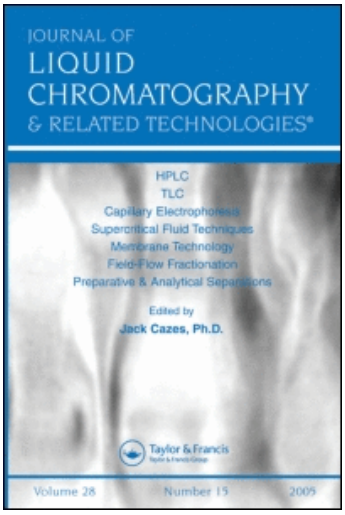

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An Improved Microanalytical Procedure for the Quantitation of Procainamide and N-Acetyl Procainamide in Serum by High-Performance Liquid Chromatography

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AN IMPROVED MICROANALYTICAL PROCEDURE FOR THE QUANTITATION OF PROCAINAMIDE AND N-ACETYL PROCAINAMIDE IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatographic method for analysis of procainamide (PA), and N-acetyl procainamide (NAPA) is presented. Sample preparation employs a simple base-acid double extraction procedure and analysis is carried out on a reverse phase chromatographic system using a μBondapak C_{18} column and buffered aqueous acetonitrile as the mobile phase. The extraction procedure gives quantitative recovery of both PA and NAPA, and chromatographic results show that drug levels of as low as 0.3 mg per liter of serum can be conveniently analyzed without significant background interferences. The small volume (0.2 ml) of serum needed to perform an analysis makes this method suitable for pharmacokinetic studies in humans and animals as well as for clinical therapeutic drug monitoring studies.

INTRODUCTION

Procainamide (PA) has been extensively used as an anti-arrhythmic agent (1). The proposed therapeutic concentration range in serum is 4 to 8 mg/liter, and the incidence of its toxicity increases when concentration exceeds 16 mg/liter (2). Recent reports (2-4) have indicated that N-acetyl procainamide

(NAPA), the major metabolite of PA, also possesses similar antiarrhythmic properties but appears to be less toxic (5). Due to variations in drug absorption, metabolism, and elimination characteristics, serum concentrations of PA and NAPA in individuals receiving similar dosages of PA can differ widely (6,7). Thus, in order to achieve therapeutic concentrations and to minimize toxic effects of these drugs in a given patient, careful monitoring of the serum levels of both PA and NAPA is recommended. In pharmacokinetic studies of drugs in humans or animals, large numbers of individual blood samples are usually needed. Hence, analytical methods developed for such studies utilizing a very small quantity of serum per analysis are desirable.

Measurement of PA and/or NAPA in serum has been done by fluorometric (8), thin layer (TLC) (5,9), gas-liquid (GLC) (2,3,10), and high-performance liquid chromatographic (HPLC) (11-15) methods. Fluorometric and TLC detection methods often lack specificity and/or linearity of concentration response (5,8) and GLC appears to suffer from some adsorption problems in the chromatographic column (2). The high specificity and sensitivity attainable by HPLC makes it the apparent method of choice for quantitative measurements of PA and NAPA in serum.

The present report describes a HPLC assay for serum levels of PA and NAPA. A sample preparation procedure which requires small amounts of serum is presented and its advantages over the existing procedures are discussed.

MATERIALS AND METHODS

Reagents:

All organic solvents were of Chrom AR grade (Mallinckrodt, St. Louis, MO.). Sodium acetate, 0.1 M sodium hydroxide, and glacial acetic acid were obtained from Fisher Chemical Co. (Fairlawn, N.J.). Procainamide HCl and N-acetyl procainamide standards were supplied by Squibb & Son, Inc. (Princeton, N.J.) and Arnar-Stone Laboratories (Mount Prospect, IL.), respectively. The internal standard, procaine, was of U.S.P. grade from Mallinckrodt. Control serum and sera from patients on PA therapy were obtained from Veterans Administration Hospital, Little Rock, Ark.

Stock solutions of procaine (PRO), (2,10 and 100 mg in 100 ml water) and stock solutions of PA and NAPA (2,10 and 100 mg in 100 ml water) were prepared by serial dilution and stored at 4°C. No decomposition was observed over several weeks.

Chromatographic System:

Analyses were carried out on a liquid chromatograph consisting of a solvent delivery system (Model M6000, Waters Associates, Milford, MA.), an injector (Model U6K, Waters Associates), a reverse-phase analytical column (μ Bondapak C_{18}, Waters Associates), a variable wavelength ultraviolet detector (Model LC-55, Perkin-Elmer Co., Norwalk, CO.) operated at 275 nm, and a 1 mV chart recorder (Model A-25, Varian Aerograph, Walnut Creek, CA). The mobile phase was buffered aqueous acetonitrile: 85% 0.02 M sodium acetate adjusted to pH 4.5 with acetic acid - 15% acetonitrile. The system was operated at room temperature and the mobile phase flow rate was set at 1.5 ml/min.

Procedure:

A 0.2 ml portion of blank serum was pipetted into a 10 cm x 10 mm test tube with a Teflon-lined cap. An accurate volume of PA and NAPA stock solution was added with a 25 µl or 100 µl syringe followed by 0.2 ml of 0.01 M sodium hydroxide - 1.0 M sodium chloride solution and 0.8 ml of methylene chloride. The test tube was shaken briefly and an appropriate volume of a PRO stock solution added. The solutions were mixed vigorously for one minute on a vortex type mixer. The partition phases were separated by centrifugation at 1500 x g for 3 minutes. The aqueous phase was aspirated and discarded. A fresh portion (0.4 ml) of the sodium hydroxide solution was added. The test tube contents were again mixed, centrifuged, and the aqueous phase discarded. Without disturbing the methylene chloride gel layer, the tube was carefully rinsed twice with 0.5 ml portions of distilled water to remove the last trace of sodium hydroxide. Finally, 0.4 ml of the mobile phase solution was added and the mixture was vortexed for 30 seconds and centrifuged. The aqueous phase (25 µl or 50 µl) was injected directly into the chromatograph.

RESULTS

A chromatogram of PA, NAPA and PRO standards is shown in Figure 1. The solute peaks are completely resolved and are symmetrical. Detector response, as measured from peak heights, was linear up to at least 3 µg for each solute. The minimum detection limits were 2 ng for PA and 3 ng for NAPA.

A series of serum samples containing 0.3 to 4.0 mg each of PA, NAPA and PRO per liter were prepared and analyzed. Chromato-

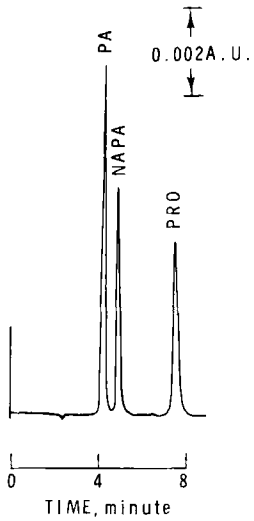

FIGURE 1

Chromatogram of a standard mixture of PA, NAPA, and PRO (50 ng each). Column: μ Bondapak C_{18}; Mobile phase: 0.02 M sodium acetate and acidic acid, pH 4.5/acetonitrile (85:15,v/v); Wavelength: 280 nm, Flowrate: 1.5 ml/min.; Injection volume: 25 μl.

graphic results showed that extraction efficiency was independent of the solute concentration, as the peak height versus concentration plots were linear (Figure 2). The apparent net recoveries (neglecting small volume changes occurring during sample preparation) from these samples were 95% for PA and 90% for NAPA and PRO. The mean analytical recoveries (relative to the internal standard) from eight 10 mg/liter samples were 103 ± 2% (mean ± SD) for PA and 100 ± 1.5% for NAPA. For 0.5 mg/liter samples, the recovery values were 104 ± 3% and 102 ± 4% for PA and NAPA, respectively.

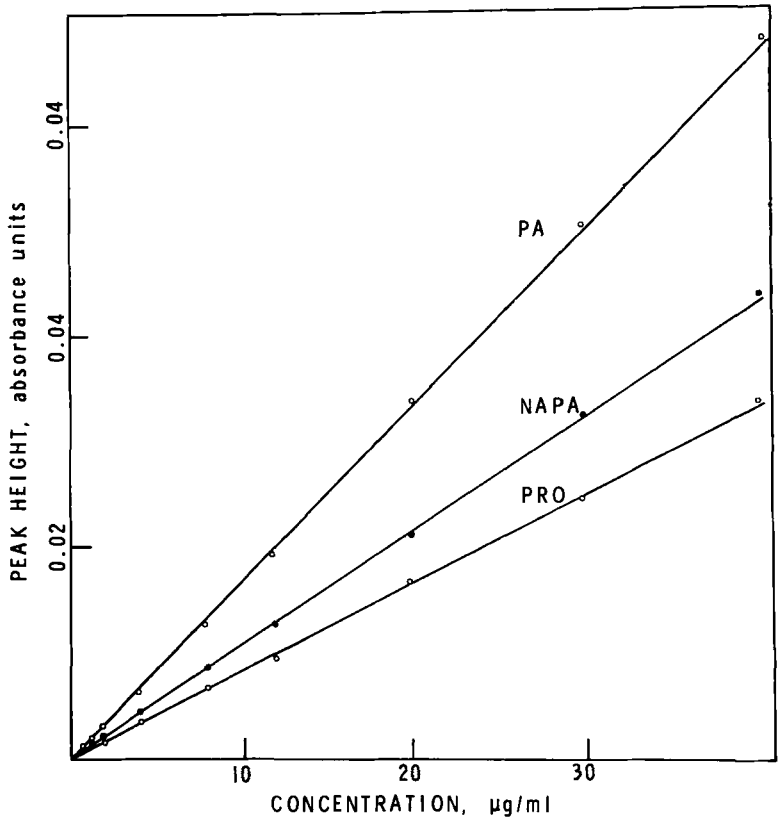

FIGURE 2

Plots of peak height as a function of plasma concentrations of PA, NAPA, and PRO.

Figure 3 shows the results of a kinetic study of PA in humans. A normal volunteer subject was given a single dose of 6.5 mg/kg of PA HCl orally. Blood samples were drawn at 0, 30, and 60 minutes, and then hourly for the next seven hours. The serum concentrations of PA and NAPA were determined. The log [PA] versus time plot shows that the peak serum level of PA (2.15 mg/liter) was reached by two hours. After the distribution phase (2 to 4 hours), PA

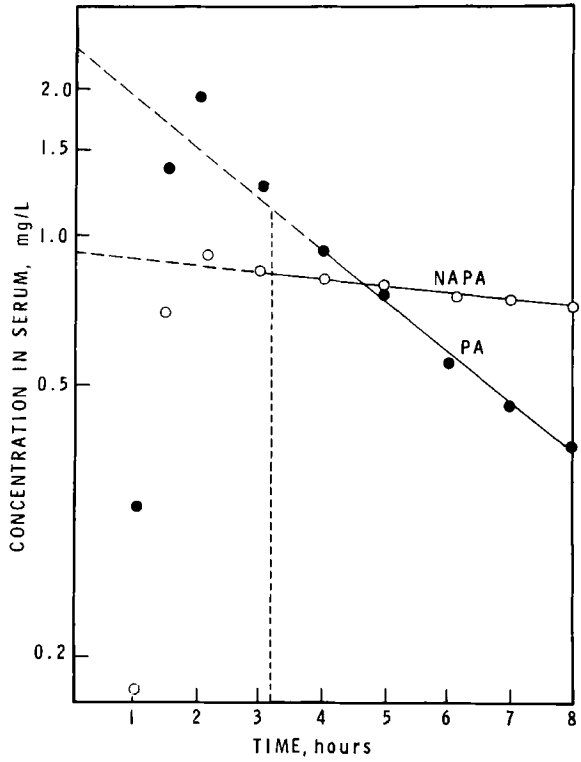

FIGURE 3

Log serum concentrations of PA and NAPA as a function of time after the subject was given 6.5 mg/kg of PA orally.

was eliminated following first order kinetics with the apparent elimination half life, T 1/2, measured directly from the graph, of 3.2 hr. The [NAPA] versus time plot shows that NAPA began to appear in serum within 1 hour and also reached peak level (0.95 mg/liter) by 2 hours. The disappearance rate, however, was much slower than that of PA, apparently influenced by the continual conversion of PA into NAPA, and/or decreased urinary excretion rate of NAPA. This study compared well with the

results previously published using a different analytical method for PA and NAPA (6).

Serum concentrations of PA and NAPA in several patients were determined. Each patient studied had been receiving 6-7 mg/kg oral dose of PA-HCl every 4-6 hours for several days and blood samples were taken 3 hours after the latest dose. Some representative chromatograms are illustrated in Figure 4. Figure 4a is a chromatogram obtained from a patient with normal renal function (BUN and creatinine normal). Figure 4b chromatogram was obtained from a patient with severely impaired renal function. The high

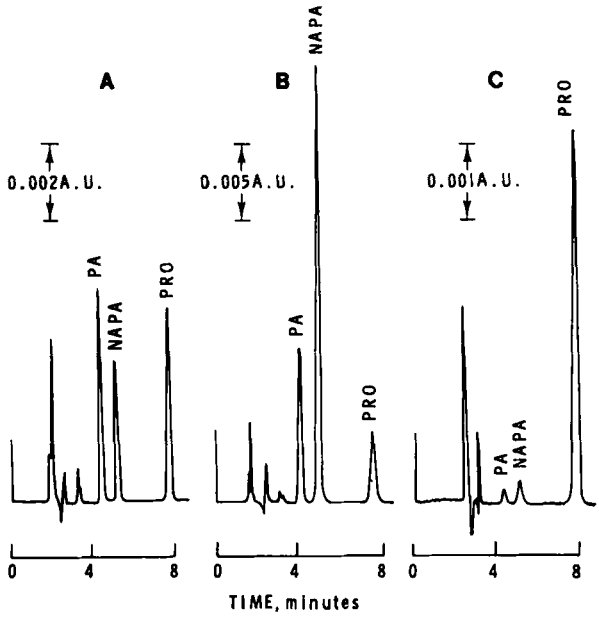

FIGURE 4

Chromatogram of sera extracts: Patient's serum (A) calculated to contain 3.4 mg/liter PA and 2.5 mg/liter NAPA; (B) 4.9 mg/liter PA, and 9.1 mg/liter NAPA; and (C) 0.1 mg/liter PA and 0.3 mg/liter NAPA.

NAPA/PA ratio is consistent with previous reports of the inability to eliminate NAPA by patients with renal insufficiency (6,11). Very low concentrations of PA and NAPA were found in an arrhythmic patient who also had jaundice (Figure 4c). In this particular case, absorption of PA may have been a major problem.

DISCUSSION

HPLC is a recognized method for the determination of PA and NAPA in serum and various preparation or clean-up procedures have recently been proposed (11-15). The charcoal adsorption (13) and ether extraction (12) techniques were found to give low solute recoveries. Better quantitative results were obtained with procedures using more polar organic extractions (14, 15). However, solute dissolution difficulties attributed to "glass adsorption" (14) appeared to be a common problem. In addition, gel frequently formed in the partition phases during these types of extractions, interfering with consistent quantitative recovery of solute.

In this sample preparation procedure, the basic extraction principle was adopted but several modifications were made. Sodium chloride was first added to serum as a salting-out agent. The extraction efficiency for PA using a methylene chloride/0.01 M sodium hydroxide (2:1 v/v) partition system was approximately 80%, and appeared to decrease with increased hydroxide concentration (2). The presence of a salting-out agent in the system was found to markedly improve the extraction efficiency. Secondly, ample time (1 minute or more) was allowed for mixing to ensure extraction

equilibrium as well as to promote a stable or consistent gel in the organic layer. This gel prevented disturbance of the organic layer during the washing steps, thus minimizing sample losses. Thirdly, to remove residual protein and colored impurities from the methylene chloride layer, an additional sodium hydroxide extraction step was found necessary. Finally, PA, NAPA, and PRO were extracted directly into a measured volume of the mobile phase. This eliminated the transfer, evaporation, and reconstitution steps commonly used by other methods (11-15). Quantitative extraction was achieved since the solutes were highly soluble in the acidic buffer. Sample losses attributed to solvent transfer and the "glass adsorption" (14) problems were prevented. Samples prepared by this method were clear and ready for HPLC analysis.

In principle, the clean-up procedure described is applicable to serum samples of smaller sizes. However, the 0.2 ml sample size appeared to be the practical lower limit for routine preparations without resorting to special low-volume apparatus for precision and accuracy.

PRO, which exhibits similar extraction and chromatographic properties as PA and NAPA, was selected as the internal standard. However, caution must be exercised as PRO is susceptible to rapid hydrolysis by esterases present in serum (13). In our procedure, hydrolysis was prevented by deactivating the enzymes with the sodium hydroxide solution before the internal standard was added. Once extracted into the organic phase, PRO remained stable throughout the experiment.

This HPLC method is relatively simple, accurate, and specific for simultaneous analysis of PA and NAPA in small serum samples. Thus, it can be used as a reliable clinical chemistry laboratory procedure as well as for pharmacokinetic studies of procainamide and N-acetyl procainamide in humans and/or animals.

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