Improved High-Performance Liquid Chromatographic Assay for the Determination of Procainamide and Its *N*-Acetylated Metabolite in Plasma: Application to a Single-Dose Pharmacokinetic Study

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Abstract

An improved high-performance liquid chromatographic assay for the determination of procainamide and *N*-acetylprocainamide (NAPA) at concentrations observed up to 32 h after a single oral dose administration of procainamide to human subjects is reported. Following liquid–liquid extraction of plasma samples, procainamide, NAPA, and the internal standard (*N*-propionylprocainamide) are separated on a reversed-phase C_8 column with retention times of 4.0, 6.7, and 13.2 min, respectively. The ultraviolet detection limit (wavelength, 280 nm) of procainamide and NAPA is 2 ng/mL (signal-to-noise ratio, 3:1), and the quantitation limit is 4 ng/mL (signal-to-noise ratio, 5:1). Intra- and interday coefficients of variation are less than 8% in the range of 20–500 ng/mL.

Introduction

Procainamide is an effective class IA antiarrhythmic agent used in the treatment of a variety of atrial and ventricular arrhythmias (1–4). Monitoring plasma concentrations of procainamide and of its active metabolite, *N*-acetylprocainamide (NAPA), is dictated by the wide intersubject variability in genetically-determined *N*-acetyltransferase activity involved in the formation of NAPA and by the narrow therapeutic indeces of both procainamide and NAPA (5,6). Consequently, several gas chromatographic assays, high-performance liquid chromatographic (HPLC) assays, and immunoassays have been developed (7–19).

Although these assays are suitable for therapeutic monitoring purposes, most of them lack the sensitivity required for the assessment of procainamide pharmacokinetics following a single oral dose of the drug. One exception is the assay developed by Jamali et al., which appeared suitable for pharmacokinetic studies of procainamide in humans (17). The assay was validated between 50 and 5000 ng/mL, whereas the predicted limit of sensitivity for both procainamide and NAPA was 5 ng/mL. Achievement of such a detection limit was a major improvement compared with previously described assays. On the other hand, chromatographic resolution and elution characteristics of procainamide and NAPA were not ideal. In fact, both peaks were tailing, even though the retention times of procainamide and NAPA were relatively short (6.3 and 11.7 min, respectively). Consequently, quantitation at low concentrations of the products was very difficult. Therefore, the objective of our study was to improve previously reported assays of procainamide and NAPA in order to allow quantitative measurement of these products in plasma for at least 32 h (estimated minimal concentration, 5 ng/mL) following administration of a single oral dose (500 mg) of procainamide hydrochloride to healthy volunteers.

Experimental

Reagents and Standards

Procainamide hydrochloride, the hydrochloride salt of NAPA, and *N*-propionylprocainamide were obtained from Sigma Chemical (St. Louis, MO). HPLC-grade methanol and methylene chloride were purchased from Fisher Scientific (Montreal, Quebec, Canada). Triethylamine (TEA) and glacial acetic acid were both analytical-grade. Stock solutions of procainamide (100 µg/mL) and NAPA (100 µg/mL) were prepared by dissolving appropriate amounts of the salts in distilled water. *N*-propionylprocainamide (10 mg) was dissolved in ethanol (5 mL) prior to dilution with water to a final volume of 100 mL (100 µg/mL). Stock solutions were stored at –20°C for 10 weeks without degradation. Working solutions were prepared using double-distilled, deionized water.

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Apparatus

The HPLC system (Shimadzu, Tokyo, Japan) consisted of a model LC-600 pump, an SIL-9A automatic injector, and a SPD-6A variable ultraviolet (UV) light detector set at 280 nm. Peak heights were determined by a Shimadzu CR501 Chromatopac integrator.

Procedure

Separation was carried out at ambient temperature using a C_8 column (Ultrasphere octyl, 250 × 4.6-mm i.d., 5-µm film thickness, Beckman Instruments, Fullerton, CA). The mobile phase, pumped at a flow rate of 1.3 mL/min, consisted of water–methanol–acetic acid–TEA (78:22:1:0.01, v/v) adjusted to pH 5.5 with NaOH.

Table I. Peak Width Ratios and Retention Times of Procainamide, NAPA, and *N*-Propionylprocainamide Using Various Stationary Phases

	Procainamide		NAPA		*N*-Propionylprocainamide	
Stationary phase	Peak width ratio*	Retention time (min)	Peak width ratio*	Retention time (min)	Peak width ratio*	Retention time (min)
C_8 Ultrasphere	1.60	4.0	2.50	6.7	2.25	13.2
C_{18} Ultrasphere	2.50	3.2	3.00	6.1	2.90	12.1
Phenyl-SB Zorbax	2.20	10.9	2.67	24.0	2.53	39.8
TMS Zorbax	3.50	8.0	2.80	11.2	3.25	17.1

* The peak width ratio was determined by dividing the peak width at 5% of maximal peak height by the peak width at 50% of maximal peak height.

Calibration curves

Aliquots of procainamide and NAPA working solutions were added to blank human plasma to obtain final concentrations ranging from 4 to 100 ng/mL (4, 6, 10, 16, 20, 25, 30, 40, 50, and 100 ng/mL) for the low concentration standard curve and from 200 to 2500 ng/mL (200, 250, 300, 400, 500, 700, 1200, 2000, and 2500 ng/mL) for the high concentration standard curve. Calibration curves (linear regression analysis) were constructed by plotting the peak height ratios (procainamide or NAPA/internal standard) versus their corresponding plasma concentrations. In the low concentration standard curve (4–100 ng/mL), the added amount of the internal standard was 50 ng; for the high concentration curve (200–2500 ng/mL), the amount added was 500 ng.

Sample preparation

Due to the wide range of human plasma concentrations to be ana-

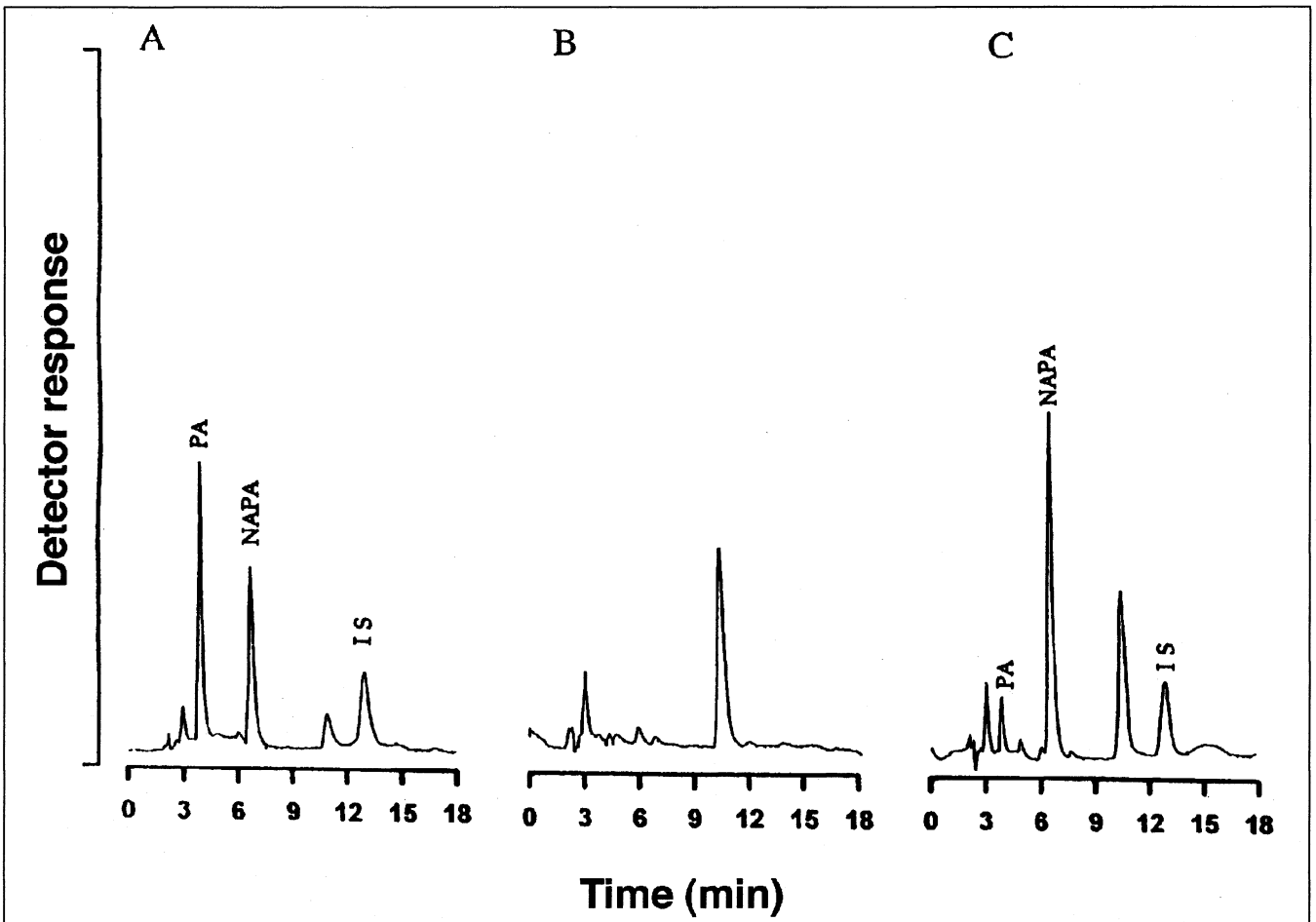

Figure 1. Chromatograms of (A) a plasma sample extract spiked with 60 ng of procainamide (PA), 60 ng of NAPA, and 50 ng of *N*-propionylprocainamide (internal standard); (B) a blank plasma extract; and (C) a plasma extract from a subject collected 24 h after administration of a 500-mg single oral dose of procainamide hydrochloride. The sample shown in Figure 1C contained 12 ng/mL of procainamide and 112 ng/mL of NAPA.

lyzed, plasma samples obtained from 0 to 12 h and from 24 to 32 h following a single oral dose of procainamide hydrochloride were processed slightly differently. A 0.5-mL amount of water and 100 µL of the internal standard at a concentration of 5 µg/mL were added to plasma samples (0.5 mL) from the 0–12-h period. Samples (1 mL) from the 24–32-h period were spiked with 100 µL of the internal standard at a concentration of 500 ng/mL. Thereafter, 0.2 mL of 4M NaOH and 4 mL of methylene chloride were added. The mixture was vortexed for 10 s and centrifuged for 5 min at 2000 *g*. The organic layer was then separated, and the liquid–liquid extraction procedure repeated. The combined organic extracts were dried by the addition of anhydrous sodium sulfate (1–2 g), filtered, and then evaporated at 50°C. Prior to complete evaporation, the tubes were placed on ice, and evaporation was completed to dryness under a stream of nitrogen. The residue was dissolved in 100 µL of mobile phase, and 45 µL of the resultant was injected into the HPLC system.

Accuracy and precision

Intraday variability in the analysis of procainamide and NAPA was assessed by repeated analysis (six replicates) of blank human plasma samples spiked with the products at concentrations of 20, 300, or 1500 ng/mL. The accuracy was expressed as the mean ratio of observed and spiked concentrations. The precision was calculated as coefficients of variation (CV [%]). Interday precision was determined by the analysis of samples containing 20, 300, or 1500 ng/mL of procainamide and NAPA on six consecutive days. For these analyses, the amount of internal standard added to the samples was fixed at 50 ng (20 ng/mL) or 500 ng (300 and 1500 ng/mL).

Statistical analysis

All data were expressed as the mean plus or minus the standard deviation (SD). CVs were calculated as the SD times 100 divided by the mean.

Results and Discussion

Chromatographic analysis

As a first step to improve the assay of procainamide and its *N*-acetylated metabolite, we modified methanol proportions and the pH of the mobile phase using a C_{18} column as reported previously (17). Increasing methanol content or decreasing the pH tended to decrease retention times and resolution of the peaks. To obtain good resolution and sharp peaks, the presence of ion-pairing agents such as acetic acid and TEA was required. Ion-pairing agents also allowed good separation from potentially interfering plasma extract peaks. Table I reports peak width ratios (width at 5% peak height divided by peak width at 50% peak height) and retention times of procainamide, NAPA, and the internal standard observed using various stationary phases (C_8, C_{18}, trimethylsilane [TMS], and phenyl columns). In these experiments, the mobile phase consisted of water–methanol– acetic acid–TEA (78:22:1:0.01 [v/v] adjusted to pH 5.5 with NaOH). Smaller peak width ratios, indicating an increase in peak sharpness, were observed in the presence of the C_8 column. Moreover, retention times for procainamide, NAPA, and

Table II. Linear Regression Analysis Parameters for Quantitation of Procainamide and NAPA in Human Plasma

Low concentration curve (4–100 ng/mL)	Procainamide	NAPA
Slope	0.05883	0.04105
(95% confidence limits)	(0.05645 – 0.06121)	(0.03947 – 0.04263)
y-Intercept	-0.09094	-0.04105
(95% confidence limits)	(-0.15458 – -0.02730)	(-0.09200 – -0.00746)
Correlation coefficient	0.99914	0.99910
High concentration curve (200–2500 ng/mL)	**Procainamide**	**NAPA**
Slope	0.00419	0.00248
(95% confidence limits)	(0.00408 – 0.00430)	(0.00240 – 0.00256)
y-Intercept	0.06719	0.11246
(95% confidence limits)	(-0.06446 – -0.19885)	(0.01954 – 0.20538)
Correlation coefficient	0.99956	0.99938

Table III. Accuracy and Precision of the Quantitation of Procainamide and NAPA in Human Plasma

	Procainamide (two replicates)			NAPA (two replicates)		
Spiked concentration (ng/mL)	Observed concentration (ng/mL)	CV (%)	Accuracy (%)	Observed concentration (ng/mL)	CV (%)	Accuracy (%)
Low concentration curve						
4	4.3	6.6	108.6	4.0	2.8	100.1
6	6.6	1.5	110.1	5.6	8.1	92.7
10	9.4	2.1	94.4	9.8	6.5	97.8
16	15.7	2.8	98.2	16.6	0.7	103.7
20	20.0	0.6	100.0	19.4	2.7	97.1
25	24.9	2.3	99.5	24.8	2.0	99.2
30	30.3	3.4	101.1	31.4	0.4	104.8
40	38.6	0.4	96.6	40.2	0.1	100.5
50	51.1	4.0	102.1	49.2	0.7	98.5
100	103.4	4.7	103.4	104.3	1.2	104.3
High concentration curve						
200	178.6	1.5	89.3	168.4	4.7	84.2
250	222.2	3.0	88.9	214.5	7.3	85.8
300	305.8	3.8	101.9	297.1	3.0	99.0
400	416.0	2.9	104.0	404.6	2.5	101.2
500	519.8	7.0	103.9	515.4	2.0	103.1
700	736.4	3.9	105.2	741.1	0.6	105.9
1200	1161.4	6.9	96.8	1247.5	1.5	104.0
2000	2010.0	1.1	100.5	1982.3	6.2	99.1
2500	2493.8	2.2	99.8	2488.3	3.8	99.5

N-propionylprocainamide were shorter with the C_8 stationary phase than with the TMS and phenyl columns. These results suggested that the C_8 column possessed characteristics that are required for good resolution of procainamide and its congeners.

Table IV. Intraday and Interday Variability in the Analysis of Procainamide and NAPA in Human Plasma

Compound	Spiked concentration (ng/mL)	Observed concentration (mean ± SD) (ng/mL)	CV (%)	Accuracy (%)
Intraday variation				
Procainamide (six replicates)	20	21 ± 4	5.9	103.7
	300	302 ± 14	4.5	100.7
	1500	1538 ± 40	3.0	102.5
NAPA (six replicates)	20	21 ± 1	4.4	102.8
	300	309 ± 6	2.0	103.0
	1500	1539 ± 18	1.2	102.6
Interday variation				
Procainamide (six replicates)	20	20 ± 2	6.0	100.7
	300	300 ± 17	4.2	100.1
	1500	1498 ± 55	5.8	99.9
NAPA (six replicates)	20	20 ± 1	7.8	100.6
	300	306 ± 10	5.5	102.4
	1500	1523 ± 54	4.2	101.9

Figure 1A represents the analysis of a human plasma sample extract spiked with 60 ng of procainamide, 60 ng of NAPA, and 50 ng of *N*-propionylprocainamide (internal standard). The retention times of procainamide, NAPA, and the internal standard were 4.0, 6.7, and 13.2 min, respectively. No interference with plasma constituents or other metabolites of procainamide such as desethylprocainamide and desethyl-*N*-acetylprocainamide (3.3 and 5.0 min), procainamide hydroxylamine (2.3 min), and nitroprocainamide (14.9 min) was observed. In addition, the extraction procedure did not allow for the recovery of other metabolites such as *p*-aminobenzoic acid and *N*-acetyl-*p*-aminobenzoic acid. Figure 1B shows a chromatogram of a processed blank human plasma sample, and Figure 1C illustrates a chromatogram obtained by the analysis of treated plasma samples from a subject who received a single oral dose of procainamide.

Linearity and sensitivity

Typical linear regression curves used to determine plasma concentrations of procainamide and NAPA in the range of 200–2500 ng/mL were $y = 0.00419x + 0.06719$ (correlation coefficient [r] > 0.999) and $y = 0.00248x + 0.11246$ ($r > 0.999$),

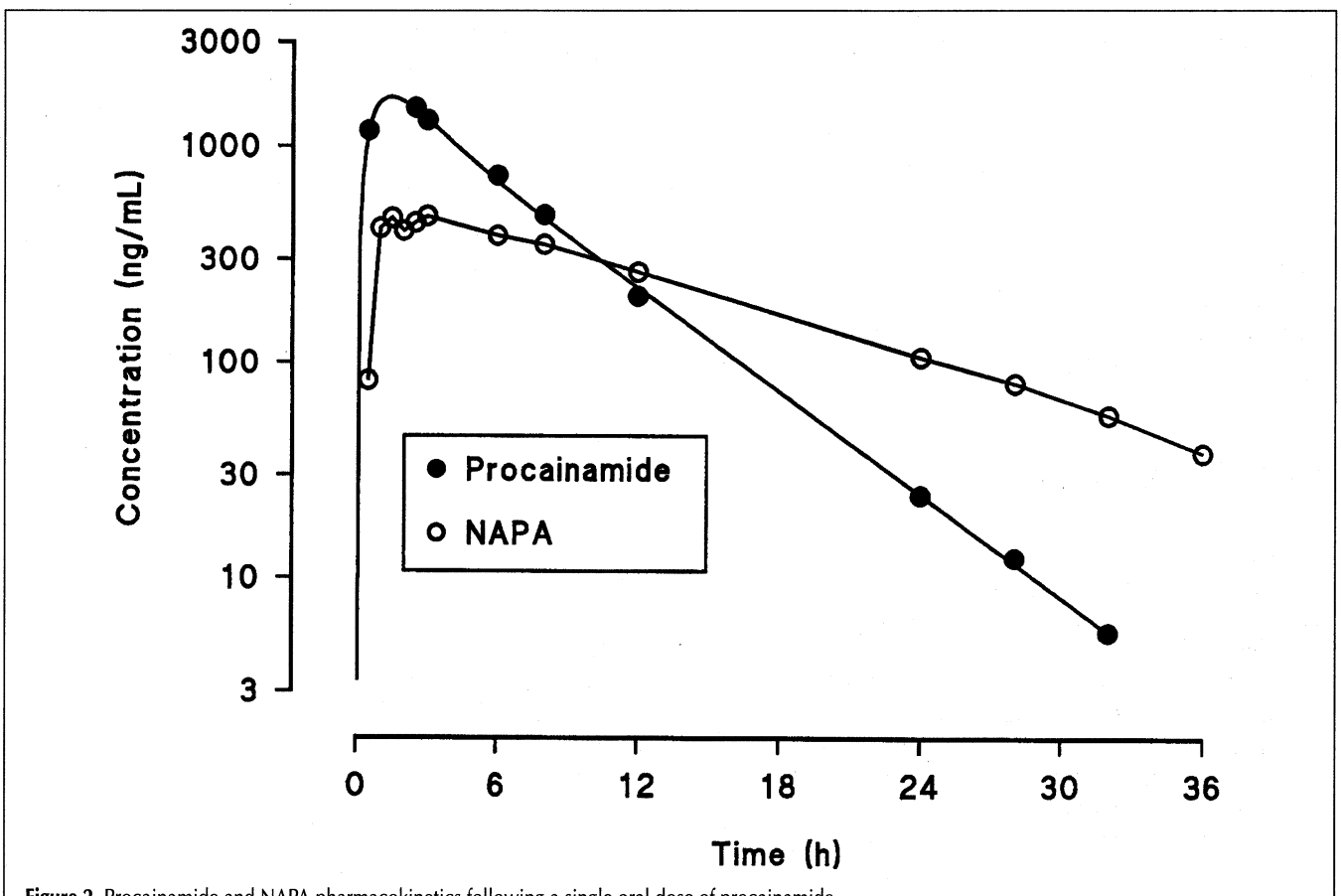

Figure 2. Procainamide and NAPA pharmacokinetics following a single oral dose of procainamide.

respectively (Table II). The values for concentrations of procainamide and NAPA in the range of 4–100 ng/mL were $y = 0.05883x - 0.09094$ ($r > 0.999$) and $y = 0.04105x - 0.04973$ ($r > 0.999$), respectively (Table II). Values for the accuracy and precision of the assay are reported in Table III. The detection limit of procainamide and NAPA determined by a signal-to-noise ratio of 3:1 was 2 ng/mL, and the quantitation limit determined by a signal-to-noise ratio of 5:1 was 4 ng/mL (20). These limits allowed measurement of procainamide and NAPA concentrations for up to 32 h following a single oral dose of 500 mg procainamide hydrochloride.

Plasma samples spiked with procainamide and NAPA at concentrations of 20, 300, and 1500 ng/mL were analyzed in replicates of six to determine intraday and interday precision. As shown in Table IV, day-to-day and within-day variabilities were less than 8%. Precision at the lowest concentration (50 ng/mL) of procainamide and NAPA tested by Jamali et al. was in the range of 7–11% (17). Improved precision observed in our assay is most likely related to the better elution characteristics of procainamide and NAPA. Elution characteristics were improved by the selection of a C_8 column instead of a Novapac stationary phase as well as a slight modification of the mobile phase (17).

Applications

Plasma samples were obtained from a healthy volunteer prior to dosing and at subsequent time points following a 500-mg oral dose of procainamide hydrochloride. After collection, samples were stored at –20°C until analyzed by the method described herein. Figure 2 shows the concentration time profile for procainamide and NAPA. The elimination half-life ($t_{1/2\beta}$) for procainamide, as calculated from the slope of the terminal linear portion of the curve, was 3.78 h.

Conclusion

In conclusion, a sensitive procedure for the simultaneous analysis of procainamide and NAPA in plasma is reported. This method is sensitive, simple, uses a fast, easy extraction procedure, and possesses excellent linearity and precision characteristics in the range of 4–2500 ng/mL. This assay can be useful to quantitate procainamide and its metabolite, NAPA, in plasma during single-dose pharmacokinetic studies for up to 32 h following drug administration.

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