, norepinephrine, nitroprusside, nitroglycerine, phenytoin, penicillin, gentamicin and cephalosporin. In addition, all patients had received lidocaine prior to receiving procainamide and some had received quinidine prior to lidocaine.

Blood samples were obtained by venipuncture, centrifuged at 380 *g* for 10 min and the serum removed and frozen at −20° until assayed. To minimize bias, the order of the samples was randomized prior to analysis and the analyst was unaware of the results of one method while performing the other.

### *EMIT assay*

All components for analysis, including standards, controls and equipment were part of the assay system supplied by Syva for evaluation of the procedure for PA and NAPA analysis. The immunoassay procedure was performed according to the protocol provided by Syva which has been reported previously in abstract form [4, 5]. The procedure employed a Model 300-N Gilford microsample spectrophotometer with an automatic sampling system (Gilford Instrument Labs., Oberlin, Ohio, U.S.A.). The spectrophotometer was coupled to a Model 2400 printer-calculator (Cavro Scientific Instruments, Los Aitos, Calif., U.S.A.). Samples were dispensed and diluted with a Model 1500 pipettor-dilutor (Cavro Scientific Instruments). Briefly, the procedure consisted of taking a 50-$\mu$l sample, diluting with buffer, adding antibody and drug-labeled glucose-6-phosphate dehydrogenase (G6PDH), then measuring spectrophotometric absorbance changes over 45 sec. This assay can be done quite rapidly because no sample preparation is required. Once calibration curves for both PA and NAPA are constructed, the analysis of a single sample for both compounds can be accomplished in 10 min.

Specific antisera were obtained from sheep previously immunized with derivatized procainamide covalently linked to a protein carrier. Drug-labeled enzyme was prepared by linking derivatized procainamide to G6PDH. When

antibody binds to procainamide-labeled G6PDH, the activity of the enzyme is reduced. Free drug in the test sample decreases this antibody-induced inactivation of the enzyme. The subsequent increase in enzyme activity is directly related to the amount of procainamide. Spectrophotometric absorbance changes (340 nm) reflecting the conversion of NAD to NADH are measured as the analytical endpoint. The range of drug concentrations which may be measured by this assay is 1.0—16 mg/l for both PA and NAPA. Evaluation of the assay system by the manufacturer showed that concentrations of NAPA greater than 40 mg/l must be present in the sample to produce interference in the EMIT PA assay; PA will not cross-react with the EMIT NAPA assay until levels greater than 100 mg/l are present [10]. Other compounds of similar chemical or pharmacological properties were tested and found not to cross-react with either the PA or NAPA reagents at concentrations less than 100 mg/l.

Calibration curves were constructed by analyzing duplicate samples containing PA and NAPA at concentrations of 1, 2, 4, 8 or 16 mg/l and plotting the data points on graph paper supplied by the manufacturer and which has been specially matched with the reagents. Data points were plotted as change in absorbance versus log drug concentration. Curves were linear over the range of concentrations measured and all data points were within the 95% confidence interval for the regression line. Within-run precision for the EMIT assays was determined by analyzing 20 samples of two sera which had been fortified with 4 mg/l PA or 4 mg/l NAPA. The mean value for PA was 4.05 mg/l, ± 0.20 S.D. and a coefficient of variation (C.V.) of 5%. For NAPA the mean value was 3.8 mg/l, ± 0.08 S.D. and a C.V. of 2%. Day-to-day precision was somewhat less as the coefficients of variation for PA and NAPA increased to 11% and 5% respectively.

*HPLC assay*

Assay by HPLC involved double extraction of serum samples with ethyl acetate, the use of an internal standard, and reversed-phase liquid chromatography.

*Chemicals and reagents*

All reagents were of analytical or reagent grade. Procainamide HCl was obtained from K & K Labs. (Plainview, N.Y., U.S.A.). N-Acetyl-procainamide and the internal standard, *p*-amino-N-(2-dipropylaminoethyl)benzamide HCl (the dipropyl analog of PA) were obtained from E.R. Squibb and Sons (Princeton, N.J., U.S.A.). All calculations of PA and NAPA amounts were in terms of free base. Working solutions of PA and NAPA and the internal standard were made in distilled water and stored at 4°.

Ethyl acetate used in the extraction was glass-distilled in our laboratory. All glassware used in the assay was silanized with Siliclad$^{R}$ (Clay-Adams, Parsippany, N.J., U.S.A.), a water-soluble silanizing agent.

*Sample preparation*

Ethyl acetate extraction of plasma samples was performed as follows. To a 60 × 125 mm PTFE-lined, screw cap culture tube, was added 0.5 ml plasma or serum, 0.5 ml internal standard (24 mg/l), 0.1 ml 2 *N* NaOH, and 3.0 ml ethyl

acetate. The sample was vortexed 30 sec and centrifuged for 5 min at 360 *g*. The organic (upper) phase was pipetted into another 16 × 125 mm culture tube, and the aqueous phase was reextracted with another 3.0 ml of ethyl acetate. The organic phase from this second extraction was then combined with that from the first. One drop of concentrated HCl was added and the mixture vortexed 5 sec. Samples were placed in a water bath (40°), and the solvent evaporated to dryness under a gentle stream of nitrogen. The residue was frozen until ready for assay or was reconstituted immediately by adding 0.5 ml mobile phase. The reconstituted sample was allowed to equilibrate at room temperature for 30 min followed by vortexing 5 sec. A 50-μl aliquot was injected into the liquid chromatograph for analysis.

*Liquid chromatography*

A Waters Model 6000A high pressure liquid chromatograph was used equipped with a Model U6K loop injector and a Model 440 fixed wavelength (254 nm) ultraviolet absorbance detector (Waters Assoc., Milford, Mass., U.S.A.). A 30 cm × 3.9 mm I.D. μBondapak $C_{18}$ reversed-phase column (Waters Assoc.) was used. In addition, a 0.5-μm HPLC in-line filter (Alltech Assoc., Arlington Heights, Ill., U.S.A.) and a 8.0 × 0.6 cm guard column packed with Co:Pell$^R$ ODS pellicular packing (Whatman, Clifton, N.J., U.S.A.) were fitted between the injector and the analytical column to prolong the analytical column life.

The mobile phase was acetonitrile in distilled water (100 ml/l) with acetic acid (40 ml/l) and sodium acetate (4 g/l). The flow-rate was 2 ml/min at an approximate pressure of 2000 p.s.i. The mobile phase was filtered through a 0.45-μm filter before use.

Retention times for PA, NAPA and the internal standard were 3.3 min, 4.8 min and 7.6 min, respectively. The total chromatographic analysis time was approximately 12 min for the three compounds. The ratios of the area under the peaks for standards and unknown to peak area of the internal standard were used for quantitation. Minimum detectable amount, defined as that amount of drug producing a detector response twice that of background noise, was 4 ng (20-μl samples of 0.2 mg drug/l) for both PA and NAPA.

Calibration curves were constructed by extracting and analyzing pooled normal human sera which contained PA and NAPA at concentrations of 2, 5, 7, 10 or 15 mg/l and fitting the data points by linear regression. Curves were linear over this range of concentration with correlation coefficients typically being $\geq 0.950$ for both drugs. Within-run precision for the HPLC method was determined by the analysis of five samples fortified at 2 mg/l PA and NAPA and five samples fortified at 10 mg/l PA and NAPA. At the 2 mg/l concentration the coefficients of variation for PA and NAPA were 4% and 6% respectively and at the 10 mg/l concentration the coefficients of variation for PA and NAPA were 4% and 1% respectively. Day-to-day precision was somewhat less as the coefficients of variation for PA and NAPA at 2 mg/l increased to 11% and 7% respectively.

## RESULTS AND DISCUSSION

Figs. 1 and 2 are scattergrams which compare values obtained by HPLC and EMIT. The results from the two methods for both PA and NAPA were in close agreement. A statistical comparison of these data by least squares analysis and linear regression shows that for PA, the correlation coefficient was 0.895, and the least square values of slope and intercept were 1.110 and −0.400, respectively. The correlation coefficient for N-acetylprocainamide was 0.964, and the slope and intercept values were 0.942 and 0.248, respectively.

The extraction procedure used for HPLC assay was a modification of several published methods [6–9]. Both *n*-propanol–chloroform (1:9) and methylene chloride were tried as the extracting solvent for PA and NAPA, according to the procedures of Carr et al. [6] and Rocco et al. [7], and rejected in favor of ethyl acetate. Extraction with ethyl acetate gave a more consistent recovery because it did not form emulsions. In addition, ethyl acetate is the top layer in a solvent–water mixture and can be transferred more rapidly and with greater precision than can methylene chloride or chloroform, which are more dense than water. Two extractions with ethyl acetate increased both recovery and precision (C.V.) by 5% over one extraction for PA and increased recovery, but not precision, of NAPA by 27%.

PA has been found to be non-specifically bound to plasma proteins [11] and absorbed on glassware [6, 12] which has resulted in poor extractability and non-linear calibration curves. When silanized glassware or polypropylene tubes

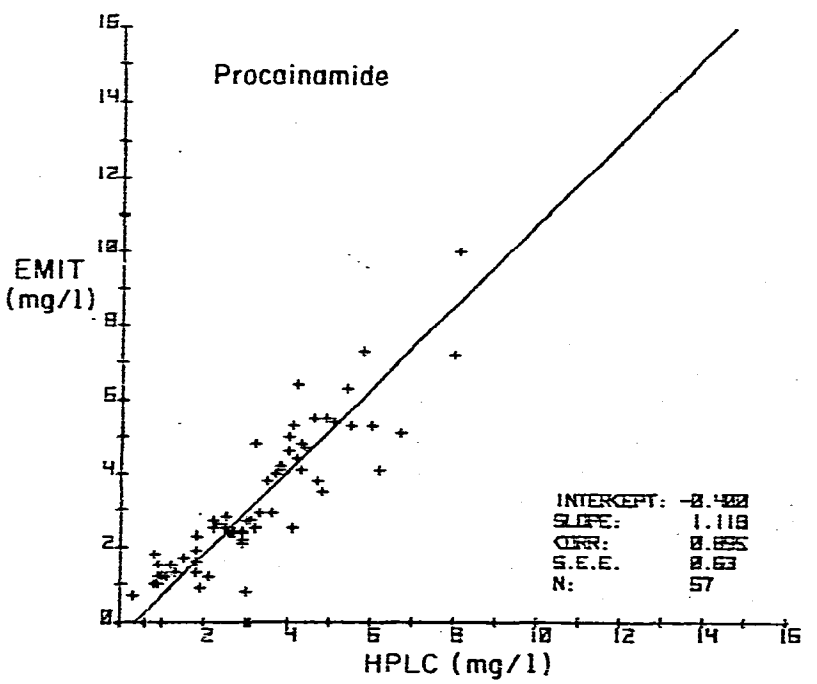


Fig. 1. Scattergram comparing serum procainamide concentrations determined by EMIT with those determined by HPLC.

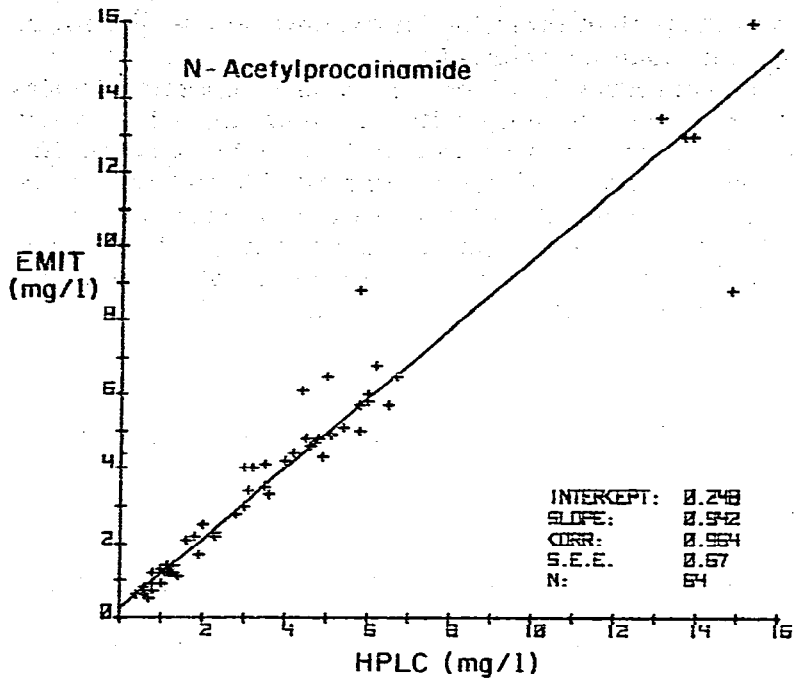


**Fig. 2. Scattergram comparing serum N-acetylprocainamide concentrations determined by EMIT with those determined by HPLC.**

were used for extraction and concentration, this problem was overcome. In the present study, silanizing all glassware prior to use increased recovery of PA from 58 to 85% and precision (C.V.) from 9 to 5% (10 samples fortified at 5 mg/l). For NAPA, recovery and precision remained unchanged at 96% and 3% (C.V.) respectively. Although recovery of PA can be increased by the use of appropriate solvents and silanizing all glassware, nonspecific binding may still be a potential source of error.

The acetonitrile, sodium acetate-buffered eluting solvent was chosen as the mobile phase for the PA and NAPA HPLC assay because it gave sharp, symmetrical peaks with good separation of PA, NAPA and the internal standard. Methanol in water, which was used as the mobile phase by Rocco et al. [7] gave poorer resolution and broader peaks over the range of methanol:water ratios evaluated.

This study attempted to rigorously challenge the specificity of the two assay methods since all samples were obtained from patients who were receiving an average of six other drugs in addition to PA. In fact, all patients received lidocaine prior to PA and most had received quinidine. There was no apparent interference by any of these other medications in either assay as evidenced by the absence of artifacts in the pre-drug (PA) control samples. The somewhat lower correlation between the two methods for PA may be due to (1) the known incomplete and somewhat variable recovery of PA from plasma which may result in an error in the HPLC procedure or (2) the presence of heme pig-

ments, lipids or bilirubin in the patient sera which is a known source of error in any enzyme multiplied immunoassay technique [13].

In conclusion, in the clinical setting in which a number of pharmacological agents are being administered concomitantly with PA, both the HPLC assay and the EMIT assay described herein were shown to be sensitive and specific techniques for the therapeutic drug level monitoring of PA and the active metabolite NAPA. The HPLC assay has the advantage of allowing simultaneous determination of both PA and NAPA but has the disadvantage of requiring prior extraction with organic solvent. EMIT has the advantage of being a simpler, more rapid assay but requires separate tests for PA and NAPA.

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