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HPLC determination of valproic acid in human serum

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A HPLC method for the determination of valproic acid (VA) in human serum using diazepam as internal standard (I.S.) is described. The eluates were separated with a C18 250 × 4.6 mm internal diameter reversed phase column maintained at a temperature of 50 °C. A mobile phase consisting of acetonitrile and 0.05 M phosphate buffer (pH 3.0) 45 : 55 v/v was used at a flow rate of 1.2 ml/min. Wavelength was switched from 360 nm to 210 nm during valproic acid retention. The method was linear over a concentration range of 20 to 160 μg/ml for valproic acid. Recovery was greater than 94% over a concentration range of 20 to 120 μg/ml and the limit of quantitation was 1 μg/ml. The intra day and inter day relative standard deviation (R.S.D.) measured at 20, 60, 80 and 120 μg/ml ranged from 1.46 to 5.34% and 0.83 to 5.03% respectively. The method is simple, rapid, accurate and sensitive and it was used for Therapeutic Drug Monitoring (TDM) in Indian epileptic patient population. The results obtained with this method correlated well with clinical practice.

1. Introduction

A comprehensive therapeutic monitoring of anticonvulsant therapy with valproic acid demands the availability of suitable methods for measuring VA concentrations. VA is routinely monitored by gas chromatography (GC) or by an immunological assay. GC analysis is hampered by the fact that it is necessary first to extract the drug with an organic solvent and immunological techniques are expensive.

The optimum procedure for the assay should provide reliable, rapid and precise quantitation.

VA is a branched chain carboxylic acid (attachment of a suitable chromophore or fluorophore to the carboxylic acid is necessary) and has poor UV absorption. Derivatization for detection enhancement [1–4] or other technical alterations (i.e. a colorimetric procedure based on variation in colour of a solution of bromocresol purple [5] or controlled evaporation using the Technicon Evaporation-to-Dryness Module) seems to be necessary [6]. In contrast, since VA is a volatile compound, extreme care must be taken to avoid losses due to volatilization when concentrating sample extracts. In general, when compounds have a low ultraviolet absorbance, one would attempt to derivatize them for detection enhancement and/or to evaporate the extract for their enrichment. Obviously, the volatility of VA precludes any evaporation procedure.

Though many HPLC methods like micelle mediated pre column derivatization [7], fluorescence polarization assay [8], derivatization [10], fluorimetric detection [11–12] and colorimetric procedure [10] are reported, they are complex, expensive and time consuming.

Therefore, we developed a simple method, which is rapid, accurate and sensitive for estimation of VA on C 18 column and evaluation of its performance for monitoring drug levels in epileptic patients.

2. Investigations, results and discussion

Typical chromatograms corresponding to blank serum and VA spiked standard (control) serum are shown in Figs. 1 and 2. A chromatogram of a serum sample from epileptic patient (under treatment of valproic acid) is shown in Fig. 3. Both the analyte and I.S. were well separated with retention times of 7.1 and 8.69 min respectively with no endogenous interfering peaks. System suitability parameters for the method were as follows: Theoretical plates for VA and I.S. were 12776 and 9279 respectively. Tailing factor was less than 1.05 for both VA and I.S. and resolution between VA and I.S. was 5.15.

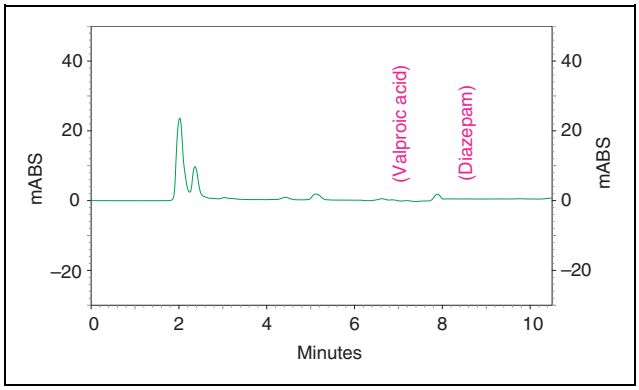

Fig. 1: Typical HPLC chromatogram for analysis of valproic acid: Blank serum

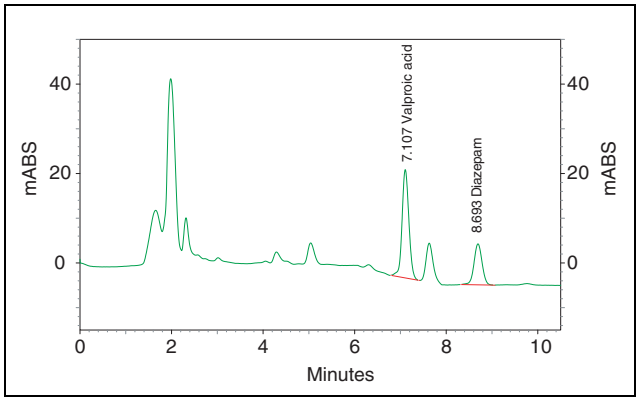

Fig. 2: Typical HPLC chromatogram for analysis of valproic acid: Blank serum sample spiked with 160 μg/ml

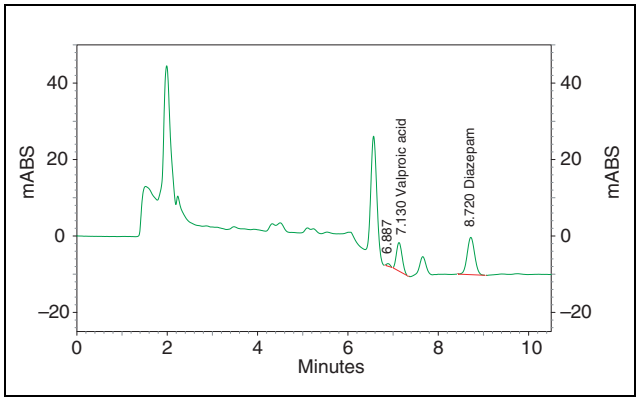

Fig. 3: Typical chromatogram of a epileptic patient serum sample presently under the treatment of valproic acid, collected at C^{ss}_{min}

The peak area ratio of VA to I.S. was used for quantification of VA in serum samples. The calibration curves were linear in the concentration range 20–160 μg/ml. The calibration equation is $y = mx + c$, where y represents the peak area ratio of VA to I.S., x represents the concentrations of valproic acid, m is slope of the curve and c is the intercept. The equation of the calibration curve obtained from 7 points was $y = 0.0150182x - 0.214819$ ($r^2 = 0.996$).

The LOQ, established by determining concentration of four spiked calibration standards having reproducibility with RSD < 20% and accuracy of 93–102% was found to be 1 μg/ml. Using this method, it is possible to further increase the sensitivity by increasing the serum/injection volume.

The intra day precision of the assay was determined by analyzing four spiked serum samples at each concentration on the same day. Inter day precision was determined by analyzing spiked serum samples on four different days. The inter-day RSD ranged from 0.938–5.032, 0.959–3.248, 2.415–4.209 and 0.830–3.683 for 20, 60, 80 and 120 μg/ml respectively. The intra-day RSDs were 5.340, 2.257, 2.235 and 1.468 for 20, 60, 80 and 120 μg/ml respectively. These values were within the limits (<15%) specified for inter day and intra day precision.

The recovery from serum was estimated at 20, 60, 80 and 120 μg/ml concentrations. Proteins in serum samples (in six replicates) containing VA and I.S. were precipitated and analyzed. Six samples containing similar concentration of VA in methanol were directly injected and peak areas were measured. Absolute recovery was calculated by comparing the peak areas for direct injection of pure VA with that obtained from serum samples spiked with the same amount of VA and processed similarly. The absolute recoveries ranged from 91.18–98.16% (Table). The accuracy of the method was verified by comparing the concentrations of VA measured in spiked serum with the actual concentrations added.

A TDM study was conducted in Indian epileptic patients to investigate the serum drug levels of valproic acid for assessing the validity of therapeutic ranges. More than 40 serum samples were collected from patients who were under the treatment of valproic acid. The drug serum levels were classified as sub therapeutic level, within therapeutic range and toxic level. The results correlated clinically and were well accepted by neurologists.

3. Experimental

3.1. Materials

Valproic acid and diazepam pure samples were gifted by INTAS Pharmaceuticals Pvt. Ltd, Ahmedabad India. Orthophosphoric acid, methanol and acetonitrile (HPLC grade) were procured from Rankem, Ranbaxy laboratories, India. Potassium dihydrogen orthophosphate was procured from SD fine chemicals, Mumbai, India. Millipore water was used during entire HPLC procedure.

3.2. Standard solutions

Stock solutions of 1 mg/ml of VA and 0.01 mg/ml of diazepam were prepared in methanol and acetonitrile respectively and stored at −4 °C. VA stock solution was further diluted with methanol to the required concentrations (20, 40, 60, 80, 100, 120 and 160 μg/ml). Calibration samples were prepared by spiking 250 μl blank serum with appropriate amounts of drug on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control human serum in bulk of appropriate concentrations (20, 60, 80 and 120 μg/ml) and stored at −20 °C.

3.3. Extraction procedure

To 250 μl serum samples, acetonitrile solution of diazepam equivalent to 2.5 μg was added as I.S. and shaken well. Then an equivalent amount of (250 μl) acetonitrile was added for protein precipitation and mixed on a cyclomixer for 1 min and centrifuged at 4000 rpm using a REMI centrifuge (R8C laboratory centrifuge, REMI motors, Mumbai, India) for 10 min. 50 μl of the supernatant were injected into the HPLC column.

3.4. Chromatographic conditions

The HPLC system (Shimadzu, Kyoto, Japan) consisted of a SCL-10 A VP system controller, LC-10 AT VP liquid chromatography pumps A and B, SPD-10 A VP UV-Vis detector, SIL-10 AD VP auto injector with peltier rack maintained at 4 °C and CTO-10 AS VP column oven. The column used was HYPERSIL 5 μ C 18 BDS (Stainless steel column of length

Table: Absolute recovery and accuracy of determination of valproic acid in human serum

Concentration (μg/ml)	Absolute recovery (%)		Accuracy (%)	
	Mean ±S.D. (n = 6)	Range (min–max)	Mean ±S.D. (n = 6)	Range (min–max)
20	94.32 ± 1.23	93.09–95.55	97.23 ± 0.91	96.32–98.14
60	97.07 ± 1.09	95.98–98.16	99.97 ± 2.03	97.94–102.00
80	93.19 ± 2.01	91.18–95.20	100.12 ± 1.03	99.09–101.15
120	95.23 ± 1.91	93.32–97.14	96.09 ± 2.12	93.97–98.21

25 cm and internal diameter of 4.6 mm) (North America, USA) maintained at 50 °C. The mobile phase consisting of acetonitrile and 0.05 M potassium dihydrogen ortho phosphate (pH adjusted to 3 (polmon pH/mv/temp meter LP-135) with ortho phosphoric acid) (45 : 55 v/v) was used at a flow rate of 1.2 ml/min. The eluate was monitored at 360 nm from 0 to 10 min except monitoring the run at 210 nm from 6 to 7.99 min. Wavelength switching was done to reduce the runtime (within 9 min) and to obtain prominent elution of I.S. and valproic acid at 360 and 210 nm respectively without any interference. BDS column and temperature maintenance were opted to obtain sharp peaks and to reduce the run time. The sensitivity was set at 0.001 A.U.F.S.

3.5. Linearity and limit of quantitation

The calibration samples were prepared by spiking 250 µl control serum with appropriate amounts of VA and I.S. on the day of analysis. The lower limit of quantitation (LOQ) was defined as the lowest concentration at which the relative standard deviation from the nominal concentration were less than 20%.

3.6. Precision

Samples for the determination of precision were prepared by appropriately spiking control serum in bulk to get concentrations of 20, 60, 80 and 120 µg/ml. At each concentration 250 µl aliquots were distributed into screw-capped tubes and stored at −20 °C. Four replicates at each concentration were processed as described, in the sample preparation on day 0, 1, 2 and 3 to determine the inter day and intra day reproducibility. The precision of the method at each concentration was calculated as the RSD.

3.7. Recovery and accuracy

The recovery from serum samples was determined by comparing the amount of VA from serum samples with that of recovery standards, which were processed similarly without serum matrix (using methanol instead). The accuracy of the procedure was determined by expressing the mean calculated concentration as a percentage of the spiked/nominal concentration.

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