Measurement of Diphenylhydantoin in 0.1-ml Plasma Samples: Gas Chromatography and Radioimmunoassay Compared

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Concentrations of diphenylhydantoin in 364 plasma samples have been measured both by radioimmunoassay and with a recently developed gas-chromatographic method, which requires only 0.1 ml of plasma per determination. There was an excellent correlation between values obtained by the two methods (r = 0.986), and in only 11 plasma samples did the results differ by more than 20%. Of the investigated samples, 105 were obtained from uremic patients. For these, an equally good agreement was obtained between the two methods. Within-assay variance was 3.1% for the immunoassay and 3.3% for the gas-chromatographic procedure. Without automatic pipetting equipment, the radioimmunoassay procedure took twice as long as the chromatographic assay, and the cost of chemicals was considerably higher. Nevertheless, the better sensitivity of the radioimmunoassay makes it of great value, especially in children, because plasma samples of 10 to 20 μl can be used.

Additional Keyphrases: Micro-scale gas chromatography • flash methylation • pediatric chemistry • uremia • epilepsy

There is an increasing need for sensitive, precise, and specific methods for measuring the concentrations of drugs in plasma, both for research purposes and for monitoring therapy (1, 2).

A radioimmunoassay for diphenylhydantoin (DPH) was developed by Tigelaar et al. (3) but was unspecific in that the main metabolite 5-(4-hydroxyphenyl)-5-phenylhydantoin was detected as sensitively as DPH. This technique also required the antiserum to be used at a final dilution of only eightfold and the lower limit of detectability was relatively high (300 μg/liter of plasma). Although adequate for clinical purposes—the range of therapeutic plasma concentrations is 10–20 mg/liter—this is a relatively low sensitivity for an immunoassay procedure. Cook et al. (4) developed an immunoassay that was specific for DPH and showed little cross reactivity with either 5-(4-hydroxyphenyl)-5-phenylhydantoin or with drugs structurally similar to DPH, such as primidone or phenobarbital. The antiserum was used at a dilution of about 14,000-fold, and the limit of sensitivity was of the order of 1 μg/liter of plasma. To evaluate the use of this radioimmunoassay in clinical practice, we decided to compare it with the gas-chromatographic micromethod used for the routine determination of plasma DPH concentrations in this hospital.

Materials and Methods

Plasma Samples

The plasma samples to be analyzed were divided into three groups:

a. Retrospective. All plasma samples coming into the laboratory for routine DPH analysis during one month were analyzed on arrival by the gas-chromatographic method. They were analyzed retrospectively by the immunoassay techniques after a mean storage time at −20 °C of 16 days. DPH in plasma is stable at −20 °C for at least three months (unpublished data from this laboratory).

This group comprised 131 plasma samples, of which 11 samples were also analyzed by both methods on a second occasion.

b. Plasmas from uremic individuals. 105 plasma samples were collected during a year and stored at −20 °C; they were from a group of six patients who were receiving DPH and who also had impaired renal function. The creatinine clearance in these patients varied between 6 and 18 ml/min. Five of these patients had received a single intravenous dose of DPH (2.0 mg/kg body wt), to explore the effect of impaired binding of the drug to plasma proteins on its distribution kinetics (5). The other patient was receiving DPH for the treatment of grand mal epilepsy.
In patients with impaired renal function, concentrations of both conjugated and unconjugated 5-(4-hydroxyphenyl)-5-phenylhydantoin in plasma are known to increase more than in patients with normal renal function (6). If the immunoassay were to measure the metabolites of DPH to any extent, then analysis of such plasma samples provides a good test for the specificity of the immunoassay (by comparison of results with those obtained gas chromatographically).

c. Prospective. In a prospective study, we assessed the practicability of the radioimmunoassay for the routine measurement of plasma DPH in a laboratory not automated for immunoassay work. During four weeks all plasma samples entering the laboratory for DPH analysis were assayed concurrently by gas chromatography and radioimmunoassay. Analyses were started at the same time of day and the time required for the complete analyses was recorded. The plasma samples (n = 128) were analyzed in batches of 20.

Radioimmunoassay of DPH

The radioimmunoassay was done as described by Cook et al. (4), with a few modifications. The affinity constant of the antiserum was $3.5 \times 10^9$ liter/mol. The plasma samples were diluted 10 000-fold in buffer before analysis and if necessary were re-analyzed on the next occasion at a 1000-fold dilution. The standard DPH samples were prepared from an alcoholic solution (1 g/liter) and diluted serially in buffer so as to give the following quantities per 0.5 ml: 2000, 1000, 500, 200, 100, 50, and 20 pg. Both standard samples and unknown plasma samples were analyzed in triplicate. In addition, two different volumes of the diluted plasmas—0.1 and 0.4 ml—were analyzed. A quality-control solution (20 mg of DPH per liter) was analyzed in each assay as above. (All concentrations of DPH mentioned in this paper refer to the free drug, rather than its sodium salt.)

The range of the standard curve at a plasma dilution of 10 000-fold enables DPH concentrations between 5 and 200 mg/liter to be measured and at a 1000-fold dilution concentrations between 0.5 and 20 mg/liter.

Gas Chromatography of DPH

We used an improved version of the method of Berlin et al. (7), a microadaptation of the principles outlined in the method by MacGee (8), including on-column methylation of DPH by trimethylaminium hydroxide and use of the internal standard 5-(4-methylphenyl)-5-phenylhydantoin. The procedure, which omits one of the extraction steps in the original procedure (7), was:

To a 100-μl sample of plasma in a 3-ml tapered glass-stoppered tube, was added 25 μl of NaH$_2$PO$_4$ (3 mol/liter) and 2.00 ml of toluene containing 2.40 μg of the internal standard. The tube was gently shaken for 5 min and centrifuged. The toluene (top) layer was transferred to a similar glass tube and 25 μl of a trimethylaminium hydroxide solution (1 mol/liter) in water/methanol (equal volumes) was added. The tube was shaken vigorously for 1 min (vortex-type mixer) and centrifuged. One microliter of the trimethylaminium hydroxide (bottom) layer was analyzed on a 1.5 m × 3 mm (i.d.) glass column packed with 3% OV-17 on Gas Chrom Q, 100–120 mesh (Applied Science Labs Inc., State College, Pa. 16801) kept at 220 °C. The injector and detector temperatures were 270 and 265 °C, respectively. The flow rates were: N$_2$, 25 ml/min; O$_2$, 200 ml/min; and H$_2$, 25 ml/min.

Under the above conditions the retention times for DPH and the internal standard were respectively 2.7 and 3.8 min (compared to 8 and 10.9 min in the original publication).

Standard curves were prepared by adding pure DPH to drug-free plasma to produce concentrations of 5, 10, 15, and 20 mg/liter. All standard samples and unknowns were processed in duplicate.

For the plasma samples from uremic patients, which generally contained low concentrations of DPH (5), 400-μl samples were analyzed and calculated from standard curves covering concentrations from 0.25 to 4 mg/liter. The lower limit of sensitivity was 0.5 mg/liter for 100-μl samples, 0.2 mg/liter for 400-μl samples.

Results

Reproducibility

The radioimmunoassay gave good repeatability in the analysis of plasma DPH concentrations. A quality control containing 20.0 mg of DPH per liter gave a mean value of 19.00 mg/liter, with a standard devia-

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**Fig. 1.** Comparison between plasma DPH concentrations as measured by gas chromatography and radioimmunoassay in the retrospective study (131 plasmas)

The dotted lines join plasma concentrations of DPH in those samples where the analyses were repeated by both methods. The open circles indicate the first analysis, and the open squares indicate the second analysis.
tion of 1.02 mg/liter, in 22 determinations. A control plasma containing 2 mg of DPH per liter gave a mean value of 2.00 ± 0.103 mg/liter (SD) (n = 8). Within-assay variance was 3.1% for the immunoassay and 3.3% for the gas-chromatographic procedure.

Correlations

Plasma DPH concentrations as measured by radioimmunoassay and by gas chromatography correlated excellently in all the three parts of the study. In the regression analyses the gas-chromatographic value was arbitrarily chosen as the “independent” variable (x-value).

Retrospective study. In the retrospective study the correlation coefficient (r) was 0.983. The equation for the line was y = 0.995x + 0.5. In 11 of the 131 plasma samples there was an apparent disparity between the DPH concentrations found by the two methods. These samples are indicated by arrows in Figure 1. The disparity was largely due to differences in the chromatographic results on the two occasions.

Uremic study. There was a good correlation between the plasma DPH concentrations obtained by the two methods (r = 0.993, n = 105). The equation of the line was y = 0.94x + 0.04 (Figure 2). The plasma samples all contained less than 5.0 mg of DPH per liter.

Prospective study. There was also a good correlation between the plasma DPH concentrations measured by the two methods in the prospective study (r = 0.986, n = 128, y = 1.02x + 0.04).

Other Considerations

Assay times and costs. The average time for immunoassay of 20 unknown plasma samples was 8.2 h (not including counting time), while the average time for determination of the same samples by the gas-chromatographic procedure was 4.6 h. Throughout the immunoassay procedure, pipetting was done by hand (using Oxford samplers). The cost of the chemicals (not including the cost of 3H-DPH and antiserum) for the immunoassay as performed was $1.50 per plasma sample (for an assay of 20 plasma samples), and the chief cost was that of liquid-scintillation fluid. The cost and time analysis of the immunoassay were based on triplicate analysis of all samples, and furthermore each unknown plasma sample was analyzed at two dilutions as described in the experimental part. The cost of the gas-chromatographic analysis for DPH was less than $0.25 per plasma sample.

Interference from other drugs. The patients from whom these plasma samples were taken were also being treated with various other anti-epileptic, sedative, and cardiovascular drugs, but in no plasma sample could any disparity between the two methods be ascribed to an interfering drug. In the retrospective and prospective studies combined, 109 plasma samples were from patients taking phenobarbital, 48 from patients receiving carbamazepine, 38 from pa-

tients receiving primidone, 25 from patients receiving ethosuximide, and 37 from patients receiving diazepam or nitrazepam. Many patients were receiving more than one drug at the same time (the number of drugs taken averaged 1.96 per patient), including such drugs as digoxin, furosemide, thiazide diuretics, propranolol, orphenadrine, chlorpromazine, thiorida-

zine, and haloperidol.

Discussion

Measurement of diphenylhydantoin is becoming increasingly important, and recent studies have shown that clinical care is improved considerably if DPH concentration is monitored (9). Most current analyses are gas chromatographic, but we have shown here that the radioimmunoassay developed by Cook et al. (4) gives results identical to those found by gas chromatography in the large majority of analyses. The few discrepancies exceeding 20% seemed to be due to nonsystematic errors rather than specificity problems. No interference has been found in either of the two assay procedures from any of the drugs usually given with DPH, nor have we found any interference from the metabolites of DPH. There was 2% cross-reaction of 5-(4-hydroxyphenyl)-5-phenylhydantoin (added in vitro) with the antiserum used in this study. The cross reactivity to other known metabolites of DPH in man has not been tested in vitro, owing to the lack of reference compounds. There was a cross reaction between 5-(3-hydroxyphenyl)-5-phenylhydantoin and this antiserum of 16%. However, 5-(3-hydroxyphenyl)-5-phenylhydantoin is a DPH metabolite in the dog, but not in man (10). It
seems unlikely, therefore, that any of the metabolites of DPH in man will interfere with the immunoassay of DPH. This is in contrast to a previous immunoassay for diphenylhydantoin developed by Tigelaar et al. (3), in which the antibody used had a 100% cross-reactivity with 5-(4-hydroxyphenyl)-5-phenylhydantoin and thus limited the value of this assay.

As done in this study, the immunoassay procedure took twice as long to perform as the gas-chromatographic assay, but if the immunoassay were in constant use an automatic pipetting station (such as the Micromedic) would result in much time saving. In routine use it should be possible to accept a smaller range for the immunoassay (e.g., 3–36 mg/liter) and thus to analyze only one volume of the diluted plasma. This would essentially halve both labor and costs.

The cost of the immunoassay was also greater than that of the gas-chromatographic procedure. It should be pointed out that the cost quoted is just for the chemicals involved (except 3H-DPH and antiserum) and does not include costs for equipment or labor. Capital costs of the immunoassay are also likely to be greater, because a liquid-scintillation counter is usually much more costly than a gas chromatograph. However, one advantage of the immunoassay is the possibility of using small (capillary-blood) samples, which makes it ideal for pediatric practice. It would also appear to be useful in pharmacokinetic studies in either animals or man where plasma concentrations of DPH of less than 0.5 mg/liter are to be anticipated.

This work was supported by Grant 04X-3902 from the Swedish Medical Research Council. The antiserum and radio-labeled diphenylhydantoin were prepared under Contract No. PH-43-NIGMS-65-1057 between the Pharmacology/Toxicology Program, NIGMS, NIH, USPHS, and Research Triangle Institute.

References