controlled-release

mmol/L, 250 mmol/L, and 7.7, and a smoother variation of AAP activity above these values, the most important factor being the substrate concentration. This weak variation, demonstrated by the small values for the total variance in subsets 4 and 5 (Table 2), is confirmed by pseudo-three-dimensional plots showing a broad plateau in the optimal area (Figure 2).

We confirmed the need of pretreatment of urine in the method of Jung and Scholz (8) (Table 1). But after multivariate optimization of the method, this pretreatment became unnecessary—that is, better optimization overcomes the inhibitors' influence.

Our re-assessment of optimal conditions when untreated urine is used confirmed the previously reported optimal condition for treated urines.

We conclude that multivariate studies are required if a method involving interdependent reaction parameters is to be truly optimized. For measurement of AAP activity this procedure allows us to determine a broad plateau optimum area for the three parameters studied. In these areas the method is more resistant to concentration variations and inhibitors' influence. This improved procedure leads to a great practical advantage: urine pretreatment is no more a prerequisite to accurate AAP determination, and complete automation of the AAP measurement is then possible.

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References

More-Sensitive Enzyme-Multiplied Immunoassay Technique for Procainamide and N-Acetylprocainamide in Plasma, Serum, and Urine
Paul R. Henry and Rohini A. Dhruv

A commercially available (Syva Co.) enzyme-multiplied immunoassay technique (EMIT) for the quantitative determination of procainamide (PA) and N-acetylprocainamide (NAPA) was modified to allow automated quantitative analysis of approximately 100 samples per day, in a working range of 0.1 to 2.0 μg/mL. Such a test was needed to evaluate the pharmacokinetic characteristics of controlled-release dosage forms characterized by long half-lives at low plasma concentration. Analytical recovery of PA and NAPA from serum, plasma, and urine was satisfactory, but at extreme ratios for PA:NAPA the accuracy of determining the lower-concentration component became unsatisfactory. In fact, however, we found no such ratios in 5400 clinical samples assayed by this procedure.

Additional Keyphrases: pharmacokinetics  •  controlled-release medication  •  anti-arrhythmic drugs

The drug procainamide (PA), used in the treatment of individuals with cardiac arrhythmia, is therapeutically effective at concentrations of 4 to 8 μg/mL in plasma (1). Its major metabolite, N-acetylprocainamide (NAPA), is equally effective against cardiac arrhythmias. Toxic effects are observed when the total concentration of PA and NAPA exceeds 12 μg/mL (2). Thus, determination of concentrations of PA as well as NAPA in plasma has been essential in individuals being treated with PA, and an enzyme-multiplied immunoassay technique (EMIT) is a well-accepted method for this purpose (3, 4).

Controlled-release formulations of PA, currently under development, are expected to maintain therapeutic concentrations of the drug in plasma for prolonged periods when administered to patients. However, typical of this type of formulation are prolonged periods during which the concent-

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trations in blood are far below the therapeutic concentration; i.e., they range from 0.1 to 2.0 μg/mL. Determination of the pharmacokinetics of such formulations is clearly inappropriate with the existing EMIT procedure described by Syva Co. (5–7), for which the recommended range for quantitative determinations is 1.0 to 16.0 μg/mL.

Here we describe a modification of the standard EMIT procedure, which can be used to quantify PA and NAPA in plasma, serum, and urine at concentrations as low as 0.1 μg/mL. Unlike previously described methods for the quantitative determination of PA and NAPA in plasma, which could detect as little as 0.1 μg/mL (8–11), this procedure requires no sample preparation by solvent extraction before assay and can be performed by commercially available automated systems (6) at a rate of 100 samples per 8 h.

Materials and Methods

Reagents

We obtained both kinds of EMIT kits from Syva Co., Palo Alto, CA: EMIT for PA, and EMIT for NAPA. Each kit contains a matched set of two reagents, A and B; six serum/plasma-based lyophilized calibrators (0–16 μg/mL); and a buffer in concentrated form. The EMIT calibrators served as the standards for determination of PA and NAPA in plasma, serum, and urine.

Reconstitute EMIT calibrators and EMIT drug assay buffer as described in the EMIT kit insert. Dilute the 1.0 μg/mL calibrator with EMIT zero control calibrator to obtain concentrations of 0.1 and 0.2 μg/mL. Similarly, dilute 2.0 μg/mL calibrator to obtain a concentration of 0.6 μg/mL. Verify that the PA and NAPA calibrators and the zero control diluent have similar lot numbers and thus are compatible with the EMIT Reagents A and B. Reconstitute Reagents A and B as follows: dissolve Reagents A and B each in 3 mL of doubly distilled water, then add 15 mL of reconstituted EMIT drug assay buffer to each reagent. Equilibrate the calibrators and Reagent A and B solutions for at least 1 h at room temperature before use in the assay.

We also used heparinized human plasma from whole blood (Intestate Blood Bank, Philadelphia, PA) and human serum (Gibco Laboratories, Grand Island, NY; lot no. 79-0278). A human urine pool consisted of a mixture of equal volumes of fresh urine obtained from five male donors. For independent standards, we obtained procainamide HCl (E. R. Squibb & Sons, Princeton, NJ; SQ2313, lot no. 78428) and N-acetylprocainamide HCl (Aldrich Chemical Co., Milwaukee, WI; lot no. 7926 E.K.), which we used as internal controls to determine analytical recoveries of PA and NAPA from donated urine and commercial preparations of plasma and serum. All water used was doubly distilled.

Equipment

Automated Gilford 203S continuous-flow system (Gilford Instruments, Oberlin, OH), modified with a Model DU spectrophotometer (Beckman Instruments, Fullerton, CA) and updated with a Gilford power supply, digital read-out, and thermocuvette.

Refrigerated centrifuge: IEC Model DPR-6000, or equivalent.

Strip-chart recorder (Linear Instruments, Reno, NV). Input potential 2 mV, chart speed 3 cm/min.

Air displacement pipettes: Gilson Models P200 and P1000, with disposable plastic tips.

Disposable plastic sample cups and reaction cups (Gilford Instruments, Oberlin, OH).

Procedures

Sample storage and preparation. Store plasma and urine samples at −20 °C in plastic tubes stoppered with plastic caps. Just before the assay, thaw and centrifuge plasma samples at 2000 × g for 30 min at 4 °C. No centrifugation is required for urine samples, just thawing.

Equipment setup and operation. Use the standard procedure described in the Gilford 203S operator's manual for Immunoassay I, test no. 23. Choose the "User Defined" and "Enzyme" options to select Lag, Equilibration, and Read times of 40, 15, and 60 s, respectively. Sample, buffer, and Reagent A and B volumes are 100, 300, and 400 μL, respectively. Monitor the plot of A390 nm vs time with the strip-chart recorder, to verify that particulate matter in the reaction mixture does not cause nonlinear kinetics. Samples with drug concentrations exceeding the upper limit of the assay can be diluted in an appropriate diluent: control EMIT calibrator or isotonic saline for plasma and distilled water for urine samples.

Standard curve. Prepare the standard curve EMIT calibrators and dilutions of these calibrators to produce concentrations of 0, 0.1, 0.2, 0.6, 1.0, and 2.0 μg of either drug per milliliter. Calculate the standard curve by use of the instrument computer, with either a four-parameter logistic fit or a five-parameter exponential fit (12), as specified by the reagent manufacturer. The standard curve should have a calculated standard deviation of 5% or less. The computer calculates the standard deviation and the value is printed on the tape printout.

Because the Gilford 203S system has limited decimal capacity (it prints concentrations to only one decimal place), we must increase the accuracy needed at the detection limit of 0.1 μg/mL. Input standard curve concentrations × 1000 for calculation of the standard curve by the computer. Divide by 1000 the drug concentrations so calculated, to obtain the drug concentrations in the samples to three decimal places.

Results and Discussion

Analytical recovery. Control plasma, serum, and urine were each supplemented with PA or NAPA to give final concentrations ranging from 0.1 to 2.0 μg/mL, then assayed for PA and NAPA, respectively. Average recoveries of PA and NAPA from plasma, serum, and urine were acceptable at concentrations as low as 0.1 μg/mL (Table 1). In both cases, we used the modified EMIT standard curve, which had a standard deviation of 5% or less.

Sensitivity and precision. To enhance assay sensitivity, we increased the sample volume from 25 to 100 μL. This correspondingly increased the observed net reaction rate for PA and NAPA at 0.2 μg/mL by threefold, thus improving the sensitivity of the assay. Use of sample volumes exceeding 100 μg/mL may lead to problems such as cross-reactions of drug-specific antibody with unidentified plasma proteins or transport proteins and cause erroneous results (13). We have observed that a sample volume of 100 μL is adequate for the required sensitivity of 0.1 μg/mL with minimal sample interference, if the plasma samples are centrifuged before assay.

Precision and sensitivity can both be improved by increasing the time over which the reaction rate is monitored (14). As shown in Figure 1, such an increase resulted in a change in reaction rate, which was most significant during the first 60 s of both the PA and NAPA reactions, with the rate
Table 1. Analytical Recovery of PA and NAPA from Plasma, Serum, and Urine

<table>
<thead>
<tr>
<th>Expected concn, µg/mL</th>
<th>Plasma</th>
<th>Serum</th>
<th>Urine</th>
<th>Plasma</th>
<th>Serum</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>106.2</td>
<td>99.0</td>
<td>102.7</td>
<td>105.6</td>
<td>104.5</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>109.4</td>
<td>96.5</td>
<td>102.4</td>
<td>106.7</td>
<td>107.8</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>99.1</td>
<td>93.0</td>
<td>100.5</td>
<td>101.7</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>101.8</td>
<td>92.2</td>
<td>102.4</td>
<td>101.7</td>
<td>100.7</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>105.3</td>
<td>92.4</td>
<td>107.4</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Av</td>
<td>104.4</td>
<td>92.6</td>
<td>103.1</td>
<td>97.6</td>
<td>100.4</td>
<td>103.2</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.8</td>
<td>2.9</td>
<td>1.9</td>
<td>5.2</td>
<td>4.1</td>
<td>3.6</td>
</tr>
</tbody>
</table>

N.D. = not done.

doubling between the first 30 and 60 s of the reaction. Thus, we used a reaction time of 60 s to determine the reaction rate. Assay precision was determined by a study in which drug concentrations between the range of 0.1 to 1.0 µg/mL, added to control (drug-free) plasma, were assayed in duplicate and average analytical recoveries were calculated from data obtained on two separate days. Recovery of PA was 99.5% (CV 2.1%) and of NAPA was 100.4% (CV 3.0%).

Interference by nonspecific antibody binding. Quantitative determination of PA and NAPA by the E.M.I.T. procedure is based on specific antigen–antibody interaction between the drug and its analogous antibody. Nonspecific cross-reaction of the antibody with interfering components in samples will result in erroneous values. In general, the antibodies of most commercial enzyme immunoassays show relatively high specificity for therapeutic drug-monitoring purposes (13), and E.M.I.T. assays for quantification of PA and NAPA are no exception (13). However, the method we describe was for PA and NAPA at subtherapeutic concentrations, so we investigated interference by PA and NAPA in the NAPA and PA assays, respectively. To plasma samples we added both PA and NAPA at concentrations as low as 0.1 µg/mL and at ratios of one drug to the other as high as 100-fold. We then assayed the samples on three separate days, with fresh samples each day. The average analytical recoveries indicated serious interference at drug ratios of 10:1 or greater, when the lower concentration of drug present was 0.1 µg/mL. In contrast, analytical recovery of PA and NAPA in concentrations ≥0.6 µg/mL was not affected at similar 10:1 ratios of interfering drug to test drug, confirming previous observations (11). Because clinical plasma samples are not expected to have extreme PA:NAPA ratios (2)—an expectation confirmed in our laboratory for 5400 plasma and urine samples—no further discussion on the correction of high-biased values resulting from interference will be presented here. A method for such corrections was devised during the initial stages of this study, and is available from the authors upon request.

The more-sensitive, modified E.M.I.T. procedure we describe is a convenient, rapid, and accurate method capable of quantitative determination of PA and NAPA in plasma, serum, and urine samples at concentrations as low as 0.1 µg/mL. Although the increased sensitivity may not be needed in a clinical environment, it is necessary for evaluation of the pharmacokinetics of controlled-release formulations of PA, where concentrations below the lower therapeutic limit may be encountered. Samples can be assayed at a rate of approximately 100 samples per 8-h working day, because no sample preparation is required before assay. As a precaution, samples should be stored frozen at −20 °C until assay or assayed soon after collection. Repetitive freeze/thaw cycles, which may change the integrity of the samples, should be avoided.

References
9. Rocco RM, Abbott DC, Giese RW, Karger BL. Analysis of

Fig. 1. Net absorbance rate change, calculated from Δ A405 nm (0.1 µg of drug per milliter) vs reaction time.

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Excretion of Endogenous Digoxin-like Immunoreactive Factors in Human Urine Is a Function of Urine Flow Rate

Berry A. Siegfried and Roland Valdes, Jr.†

We studied the effect of varying water and salt intake on the renal excretion of endogenous digoxin-like immunoreactive factors (DLIF). DLIF were measured in human urine and serum by competitive displacement of 125I-labeled digoxin from antidigoxin antibodies. Diuresis was selectively induced in normal healthy humans by acute water ingestion, and natriuresis was preferentially induced by acute saline ingestion. We found the amount of endogenous immunoreactivity excreted in urine to be correlated with urine flow rate but not with urinary sodium excretion. Urinary excretion of DLIF, normalized to creatinine, was 3.6-fold greater at a urine flow rate of 5.5 mL/min than at 0.5 mL/min. On the other hand, saline intake increased urine flow rate 1.9-fold and increased sodium excretion threefold, but did not affect urinary excretion of DLIF. Fractional excretion of DLIF was linearly related to fractional excretion of water. This study demonstrates that normalization of DLIF values to urinary creatinine does not make DLIF excretion independent of urine flow rate and underscores the need for information on urine flow rate when DLIF measurements in urine are being interpreted.

Endogenous digoxin-like immunoreactive factors (DLIF) are present in serum of normal human subjects (1–3), the plasma of volume-expanded animals (4, 5), and the urine of normal (3) and salt-loaded (6) healthy human subjects. Studies have shown that the concentration of these factors is increased in conditions of clinical interest including renal failure, hepatic failure, and pregnancy (7) and in pregnant women with toxemia or premature labor as compared with pregnant women without these conditions (8). The increased DLIF in serum in these conditions suggest that urinary DLIF excretion may also be affected. In fact, it has been suggested that measurement of DLIF in urine may be used to predict women at risk of premature labor or toxemia (8). A prerequisite for the interpretation of such measurements is the establishment of a valid reference interval. In fact, a reference interval for 24-h DLIF excretion has been proposed for healthy adults (3). A second application of data on urinary DLIF concentration is as an indicator of urinary DLIF excretion rate (9). Advantages of measuring DLIF in the urine are their increased concentration relative to plasma (3) and ease of urine collection. However, if urine samples are to be used for DLIF measurements it is necessary to establish the effect on DLIF excretion of commonly encountered variables affecting urinary output. One such important variable is acute fluid intake, which leads to variation in urinary flow rates. Furthermore, some of the aforementioned clinical conditions are associated with serum DLIF increases and are characterized by alterations in salt and water metabolism. DLIF isolated by immunoprecipitation from human urine induce natriuresis in rats (6). Thus there may be a relation between salt and water metabolism and urinary DLIF excretion.

As yet, no one has reported the urinary excretion of DLIF as a function of time after acute water or salt ingestion. In this present study, we use competitive displacement of labeled digoxin from antidigoxin antibodies (10) to determine the effect of varying water and salt intake on the urinary excretion of DLIF, urinary water excretion (diuresis), and urinary sodium excretion (natriuresis). Our findings demonstrate that the amount of endogenous digoxin-like immunoreactivity excreted is correlated with diuresis but not with natriuresis. They also demonstrate the importance of knowing the urinary flow rate as a prerequisite for the proper interpretation of the urinary excretion of DLIF.

Materials and Methods

Human subjects and experimental protocol. Eight volunteers ranging in age from 21 to 37 years, five men and three women, participated in the study. All had normal blood pressures; were free of exogenous medications; and had no history of cardiovascular, pulmonary, or renal disorders. Each individual followed three experimental protocols in random sequence, with at least one-day interval between protocols. The experiments differed only in the amount or type of solution consumed orally. The protocol began with an overnight (11-h) fast of no food, liquid, or smoking. Urine produced overnight was discarded. Urine produced during the hour before fluid intake was collected and its total volume recorded. Liquid was given as 1 L of 9 g/L sodium chloride solution (Travenol Laboratories, Inc., Deerfield, IL) or as 1 L of distilled water, to be drunk within 10 min, or as 30 mL of distilled water to be drunk immediately and every hour thereafter for the duration of the experiment. No other liquid, food, or smoking was allowed during the rest of the

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