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Note

Rapid analysis of valproic acid by gas chromatography

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Several methods are described in the literature for the analysis of plasma valproic acid. These include gas chromatography (GC) [1—13], high-performance liquid chromatography (HPLC) [14—16], enzyme immunoassay (EMIT) [17], and fluorescence immunoassay [18]. Although a number of methods are available, certain problems exist, such as the assays being either time-consuming, expensive, non-specific, insensitive and/or difficult to conduct. Two major characteristics of valproic acid which have restricted methods development are its lack of ultraviolet (UV) absorbance at higher wavelengths (> 235 nm) and its high volatility as the free acid. Because of this lack of UV absorption, HPLC methods which were developed, required derivatization to a phenacyl ester before being detected at 246 or 247 nm [14, 15]. A sensitive HPLC method based on UV detection at 210 nm was developed but this method requires a controlled evaporation step during specimen preparation [16]. While many of the methods are effective, they require more time to conduct than is desirable.

Other methods employ GC or enzyme immunoassay (EMIT) techniques which are not as time-consuming, but which are not as reproducible. A critical comparison of GC and EMIT methods has been reported recently showing that although the two methods produced equivalent results for control samples, there was a highly significant proportional error of 26% when the same patient sample was split and analyzed by both methods [19]. The consistently higher values which were obtained by EMIT were probably due to cross reactivity of some unsaturated metabolites of valproic acid which reportedly interfere with the enzyme immunoassay. Among the GC methods, ethyl acetate [10] and isoamyl acetate [20] have been added to the extracted free acid prior to concentration by evaporation to minimize volatilization of valproic acid. To circumvent concentration by evaporation, several methods [2, 3, 5, 7, 9, 11, 12] use a small volume of extraction solvent such as chloroform, dichloromethane, ethyl acetate, or carbon disulfide. In other methods, the efficiency of extraction is increased by adding saturated potassium dihydrogen phosphate to the plasma prior to extraction [8, 12]. Even in another method, extraction has been eliminated [13], but this method employs electron capture which does not require the concentrations needed for flame ionization detection. Reproducibility has consistently been a problem in the analysis of valproic acid in plasma using methods which employed small volumes of organic solvents for the extraction of free acid. The use of chloroform or dichloromethane for extraction has been previously evaluated [9] and causes undesirable complications such as formation of emulsions, high background and residue deposition on the flame ionization detector.

Because a rapid, sensitive, yet easy, method is required for the day-to-day analysis of valproic acid, the present phenacylbromide derivatization method was developed.

MATERIALS AND METHODS

Pure valproic acid was obtained from Saber Labs. A stock valproic acid standard (9.05 mg/ml) was prepared by diluting 100 μl of pure valproic acid to 10 ml with 0.2 M ammonium hydroxide. Working valproic acid standards were then prepared by diluting 55.3 μl of the stock valproic acid to 10 ml with drug-free plasma. Aliquots (0.3 ml) of this working solution were stored at $-20°$C and used as required. Such solutions are stable for at least two months. Standard regression lines were obtained by the analysis of standards containing 200, 100, 50, 25, 10 and 5 μg/ml valproic acid in drug-free plasma.

Valproic acid controls

Control serum specimens of valproic acid were obtained from UTAK Labs. (valproic acid control, 55 μg/ml); Hyland Diagnostic serum anticonvulsant control Level I (70 μg/ml); and serum anticonvulsant control Level II (125 μg/ml).

Stock n-caproic acid was obtained from Chem Service Labs. and diluted to 1 mg/ml in 0.2 M ammonium hydroxide. A working solution of 50 μg/ml was prepared in 0.5 M ammonium hydroxide.

Phenacylbromide was diluted to 10 mg/ml in acetonitrile and may be stored for up to four weeks.

All reagents and solvents were of analytical grade.

Extraction from plasma and derivatization

The standards, controls or specimens (0.25 ml) are placed into 10-ml glass tubes, with PTFE lines and screw tops, and 0.25 ml of the working internal standard and 0.25 ml of 1 M sulfuric acid are added. The tubes are vortexed gently for 15 sec and 4 ml of pentane are added to each tube and the tubes capped and vortexed for 2 min. The tubes are centrifuged at 3000 g for 5 min and 3 ml of the upper pentane layer are transferred to a conical tube. Triethylamine (20 μl) and phenacylbromide (20 μl) are added to each tube and the tubes vortexed for 15 sec. The sample is evaporated under nitrogen to dryness by placing the tubes in a water bath at 50°C for 5 min. The residue remaining in the tube is dissolved in 50 μl of methanol and 3—5 μl are injected onto the GC column.

The instrument employed was a Shimadzu GC 6 AM gas chromatograph equipped with a flame ionization detector and a glass column (1.83 m × 5 mm O.D., 3 mm I.D.) packed with 3% OV-17 on 100—120 mesh Gas-Chrom Q (Applied Science). The analysis was carried out isothermally with a column temperature of 205°C and a detector and injector temperature of 250°C. Nitrogen was used as the carrier gas at a flow-rate of 80 ml/min. Sensitivity and range were 1 mΩ and 80 mV, respectively. The recorder output was 10 mV.

RESULTS

A standard regression line for known concentrations of valproic acid (5—200 μg/ml) added to drug-free plasma was determined and the assay is linear for these concentrations of valproic acid in plasma.

The estimation of the precision and accuracy in the analysis of valproic acid is shown in Table I. The accuracy of the method was tested by repeated analysis (n = 10) conducted with three commercial controls. The obtained range for the UTAK control (55 μg/ml) was found to be 52.35—58.04 μg/ml; that for the Hyland anticonvulsant Level I (70 μg/ml) was 67.34—73.04 μg/ml; and that for the Hyland anticonvulsant Level II (125 μg/ml) was 121.60—128.53 μg/ml. Intra-assay coefficient of variation ranged from 1.66 to 1.80%. Inter-assay coefficient of variation ranged from 2.39 to 3.31%.

Chromatograms obtained from the analysis of valproic acid in samples containing other anticonvulsants and theophylline and employing *n*-caproic acid as an internal standard are shown in Fig. 1. Chromatogram A (Fig. 1A) was obtained using Hyland TDM anticonvulsants Level II containing carbamazepine, ethosuximide, phenobarbital, phenytoin, primidone, theophylline and valproic acid. Except for valproic acid, none of these agents were detectable on the chromatogram and did not interfere with the assay. Chromatogram B (Fig. 1B) was obtained using UTAK valproic acid control containing 55 μg/ml. The peaks of the chromatogram are sharp; the internal standard elutes prior to the valproic acid. Chromatogram C (Fig. 1C) was

TABLE I

PRECISION AND ACCURACY ($n = 10$) IN THE ANALYSIS OF VALPROIC ACID

| Valproic acid controls	Target value (μg/ml)	Range (μg/ml)	Mean value (μg/ml)	Standard deviation (μg/ml)	Coefficient of variation (%)
Intra-assay					
UTAK	55	53.81— 56.44	54.76	0.97	1.77
Hyland anticonvulsant Level I	70	68.05—72.03	70.33	1.27	1.80
Hyland anticonvulsant Level II	125	122.50—127.60	124.40	2.07	1.66
Inter-assay					
UTAK	55	52.35—58.04	55.15	1.83	3.31
Hyland anticonvulsant Level I	70	67.34—73.04	70.39	2.22	3.15
Hyland anticonvulsant Level II	125	121.60—128.53	124.69	2.99	2.39
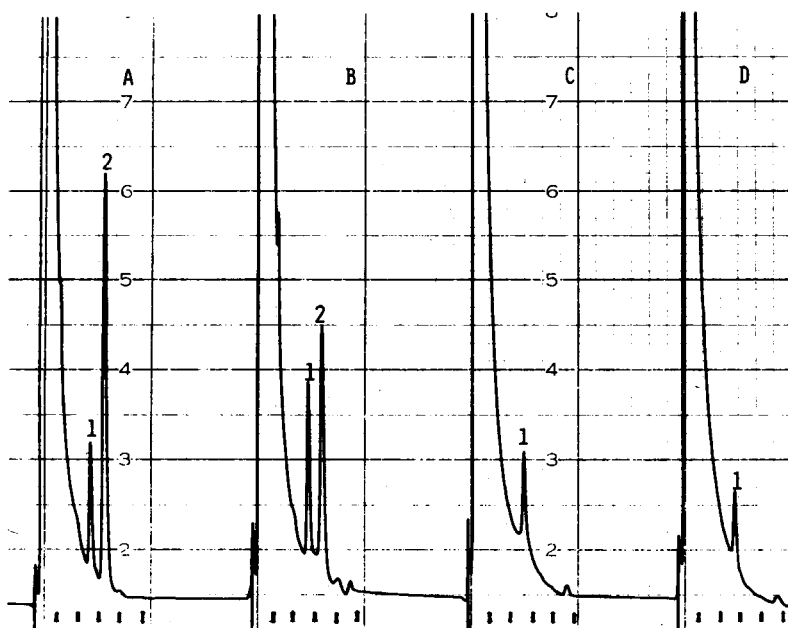
Fig. 1. Chromatograms of plasma extracts. (A) Hyland TDM anticonvulsant Level II control, containing carbamazepine (16 μg/ml), ethosuximide (125 μg/ml), phenobarbital (60 μg/ml), phenytoin (24 μg/ml), primidone (15 μg/ml), theophylline (30 μg/ml), and valproic acid (125 μg/ml); (B) UTAK valproic acid control (55 μg/ml); (C) drug-free plasma to which was added carbamazepine, ethosuximide, phenobarbital, phenytoin, primidone and theophylline (20 μg/ml of each); (D) drug-free plasma. Peaks: 1 = phenacyl derivative of n-caproic acid (internal standard); 2 = phenacyl ester of valproic acid.

obtained emploing drug-free plasma to which carbamazepine, ethosuximide, phenobarbital, phenytoin, primidone and theophylline had been added. Again, as in the Hyland anticonvulsant Level II, there was no interference from these agents; the only peak discernable is the internal standard. Chromatogram D (Fig. 1D) was obtained with drug-free plasma. The only peak is the internal standard and this indicated that plasma constituents do not interfere with the assay.

DISCUSSION

The objective of this study was to develop an analytical method for the determination of valproic acid that was suitable for a clinical laboratory. It was not only important that the assay be rapid and simple, but also that it be accurate and reproducible. A GC method was chosen over HPLC or EMIT because of lack of UV absorbance of valproic acid with the HPLC and interference by other substances with the EMIT method. Ideally, the method would not include a derivatization or evaporation step; however, this was essential to obtain the sensitivity and reproducibility that was required. Many of the GC methods in the literature are either very difficult [10], time-consuming [6] or lack reproducibility [10]. This method employs several steps described by Gupta et al. [6] with a number of modifications to simplify the method and decrease the assay time without compromising the sensitivity or reproducibility.

A single extraction of the valproic acid with pentane under acidic conditions was found to be sufficient to recover most (> 90%) of the valproic acid from the plasma. Further extraction with pentane under basic or neutral conditions was not necessary as no interfering substances were observed in the chromatograms. Derivatization of valproic acid was found to be necessary in order to obtain the desired sensitivity and to reduce the volatility of the compound during evaporation. A derivative was formed with phenacylbromide to form the phenacyl ester of valproic acid and n-caproic acid. Derivatization with phenacylbromide was selected for the following reasons: (1) the derivatization is extremely simple and rapid; (2) the phenacyl ester is less volatile than the free acid; and (3) the sensitivity with flame ionization detection is increased because of a greater number of carbon atoms (i.e. derivatization). The derivatization of valproic acid and n-caproic acid is very simple and quick requiring only the addition of triethylamine and phenacylbromide and occurs in a few seconds. The sample is immediately ready for subsequent steps.

Evaporation of the pentane was found to be necessary because of the volume of pentane required to extract the valproic acid. The pentane could be very quickly evaporated by placing the sample in a water bath at 50°C under a stream of nitrogen. By employing the stream of nitrogen, evaporation time was decreased from 1 h to 3 or 4 min. Not only does this save time, but there is less chance of decomposition of the sample. The phenacyl ester of valproic acid and of n-caproic acid is less volatile than the free acid and no loss of the sample is observed during this step.

n-Caproic acid was employed as the internal standard because it has similar properties to valproic acid with respect to extraction from the plasma by pentane and elution time during chromatography. The phenacyl ester derivative

of n-caproic acid elutes from the column during chromatography prior to the phenacyl ester derivative of valproic acid. The peaks of the internal standard are sharp and separate well from the solvent peak and the valproic acid peak. Other methods employ cyclohexane carboxylic acid which elutes after the valproic acid. The use of n-caproic acid as an internal standard decreases the assay time of each sample because it elutes before the valproic acid.

The standard curve for valproic acid was linear at the concentrations employed in the assay (5—200 μg/ml). A correlation coefficient of 0.9999 was obtained with the regression line passing through the origin. The detection limit of the assay was 5 μg/ml, and the sensitivity of the assay could certainly be increased by the use of larger volumes of plasma, but would serve no practical purpose since the therapeutic range is 55—100 μg/ml.

This method for the analysis of valproic acid is both accurate and precise. The accuracy was measured by multiple analysis of control samples. The value obtained for the controls was very close to the actual value in each case with a low coefficient of variation, as shown in Table I. This is a major criterion for an analytical method.

Another important asset of this method is the lack of interference from plasma constituents or from other anticonvulsants that might be used in combination with valproic acid.

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