High-Pressure Liquid Chromatography and Enzyme Immunoassay Compared with Gas Chromatography for Determining Phenytoin

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We compared a gas-chromatographic method for determination of phenytoin with a high-pressure liquid chromatographic technique and with enzyme immunoassay by three instrumental procedures. More than 100 sera from patients being treated with this drug were assayed by all these techniques. The coefficient of variation was the lowest (4.0%) with liquid chromatography, but all methods gave a CV of less than 10%. The correlation coefficients for all methods exceeded 0.97 when compared to gas chromatography. Operation costs varied with the number of tests per batch, reagent costs, and operator labor costs. All assays gave comparable values for the therapeutic range, so it would be plausible to use more than one method in a situation where (e.g.) satellite laboratories handle different quantities of assays. In any of these techniques, interferences from carbamazepine, mephenytoin, phenobarbital, and primidone were negligible.

Additional Keyphrases: economics of laboratory operation • intermethod comparison • drug assay • toxicology

Gas-chromatography (GC) is a widely accepted technique for measuring phenytoin in serum, but enzyme immunoassay (EMIT) and high-pressure liquid chromatography (HPLC) are gaining acceptance in the clinical laboratory (1–4). Here, our objectives were to compare and evaluate costs, analysis time, and quantitative results of these methods. The gas-chromatographic method was used as the comparison (reference) method.3

Materials and Methods

Samples. We assayed sera from more than 100 epileptic patients, at least 50 sera being assayed in each paired statistical study. All sera were stored at -4 °C until assayed.

To check the precision of each method, we prepared quality-control specimens by adding phenytoin (Applied Science Laboratories, State College, Pa. 16801) to plasma obtained from outdated blood-bank blood.

Methods

Gas chromatography. The gas-chromatographic procedure was that of MacGee (5). Trimethylsilylimidazole hydroxide ("Methelute"; Pierce Chemical Co., Rockford, Ill. 61006) was used to methylate phenytoin on the column. For the internal standard, we used 5-methyl-phenytoin (Aldrich Chemical Co., Milwaukee, Wis. 53233). A Model 5711A gas chromatograph (Hewlett-Packard, Avondale, Pa. 1934) was used, packed with 3% OV-1 on 100/120 Gas-Chrom Q (Applied Science Laboratories) in the 1.82 m × 2 mm (i.d.) glass column. Temperature was programmed from 180 to 250 °C, at 16 °C/min. Quantitation was by peak-height analysis.

EMIT: These reagents were obtained from Syva, Palo Alto, Calif. 94304. We used three different instruments to assay phenytoin by EMIT: A Model 300N spectrophotometer (Gilford Instrument Labs., Oberlin, Ohio 44074) with a heated sipper cell and a Monroe calculator/printer modified by Syva, a Model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif. 92634) with a heated sipper cell and a Monroe calculator/printer modified by Beckman Instruments, and a Model KA-150 kinetic analyzer (Perkin-Elmer Corp., Norwalk, Conn. 06856).

The reagents from Syva were prepared according to the manufacturer's specifications when the Beckman and Gilford instruments were used (6), but the reagents had to be diluted further when the Perkin-Elmer KA-150 was used (7). The volumes delivered in the pipetting stages with the Perkin-Elmer instrument were different from those in the other two procedures, but with the appropriate dilution the final concentrations at the measuring step were the same with each instrument.

High-pressure liquid chromatography. The HPLC analysis, that developed by Majjub et al. (4), involves extracting the acidified specimen containing the internal standard (5-methyl-phenytoin) with dichloromethane and evaporating the extract under nitrogen. The residue is dissolved in the mobile-phase solvent and injected into the HPLC system with a Valco injector (Glenco Scientific, Inc., Houston, Tex. 77007). The system consists of a 25 cm × 46 mm column containing Partisil 10 (Whatman, Clifton, N.J. 07014), a Model 396 Milton Roy minipump (Laboratory Data Control, Riviera Beach, Fla. 33404), and a Model 835 spectrophotometer equipped with an 8-μl flow cell (Varian Associates, Palo Alto, Calif. 94303).

Results

Specificity. On gas chromatography the resolution of phenytoin, 5-methyl-phenytoin, and phenobarbital was complete (Figure 1).

Carbamazepine, mephenytoin, phenobarbital, and primidone did not interfere in concentrations of 10, 25, 50, and 25

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3 Nonstandard abbreviations used: GC, gas chromatography; EMIT, enzyme multiplied immunoassay technique (a registered trademark of Syva, Palo Alto, Calif. 94304); HPLC, high-pressure liquid chromatography.

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mg/liter, respectively. These drugs and phenytoin are completely resolved on the high-pressure liquid chromatography system of Majub et al. (4).

**Precision.** The liquid-chromatographic procedure gave the lowest day-to-day variation with a CV of 4.0% (Table 1). The reproducibility was determined by the analysis of a drug-supplemented serum specimen assayed on different days. All methods gave CV's of less than 10%.

**Comparison.** Results from the three enzyme immunoassay procedures and the HPLC procedure were compared with the gas-chromatographic procedure. Specimens for these comparisons were obtained from patients on phenytoin therapy; 162 specimens were assayed by gas chromatography (GC), and different groups of these specimens were assayed by the other methods. For the Gilford EMIT vs. GC: n = 53, intercept (b) = 0.05, slope (m) = 1.25, and correlation coefficient (r) = 0.97; Beckman EMIT vs. GC: n = 72, b = 0.00, m = 1.17, and r = 0.99; Perkin-Elmer vs. GC: n = 56, b = 0.05, m = 1.02, and r = 0.98; HPLC vs. GC: n = 40, b = 0.20, m = 0.90, and r = 0.97.

**Analysis time and cost.** Analysis time per specimen is based on time required for 10 patients' specimens, one control, and one standard for the chromatographic systems, and six calibrators for the EMIT systems. Three primer sera are also used at the beginning of the run with the Perkin-Elmer KA-150. Reagent preparation, sample preparation, actual technologist time, and calculation of final answer is also included. The chromatographic procedures required 15 min per sample, the manual EMIT procedures required 10 min, and the automated EMIT procedure required 2 min. By increasing the number of specimens per run, the chromatographic analysis time is not decreased greatly, and a practical limit of samples per extraction is 20. The time per sample for the EMIT procedures can be shortened greatly because additional samples require about 1 min for the manual procedures and 45 s for the automated procedure.

Reagent costs with the immunoassay procedures will also vary according to size of batch (Table 2). The number of calibrators and primers must be considered in the cost, because they account for the higher costs of the EMIT systems when fewer samples are processed.

**Discussion**

The chromatographic and EMIT procedures for phenytoin show no interferences from carbamazepine, mephenytoin, phenobarbital, or primidone. Their analytical precision (<10% CV) is acceptable.

Statistically, all methods compare favorably to the GC method, although, if several methods are used in a given institution, adjustments should be made in the values so that a single set of therapeutic values can be used. High correlation coefficients indicate that the data do fit the linear regression lines.

When determining the method to be used, one must consider instrument cost and availability, reagent cost, technical expertise available for instrument maintenance, and other analyses that can be done by the equipment. As used in this study, the costs were greater for the EMIT systems, but more technical expertise was required for the chromatographic systems. At present the equipment used for EMIT is limited as to different types of drug analysis, although this equipment has a wide range of use in clinical chemistry (8). Gas chro-
matography is a widely used instrument in toxicology (9) and high-pressure chromatography is gaining wide acceptance (10). Therefore, final selection should only be made after a thorough analysis of future needs in the clinical laboratory and estimation of the number of samples to be analyzed. Laboratories with limited budgets but the need for other toxicological analyses would best select chromatography systems, whereas other laboratories may want the advantage of speed in the EMIT systems.

References

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Semiautomated Spectrophotometry of Total Phospholipids in Plasma

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A one-stage semiautomated method for estimating total phospholipids in plasma is reported. The phospholipids are extracted into aqueous ethanol, combined with a Lewis acid, and the change in pH is monitored colorimetrically. The method, compared with the acid digestion method of Gomori [J. Lab. Clin. Med. 27, 955 (1942)], gives results that are about 0.25 mmol/liter lower, but seems to be a suitable and less hazardous alternative.

Most methods for estimating total phospholipids in plasma are based on their oxidative hydrolysis to inorganic phosphate, which is then assayed (7–3). The hydrolysis stage is often long and sometimes hazardous because concentrated acid digestion mixtures are used. I have overcome this problem in the method described here by combining an extract of the phospholipid directly with ferric chloride. Ferric chloride, an electron-deficient compound, acts as a Lewis acid in solution, accepting electrons from the hydroxyl ion and the ethoxide ion. The phospholipid replaces the anions in solution, thus increasing the pH.

I tried several solvent systems for the sample extraction and reagent solvent; those described below have proved to be the most satisfactory.

Materials and Methods

In the comparison studies I used plasma from 80 adults, ranging in age from 18 to 52 years, and in build from slim to obese. All the samples were obtained after an overnight fast, but no attempt was made to control the diet.

The instruments used in the method consisted of a Technicon Sampler II (Technicon Instruments Corp. Ltd., Basingstoke, Hants., RG21 2YE, U.K.), a peristaltic pump (Watson-Marlow Ltd., Falmouth, Cornwall, TR11 4RU, U.K.), an SP 600 spectrophotometer modified for automated analysis (Fyeunicam Ltd., Cambridge, CB1 2PX, U.K.), and a Servigor recorder (Camlab Ltd., Cambridge, CB4 1TH, U.K.).

pH was monitored by the change in absorbance at 540 nm of a methyl orange solution.

A stock solution was prepared by dissolving 1 g of methyl orange in 1 liter of distilled water and filtering the solution through coarse filter paper. The reagent solution was prepared by dissolving 0.5 g of ferric chloride (FeCl₃ anhydrous) in 300 ml of ethanol, to which was added 10 ml of dilute hydrochloric acid (0.1

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