Gas-Chromatographic Analysis for Succinimide Anticonvulsants in Serum: Macro- and Micro-Scale Methods

James Bonitati

A gas-chromatographic analysis for the succinimide anticonvulsant drugs— ethosuximide, methsuximide, and phenytoin—in 1.0 ml of serum was modified to improve its reliability, speed, and precision. A separate procedure for 10–100 μl of serum was also developed. Neither method requires an initial preparation of derivatives. The working range of each method is about 10–100 mg/liter for the macro-method, 2–100 mg/liter for the micro-method. The methsuximide metabolite, N-desmethylethosuximide, is included in both methods. Concentrations of N-desmethylethosuximide in the blood of two patients with petit mal epilepsy are reported.

Additional Keyphrases: drug assay • monitoring drug concentrations in serum • therapy for epilepsy

Several gas-chromatographic methods are available for measuring the circulating concentrations of ethosuximide (2-ethyl-2-methylsuccinimide, “Zarontin”; Parke, Davis & Co., Detroit, Mich. 48232), and the other common succinimide anticonvulsants: methsuximide (N,2-dimethyl-2-phenylsuccinimide, “Celontin”; Parke, Davis & Co.), and phenytoin (N-methyl-2-phenylsuccinimide, “Milontin”; Parke, Davis & Co.) (1–9).

Although these methods appear to be specific and accurate, they tend to be tedious, lengthy, or otherwise not adapted to the rapid manual analysis of multiple samples. For example, several methods (1–3) involve extraction with large volumes of solvent, followed by either lengthy evaporation steps or the use of bulky rotary evaporators. In other methods (4, 5) the succinimides require a long time to emerge from the chromatographic column. The method of Tse and colleagues (6) is rapid, simple, and extremely sensitive, but requires a special detector for nitrogen in the gas chromatograph.

Several liquid phases are available for use in gas-chromatographic assay of the succinimides in the free state, so that derivatives of these drugs need not be made, except in schemes that include the concurrent analysis for other anticonvulsant drugs. Furthermore, methsuximide is rapidly N-demethylated in the body to N-desmethylethosuximide (7), a product that appears to be as strongly anticonvulsive as the parent compound (7). Methylation in the gas chromatograph would thus tend to complicate the measurement of methsuximide in the blood.

The method of van der Kleijn et al. (8, 9), which includes a single chloroform extraction and does not involve derivative formation, appears to offer a simple and rapid alternative to the foregoing methods. A shortcoming of this procedure, however, is the difficulty and uncertainty of obtaining sufficient serum extract in the final centrifugation step, because sera emulsify to various extents during the extraction step. Also, the syringe may become clogged when drug extract in the bottom phase is retrieved through the emulsified top phase. Finally, precision is decreased at higher concentrations of the drugs.

I have modified the method of van der Kleijn et al. for 1.0 ml of serum, to increase its reliability, speed, and precision. A scaled-down procedure for use with 10–100 μl samples is also presented. N-Desmethylethosuximide, the methsuximide metabolite, is measured in both procedures, and its concentrations in the blood of two patients are reported.

Materials and Methods

Reagents

Chloroform. Analytical reagent grade.
Phosphate buffer. Saturated aqueous solution of KH₂PO₄.

---

Neurochemistry Division, Harry M. Dent Neurologic Institute, Millard Fillmore Hospital, 3 Gates Circle, Buffalo, N. Y. 14209.
Received Nov. 3, 1975; accepted Dec. 22, 1975.
Standards

Pure samples of ethosuximide, methsuximide, and phensuximide were gifts from Parke, Davis & Co. N-Desmethylmethsuximide was a gift from Dr. A. J. Atkinson, Jr., Northwestern Memorial Hospital, Chicago, Ill.

The internal standard was fluorene (o-biphenylene-methane), obtained as an analytical-grade reagent from Eastman Kodak Co., Rochester, N. Y. 14650.

Ethosuximide standard solution. An aqueous 500 mg/liter solution.

Mixed drugs standard solution. Ethosuximide, methsuximide, phensuximide, and N-desmethylmethsuximide, each 500 mg/liter of water.

Internal standard solutions. Stock solution: fluorene, 625 mg/liter (500 μg/800 μl) in chloroform. Macromethod standard: fluorene, 62.5 mg/liter (50 μg/800 μl). Dilute the stock solution 10-fold with chloroform. Micromethod standards: Two standard solutions were used: 5 μg/800 μl, and 1 μg/800 μl, for the assay of 50 μl and 10 μl of serum, respectively. These solutions are prepared by diluting the macro-method standard 10-fold and then fivefold with chloroform. All these solutions should be stored refrigerated.

Apparatus

A Cliniscreen gas chromatograph equipped with dual flame-ionization detectors (Beckman Instruments, Inc., Irvine, Calif. 92664) was used for chromatography. The column was a 185 cm glass U-tube (2 mm i.d.) packed with 5% OV-17 liquid phase on 100–120 mesh Gas Chrom Q (Applied Science Laboratories, Inc., State College, Pa. 16801). Temperature settings were: injector port, 150 °C; detector line, 250 °C; detectors, 275 °C. Two oven temperature modes were used: isothermal heating at 180 °C for samples containing ethosuximide only, and temperature programming for multiple drug analysis: 180 °C for 1.5 min, increased to 230 °C at a rate of 10 °C/min. The carrier gas (helium) flow rate was 50 ml/min. Gas flows (ml/min) to the detectors were: air, 300; hydrogen, 45; and helium, 50. Instrument settings were: range, 1000; recorder attenuation, 1; detector suppression, 3. The response range of the 255-mm (10-inch) chart recorder was 1 mV full scale; chart speed was 12 mm (0.5 inch)/min.

Procedures

Macro-volume method. To 1.0 ml of serum in a 12-ml conical centrifuge tube add 1.0 ml of phosphate buffer, followed by 800 μl of internal standard solution (delivered with a Pipetman adjustable microliter pipet, 1000-μl model; Rainin Instruments Co., Inc., 1030 Commonwealth Ave., Boston, Mass. 02215). briskly shake on a vortex-type mixer (two tubes can be mixed at one time), and then centrifuge at 1000 × g for 5 min. Aspirate the top layer and inject 2 μl of the clear bottom layer into the gas chromatograph. Ethosuximide samples should be chromatographed isothermally. For mixed-drug samples use the temperature-programmed mode.
Prepare calibration curves for the drugs in the concentration range 25–100 mg/liter from data on extracts of drug-free pooled serum to which standard solutions are added. Plot peak-height ratios of drug to internal standard vs. drug concentration. Prepare two calibration curves, one for ethosuximide, another for the drug mixture.

Samples containing less than 25 mg of drug per liter can be assayed by concentrating a portion of the extract to a small volume (5–20 μl) at 35 °C in a stream of air.

**Micro-volume method.** In this method, 10–100 μl of serum can be assayed for ethosuximide alone, and 50–100 μl for mixed drugs. Transfer 0.1 ml of phosphate buffer to a 12-ml conical centrifuge tube. Deliver 10–100 μl of serum for the determination of ethosuximide or 50–100 μl of serum for the determination of mixed drugs. Add 800 μl of internal standard; for 10-μl samples use the 1 μg/800 μl internal standard solution and for 50–100 μl samples use the 5 μg/800 μl solution. Mix and centrifuge as in the macro-method. Remove the top layer and, with Pasteur pipets, transfer the bottom extract to clean centrifuge tubes. Evaporate the samples to 5–20 μl at 35 °C in a stream of air. Inject 1 μl of sample into the gas chromatograph.

Prepare calibration curves for the micro-volume method from data on serum containing drugs in the concentration range 25–100 mg/liter, as in the macro-method.

**Results**

**Analytical Variables**

**Chromatography and calibration.** Figure 1 shows isothermal chromatograms of ethosuximide and fluorine in serum extracts. The fact that ethosuximide elutes almost with the solvent front does not interfere with quantitation. The ethosuximide peak is more sharply defined in the micro-method however, because the solvent peak is smaller than it is in the macro-method. Figure 2 shows calibration curves for extracts of ethosuximide from serum, by both methods.

On isothermal chromatography of methsuximide, phenytoin, and N-desimethylmethsuximide, the peaks representing these compounds were broad, with relatively long retention times. Temperature programming produced sharp, well-separated peaks, with shorter retentions (Figure 3). The initial isothermal segment of the temperature program allows ethosuximide to be assayed in combination with the other drugs. Figure 4 shows mixed drug calibration curves for the macro- and micro-methods. Separate calibration curves are required for isothermal and programmed modes, because the elution temperature of the internal standard differs in the two cases.

**Precision.** Analytical precision within-run was calculated from results of 10 successive assays of the same serum sample. Day-to-day precision was calculated from at least 20 measurements during a 30-day period.

---

Fig. 3. Temperature-programmed gas chromatography of succinimide anticonvulsants in serum extracts

(left) macro-method. Analysis of 1.0 ml of serum containing 100 mg each of mixed drugs per liter. (right) micro-method. Analysis of 50 μl of serum containing 100 mg each of mixed drugs per liter. 1, ethosuximide; 2, internal standard; 3, methsuximide; 4, phenytoin; 5, N-desimethylmethsuximide

Fig. 4. Calibration curves for succinimide anticonvulsants in serum by temperature-programmed gas chromatography

(upper) micro-method. 50-μl volumes of serum standards were analyzed. (lower) macro-method. 1.0-ml volumes of serum standards were analyzed. dark circles, ethosuximide; light circles, methsuximide; dark squares, phenytoin; light squares, N-desimethylmethsuximide
Within-run precision (CV) of macro- and micro-methods for ethosuximide by isothermal chromatography was 4% and 6%, respectively; day-to-day precision was 5% and 8%, respectively.

Within-run precision for temperature-programmed chromatography of mixed drug standards in serum by the macro- and micro-methods was 5% and 7%, respectively, for all drugs; day-to-day precision was 6% and 10%, respectively, for all drugs.

**Sensitivity.** Analytical sensitivity, expressed as the minimal concentration of drug detectable in serum, was 10 mg/liter for ethosuximide and N-desethylmethsuximide, and 5 mg/liter for methsuximide and phensuximide, in the macro-method. In the micro-method, depending on the serum volume (10–100 μl) assayed, the sensitivity was 2–10 mg/liter for ethosuximide and N-desethylmethsuximide, and 1–5 mg/liter for methsuximide and phensuximide.

The sensitivity of the macro-method can be increased by transferring serum extract to a clean centrifuge tube and evaporating to 5–20 μl before injection into the gas chromatograph. Under these conditions the internal standard peak will be very large compared with the drug peaks and must be selectively attenuated during chromatography. Calibration curves for analysis of 2.5–10 mg/liter of drugs per liter of serum were linear, and they were continuous with the 25–100 mg/liter calibration curves in Figures 2 and 4.

The sensitivities of both the macro- and micro-method can be increased by injecting larger quantities of sample extract into the gas chromatograph, except for ethosuximide, because its peak is obscured by the increased size of the peak representing the solvent under these conditions.

**Analytical recovery.** Extraction efficiencies for the macro-method, measured by comparing extracted serum standards with standard chloroform solutions of the drugs, were 49.5% for ethosuximide, 67% for N-desethylmethsuximide, and >90% for methsuximide and phensuximide.

In the micro-method recoveries exceeded 90% for all drugs.

**Specificity.** In concentrations of 1.0 mg/liter none of the following commonly used anticonvulsant drugs interfered with the present methods: diphenylhydantoin (Dilantin), phenobarbital, primidone (Mysoline), and carbamazepine (Tegretol). These drugs are not eluted from the gas-chromatographic column under the present conditions of chromatography.

**Internal standards.** Two other compounds besides fluorene were examined as internal standards for the assay procedures. The succinimide derivative, 2,2-dimethyl-3-methyl-succinimide, did not separate from ethosuximide in the present chromatographic system. With naphthalene as internal standard, analytical precision was poor. Furthermore, the high chromatographic mobility of this compound required the use of low flow rates of carrier gas, which greatly lengthened the chromatographic runs, and also resulted in broadened drug peaks.

![Fig. 5. Gas chromatography of serum extracts from epileptic patients on long-term methsuximide or phensuximide therapy](image)

**Results for Patients on Methsuximide and Phensuximide**

Figure 5 shows tracings for sera from two patients with petit mal epilepsy who were on long-term anticonvulsant therapy with ethosuximide together with either methsuximide or phensuximide. Only ethosuximide and phensuximide were detected in the serum of the patient receiving these drugs. In the patient receiving ethosuximide and methsuximide, however, the methsuximide peak was absent, but a peak for its metabolite, N-desethylmethsuximide, appeared, together with the peak for ethosuximide. This is in agreement with the observations of Strong et al. (7), who found, by using a highly sensitive fragmentographic method, that N-desethylmethsuximide concentrations in serum were about 700-fold greater than methsuximide concentrations in methsuximide-treated patients. In line with this, Glazko and Dill (3) observed large increases in liver N-demethylase activity of rats that had received long-term treatment with methsuximide.

**Discussion**

**Method modifications.** The present macro-method, though based on a procedure of van der Kleijn et al. (9), differs from it as follows. The extraction solvent volume is increased from 0.1 ml to 0.8 ml, to
eliminate emulsification problems and ensure an ample quantity of extract. The larger solvent volume permits removal of the upper emulsified layer after extraction and centrifugation, thus providing a clear extract for sampling without the complication of syringe clogging. With the elimination of serum emulsification and syringe clogging, assays could be performed faster and with greater reliability.

A new internal standard, fluorene, was used. Use of lower injector port temperature (150 °C vs. 200 °C) increases the precision of the measurements in the present system. For example, repeated injections of the same sample of ethosuximide (100 mg/liter) and internal standard (50 mg/liter) at the two temperatures gave analytical precisions (CV) of 2% and 14%, respectively.

A further difference in assay conditions was the use, in the present methods, of a higher concentration of liquid phase in the chromatographic column—5% instead of 3% OV-17.

In the micro-method introduced here the volume of serum required for analysis is reduced by 10 to 100-fold compared with the macro-method, with only slight loss in precision.

Therapeutic concentrations in serum. The therapeutic concentrations of the succinimide anticonvulsants in serum are not yet firmly established. Tentative values are: 40–80 mg/liter for ethosuximide (10), and 20–30 mg/liter for methsuximide, expressed in terms of N-desmethylmethsuximide (7). These values fall within the scope of the assay methods presented here. Therapeutic values for phenusuximide are still under investigation.

Patients' sera were provided by W. R. Kinkel, M.D., and Mrs. R. Ekes.

References