Analysis of Six Anticonvulsant Drugs using Solid-Phase Extraction, Deuterated Internal Standards, and Gas Chromatography–Mass Spectrometry

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Abstract

A rapid method for simultaneously determining the anticonvulsant drugs carbamazepine, ethosuximide, phenobarbitone, phenytoin, primidone, and valproic acid is described. Blank plus single-point calibration gives reliable quantitation from therapeutic to high fatal concentrations, except for ethosuximide, for which it gives semiquantitative results. Whole blood and liver tissue samples containing deuterated internal standards were extracted using Bond Elut Certify columns. Butyl derivatives were formed using n-iodobutane and TMAH under mild conditions and were extracted into ethyl acetate as a cleanup step. Recoveries were greater than 50%, except for valproic acid (42%). Sample preparation time was less than 2 h, and the GC run time was less than 20 min per injection. At least two ion pairs formed by electron impact ionization were monitored for each drug. Intraday CVs were less than 6.28% (4.20%) and interday CVs less than 14.1% (for midtherapeutic concentrations in blood [liver], except for ethosuximide). Linearity was observed from subtherapeutic to high fatal levels for all drugs. This method has been applied to forensic cases and has significantly reduced analytical time while improving case-work quality. Results of a case study involving anticonvulsant drugs are given.

Introduction

Carbamazepine (CBZ), ethosuximide (ESM), phenobarbitone (PB), phenytoin (PHT), primidone (PRM), and valproic acid (VPA or sodium valproate NaVP) are some of the most widely used drugs for the control of the symptoms of epilepsy, and measurement of their concentrations in biological fluids is required both in clinical situations and in cases of a forensic nature.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamazepine</td>
<td>4–12 μg/mL</td>
</tr>
<tr>
<td>Ethosuximide</td>
<td>40–100 μg/mL</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>2–30 μg/mL</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>10–20 μg/mL</td>
</tr>
<tr>
<td>Primidone</td>
<td>5–12 μg/mL</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>40–100 μg/mL</td>
</tr>
</tbody>
</table>
Amberlite™ resin), with methyl derivative formation and flame ionization detection (FID), whereas Werner et al. (3) used diatomaceous earth (kieselguhr) as a solid-phase extraction (SPE) sorbent, with FID detection of the underivatized drugs. The use of these sorbents was not however extended to ESM and VPA. Several liquid–liquid extraction methods for the same four drugs have been reported (4–7), some including formation of methyl derivatives. A range of GC detectors were used.

Other liquid–liquid extraction methods also incorporate the more volatile drug ESM (8–12), but a literature search revealed that the only liquid–liquid extraction method designed to account for all six of these drugs is that described by Sengupta and Peat (13). This method is not without problems, however; packed columns were used, and VPA had to be measured using a different column and detector (FID) from the other drugs (alkali FID). The first truly simultaneous analysis of the six drugs in serum was reported in 1990 by Volmut et al. (14), who used Silipor C18 SPE sorbent and FID detection of the underivatized drugs. This method was intended for clinical use, and linearity was only established from 10 to 100 μg/mL for each drug—a suitable range for therapeutic monitoring of ESM, PHT, and VPA—but it does not cover the full range of therapeutic concentrations for the other drugs (Table I), and it certainly does not cover the range of concentrations in forensic samples, which vary from subtherapeutic to high fatal levels.

The aim of this work was therefore to develop a method suitable for the analysis of forensic samples (in particular whole blood and liver), which would meet the requirements of specificity, precision, and sensitivity. It was also highly desirable that this method should be economical (in terms of both time and money) and use similar techniques and analytical instrumentation to those already available in the ESR laboratory.

The best choice of analytical instrumentation to meet these requirements would be GC–MS. EI-MS, the most common variety of mass spectrometer used, results in highly specific “fingerprint” spectra for all of the drugs of interest. Use of deuterated internal standards results in good precision, with a linear range extending from low therapeutic to high fatal concentrations.

**Experimental**

**Materials**

Carbamazepine was obtained from CIBA, ethosuximide and phenytoin from Parke-Davis (Morris Plains, NJ), phenobarbitone from Rhone-Poulenc (Clayton, South Victoria, Australia), primidone from ICI (Auckland, New Zealand), and sodium valproate from Reckitt and Colman. Deuterated carbamazepine-d10, phenytoin-d10, and valproic acid-d4 were purchased from Scientific Technology (Canberra, Australia), and deuterated phenobarbitone-d3 and primidone-d4 were purchased from Radian Corp. (Austin, TX) and Cambridge Isotope Laboratories (Andover, MA), respectively. No deuterated analogue is currently available for ethosuximide.

Nano grade acetone, hexane, methanol, and ethyl acetate were obtained from Mallinckrodt (Paris, KY). Pronalys ethanol was obtained from Rhone-Poulenc Laboratory Products.

Butyl iodide was prepared via the Finkelstein reaction; 10 mL of butyl bromide (Unilab, Lichfield, England), 40 mL of dry acetone, and 20 g of sodium iodide (BDH, Poole, England) were stirred at room temperature overnight. The mixture was filtered, and the filtrate was washed with two 200-mL aliquots of distilled water, the lower organic layer being retained each time. The organic layer was then shaken with 200 mL of distilled water containing a single crystal of sodium thiosulfate, until colorless. A small amount of anhydrous sodium sulfate (Univar, Ajax Chemicals, Auburn, NSW, Australia) was swirled in the organic phase until the product lost the cloudy appearance (because of the presence of water). The butyl iodide product was stable when stored in a foil-covered bottle at 4°C.

Common chemicals used were AR grade or higher. Alcalase (type 2.34L) for liver digestions was obtained from Unilever (Petone, New Zealand, imported from Novo Industri A/S, Copenhagen, Denmark).

Bond Elut Certify columns (Cat. No. 1210-2051) were obtained from Varian (Harbor City, CA).

Whole blood samples were stored at 4°C and contained 2% sodium fluoride and 0.3% potassium oxalate as preservative and anticoagulant, respectively. Blank blood was obtained from a staff member, and blank human liver was from previous case samples.

Glass tubes (100 × 13-mm, Corning, Alexandria, Australia) were vapor silicaized using dimethylchlorosilane and then treated with anhydrous methanol. Screw caps contained Teflon liners.

**Apparatus**

A Hewlett-Packard (HP) 5890 series II GC with an HP 5970 mass spectrometric detector (MSD) was used. A 12-m × 0.15-mm.i.d. fused-silica capillary column with a cross-linked (5%) phenyl)-methylpolysiloxane coating of 0.4-μm thickness (SGE, Austin, TX, BPX5) was used, with splitless injection of 3-μL samples. Injection port and detector temperatures were both 280°C, and the initial oven temperature was 80°C for 1 min, then increased to 300°C at...
20°C/min. All of the drugs eluted within 20 min. Glass injection port liners were silanized, and contained a small plug of silanized glass wool. DOS ChemStation software was used with a quantitation database to detect and integrate target peaks and to calculate and compile results into a database. At least two ion pairs were monitored for each drug (Figure 1), with the exception of ESM, for which no deuterated analogue is available. The 189 ion of PHT-d10 was used as the internal reference for ESM.

A Cole Parmer (Vernon Hills, IL) 8850 ultrasonicator was used to sonicate all standard solutions and spiked samples before use.

**Standard solutions**

A drug standard containing all six anticonvulsants was prepared in ethanol from stock solutions (1 mg/mL), which in turn were prepared from the powders supplied. The mixture was evaporated under a warm nitrogen flow and made up to about 1.6 mL of 1M sodium dihydrogen orthophosphate solution (resulting in a pH of 4.5) and 100 µL of the internal standard mixture. The mixture was sonicated for 10 min and then centrifuged at 2000–3000 × g for at least 5 min.

Bond Elut Certify columns were conditioned with 2 mL of methanol, followed by 2 mL of the 1m phosphate solution, without allowing the column to run dry. The blood sample was applied and drawn through in not less than 2 min. Two washes were applied, 1 mL of 1M sodium dihydrogen orthophosphate and 1 mL of 0.01M acetic acid, and the column was then dried by drawing air through for 5 min. Elution was achieved with 2 mL of acetone.

The extract was butylated by adding 100 µL of 2.5% tetramethyl ammonium hydroxide (TMAH, in MeOH) and then 50 µL of butyl iodide to the eluent (adapted from the method of Meatherall (15)). This cloudy mixture was heated for 5 min at about 65°C, then evaporated under nitrogen at about 35°C to 200–500 µL. Complete drying was avoided to prevent loss of the volatile valproate derivative. Saturated NaCl solution (1 mL), 150 µL of 0.1M bicarbonate buffer, and 200 µL of ethyl acetate were added; and the mixture was vortex mixed at a high speed for 2 min, then quickly centrifuged to help separate the organic layer. The top (ethyl acetate) layer was transferred to an autosampler vial and analyzed using the conditions described in the Instrumentation section.

**Liver extracts.** The liver digest was prepared by adding 10 g of finely chopped liver to 40 mL of 1M Tris buffer adjusted to pH 10.5, and 400 µL of alcalase (Type 2.34L) (Adapted from the method of Dickson et al. (16)). This mixture was stirred at about 40°C for at least 1 h before use. Samples (0.2 mL) were treated similarly to blood, although with some slight changes. The digestion step results in an effective 1:5 dilution of the liver, so a lower range of calibrators (and smaller amount of internal standard) were required. Because the final pH of the digest was about 9, 100 µL of 1M orthophosphoric acid in addition to the 1M sodium dihydrogen orthophosphate was added before the extraction, resulting in a pH of about 4.5. The mixture was sonicated for 10 min and centrifuged at 2000–3000 × g for at least 10 min, whereupon a scum formed on the surface of the liquid. Avoiding the scum layer, the sample was applied (in not less than 2 min) to conditioned (as described for blood samples) Bond Elut Certify columns. Column washing and elution, derivatization, and cleanup were as described for blood samples. Because of the dilution effect of the Tris buffer, a smaller volume of ethyl acetate (e.g., 100 µL) was often used in the final cleanup extraction, and although not tested, a larger volume of liver digest (and buffering solutions) could be used to achieve a further increase in sensitivity.

**Nonextracted samples.** Calibrators were prepared directly from ethanolic standards, avoiding the extraction step. Internal standard (100 µL) and 100 µL of TMAH were added to 20 µL of the six drug-standard solution (or 20 µL of a 1:5 dilution of this standard if calibrating for liver sample analysis), and the solution was taken almost to dryness (100–200 µL of liquid remaining) using a nitrogen flow at about 35°C. Acetone (2 mL) and another 100 µL of TMAH were added, followed by 50 µL of butyl iodide, and the mixture was heated at about 65°C for

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**Analytical procedure**

Because of the excellent linearity obtainable with deuterated internal standards and the reproducibility between whole blood, liver, and nonextracted calibrators, a calibration of the assay using a zero (blank matrix with internal standard) and a single-point nonextracted calibration standard was found to be acceptable.

Sensitivity and chromatographic characteristics of these weakly acidic (except CBZ, which is neutral) drugs can be improved by derivatization. A range of derivatization techniques were trialed using methyl-, propyl-, butyl-, trimethylsilyl-, and tert-butyl dimethylsilyl- derivatizing reagents. Of these, butylation was found to be the best option. Silylation reagents gave unreproducible results for PB, and of the alkylation methods attempted the butylation procedure chosen gave the cleanest samples, with the best resolution of chromatographic peaks.

Although many of the analyses in the ESR laboratory are still performed using liquid–liquid extraction methods, SPE is increasingly used because of the cleaner samples obtained, good reproducibility, and the ability to automate the extraction procedure. Bond Elut Certify II SPE cartridges were already in use in our laboratory and are compatible with several commercially available “robots” (e.g., the Zymark Rapid Trace Workstation). In the present method, Bond Elut Certify columns, which combine a C18 sorbent with a cation exchange phase, were used.

**Blood extracts.** Screw-cap tubes were rinsed with hexane and allowed to dry. A sample of whole blood (0.2 mL) was added to 1.6 mL of 1M sodium dihydrogen orthophosphate solution (resulting in a pH of 4.5) and 100 µL of the internal standard mixture. The mixture was sonicated for 10 min and then centrifuged at 2000–3000 × g for at least 5 min.

Bond Elut Certify columns were conditioned with 2 mL of methanol, followed by 2 mL of the 1m phosphate solution, without allowing the column to run dry. The blood sample was applied and drawn through in not less than 2 min. Two washes were applied, 1 mL of 1M sodium dihydrogen orthophosphate and 1 mL of 0.01M acetic acid, and the column was then dried by drawing air through for 5 min. Elution was achieved with 2 mL of acetone.

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were measured on the same occasion as the five-point blood cal-

culated, and this curve was used to calculate results and CVs for

the intraday variation measurement. The calibration curves obtained were compared to those for nonextracted standards (seven points plus blank) and spiked liver digests (three samples plus blank).

Visual examination of matrix and nonextracted standard curves did not reveal any significant differences.

Intraday variations. Samples of blank blood and liver digest (5 mL) were spiked at three different levels, corresponding to half the lower therapeutic limit, a mid therapeutic level, and a fatal level. At least eight replicates of each spiked blood sample were measured on the same occasion as the five-point blood calibration, and this calibration curve was used to calculate results and CVs.

The spiked liver digests were analyzed on the same occasion as the seven-point nonextracted calibration curve was measured, and this curve was used to calculate results and CVs for at least nine replicates at each of the three levels.

Interday variations. The remainder of the spiked blood from the intraday variation measurement was subsampled on five separate days, and analyzed using a single nonextracted calibrator and a blank blood sample for calibration. CVs were again determined at each of the three levels.

Results and Discussion

Method development

As discussed, butylation was found to be the best derivatiza-
tion option because it generally resulted in stable derivatives and gave the cleanest samples, along with the best resolution of chromatographic peaks. The butylation reaction is essentially complete within 5 min, and only a small increase in peak areas was observed after a 15-min reaction time. Butyl derivatives were stable over a period of many days and gave improved chromatographic peak shapes and areas (see Figure 1).

Stable butyl derivatives were formed for all compounds but CBZ. Under normal GC operating conditions CBZ breaks down in the injection port and forms iminostilbene and 9-methyl-acridine. Hence, Burke and Thénot in their 1985 review (17) divided analytical methods for CBZ into three categories, those where steps are taken to avoid this breakdown, where stable derivatives are formed, and where conversion to iminostilbene (the analyte) is complete. By using a mild injector temperature (240°C) and an empty injection port liner, Volmut et al. (14) took the first approach and avoided CBZ breakdown, but here we have used the third method. Because CBZ was not butylated, the conditions were adjusted to maximize the formation of the iminostilbene: injector port temperature of 280°C, and glass wool packing in the injection port liner. Although conversion was not complete, the use of a chemically identical deuterated standard was expected to account for any variability in the iminostilbene formation.

Primidone formed both a mono- and di-substituted product. The di-substituted derivative was chosen as the target peak because it was usually larger in magnitude.

Figure 1 shows a sample chromatogram (total ion current of all selected ions, see Figure 2) from a blank blood sample spiked with drugs, the sample was prepared as described in this paper. The inset figures show the peak shape for the base ion and relevant IS ion of each drug (the base ion plus second most abundant ion are shown for ESM). Other ions are not shown, as there was little variation in peak shape between ions, extracted and nonextracted samples, or spiked samples and genuine case samples.

Choice of ions

For each drug/deuterated analogue pair, at least two ion pairs were monitored. When possible, the base ion pair and the molecular ion pair were used, but in some cases these were not free from interference. The mass spectra for the drug derivatives are shown in Figure 2. The chosen ions are labeled, and the marked ions are those giving the best CVs, that is, those having the best reproducibility and least interference. We have not observed any significant interference with the ions indicated; however, the figure shows that there are other ions that could be monitored if required.

Figure 2. Mass spectra of the butyl derivatives. The labeled peaks are those which were routinely monitored; peaks giving rise to the best CVs are marked with an asterisk.
Validation

Intraday variations. Results of the intraday variation determination are presented in Table II. For the five drugs except ESM, CVs are all below 15%.

The differences in the results for ESM clearly indicate the benefit of a deuterated internal standard. The blood intraday CVs are reasonable because calibration was performed using blood-extracted standards. However, for the liver digest samples, CVs are much higher, as a result of the difference in the behavior of ESM and PHT-d10 (the internal standard used) between extracted and nonextracted samples.

Interday variations. Samples for interday variation determination were measured using two-point calibration curves, consisting of a blank and a nonextracted standard. Although the use of a nonextracted standard is considered somewhat unusual, it is simpler and quicker to prepare than an extracted standard, and coupled with the use of deuterated standards, it gives acceptable precision for all of the analytes at therapeutic concentrations and higher. Because linearity has been demonstrated for up to midfatal concentrations, we believe that use of the nonextracted calibrator with a deuterated standard can be confidently used for analyte concentrations within the therapeutic to midfatal range.

### Table II. Validation Results

<table>
<thead>
<tr>
<th>Drug</th>
<th>Approx. recovery</th>
<th>Linear range</th>
<th>Target concentration</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>blood</td>
<td>blood liver</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(µg/mL)</td>
<td>(µg/mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(r²)</td>
<td>(µg/mL)</td>
<td></td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>158%</td>
<td>0.5-60</td>
<td>5 1 0.25 9.14 154 2.99</td>
<td></td>
</tr>
<tr>
<td>Ethosuximided</td>
<td>60%</td>
<td>10-300</td>
<td>20 4 8.66 32.4 32.4</td>
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<tr>
<td>Phenobarbitone</td>
<td>91%</td>
<td>0.5-240</td>
<td>1 0.25 2.25 2.74 2.74</td>
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<tr>
<td>Phenobarbitone</td>
<td>66%</td>
<td>0.5-60</td>
<td>1 0.5 9.31 24.3 24.3</td>
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</tr>
<tr>
<td>Primidone</td>
<td>49%</td>
<td>0.5-80</td>
<td>1 0.5 8.71 23.4 23.4</td>
<td></td>
</tr>
<tr>
<td>Valproic acid</td>
<td>42%</td>
<td>5-800</td>
<td>10 2.5 5.30 5.30 5.30</td>
<td></td>
</tr>
</tbody>
</table>

- Approximately recovery from the extraction step was calculated by comparing the peak areas of extracted and nonextracted standards. The value for carbamazepine indicates that the breakdown product was formed to a larger extent in the extracted standard, but does not reliably represent the extraction efficiency.
- The linear range is given (in µg/mL) and beneath it the r² value obtained for a linear fit of the calibration data incorporating five points plus a blank.
- The target concentration is the level of the drug, added to blood or liver digest. The levels used correspond roughly to half of the lower therapeutic limit, midtherapeutic, and midfatal concentrations. Very few data were available on liver concentrations, so it was assumed that liver and blood concentrations are similar, and the liver digest concentration is hence lower by a factor of 5 because of the volume of fluid added during the digestion process.
- Liver concentrations are micrograms per milliliter of liver digest.
- Ethosuximide concentrations were calculated using the phenytoin-d10 189 peak as the internal reference.

In general, interday CVs are higher than intraday: at subtherapeutic levels the CV for PRM is over 20%, and for CBM it is 154%. This result seems to suggest some difference in the formation of iminostilbene and iminostilbene-d5 on different occasions, contrary to expectations. The effect only becomes pronounced at low levels, however, and may possibly be overcome by use of a blood-extracted calibrator.

The CVs for ESM are broadly similar to those from the intraday liver digest determination, which suggests that their magnitude is probably due to the use of a nonextracted calibrator and is not significantly different for intraday and interday measurements.

Case study

The deceased, a 41-year-old male epileptic, returned home from an evening out at about 11:00 p.m. He went to bed at approximately 11:30 p.m. At approximately 8:45 a.m. on the following day, the deceased was discovered dead in his bed. He was lying on his stomach with his face buried in his pillow; there was a small amount of dried blood on the pillow. Over the previous month, the frequency of his seizures had increased. He was subject to frequent check-ups by his doctor; the last one being three days prior to his death. He had been taking regular medication including carbamazepine, clozapine, paracetamol, and valproic acid.

Carbamazepine was determined to be at a low therapeutic level, and valproic acid was at well below therapeutic levels. Death was considered to be due to an epileptic seizure.

Results of analysis were as follows: carbamazepine in blood (cardiac), 5 µg/mL and valproic acid in blood (cardiac), 11.3 µg/mL.

Conclusions

A sensitive and specific method for the simultaneous determination of the anticonvulsants drugs carbamazepine, ethosuximide, phenobarbitone, phenytoin, primidone, and valproic acid in whole blood and liver samples has been presented. This rapid assay gives acceptable precision for analytes with deuterated internal standards, at therapeutic and higher levels, although precision is reduced at subtherapeutic levels. The methodology is general enough to be adapted to other drugs, and the application to and validation of the method for paracetamol will also be reported (18).

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References


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