Article *NIMHANS Journal*

Simultaneous Assay of Antiepileptic Drugs in Serum using High Performance Liquid Chromatography

Volume: 06 Issue: 01 January 1988 Page: 35-39

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Abstract

A method has been developed for the determination of the most commonly used anti-convulsants namely phenobarbital, phenytoin and carbamazepine in serum using high performance liquid chromatography (HPLC). This method allows the simultaneous assay of the three drugs in serum without any interference from metabolites or endogenous compounds. This method is relatively inexpensive as compared with the widely used enzyme-multiplied immunoassay technique (EMIT), and the HPLC technique is advantageous in the quantification of drugs above and below the therapeutic range and where such knowledge is needed for purposes of management, HPLC technique scores over the EMIT.

Key words -
Antiepileptic drugs,
Serum,
High performance liquid chromatography

The objective in treating epileptic patients with anticonvulsants is to achieve control of seizures by maintaining stable drug levels in blood, through the administration of fixed daily doses of the drug. The therapeutic window between the effective and toxic levels of these drugs is very narrow and drug-level monitoring is an essential part of antiepileptic therapy [1].

Anticonvulsants have been determined by a number of analytical techniques, viz. spectrophotometry, chromatography [2] and immunological techniques [3], [4]. While, immunological methods are rapid and sensitive, they permit analysis of only one drug at a time. Chromatographic procedures on the other hand, are amenable to the analysis of multicomponents and several drugs can be assayed simultaneously. This advantage is particularly important for the determination of anti-convulsants as treatment for epilepsy usually involves administration of two or more drugs.

High performance liquid chromatography (HPLC) is a sophisticated chromatographic technique and is used extensively for therapeutic drug monitoring. An assay method using HPLC has been developed for the commonly used antiepileptic drugs (AED) namely phenobarbital (PB), phenytoin (DPH) and carbamazepine(CBZ).

Material and Methods

Patients on treatment with two or more antiepileptic drugs, who come for consultation to this centre

formed the clinical material. Blood samples were collected for drug analysis in the mornings. The serum was separated and stored at - 20° C and then taken up for analysis.

The analysis was carried out on a Tracor 955 HPLC pump, equipped with a Tracor 970A variable wavelength detector and Hewlitt-Packard 3390 integrator. Zorbax ODS (10µ m) reverse phase column (6 mm × 25 cm), obtained from Dupont Inc., USA, was used for separation of the components.

The HPLC mobile phase consisted of a mixture of 450 ml of 0.4 mM phosphate buffer (pH - 4.4), 450 ml of methanol and 80 ml of actonitrile. All the constituents of the mobile phase was filtered through 0.45µ m cellulose acetate or polytetrafluoroethylene membrane and degassed prior to use. Deionised quartz distilled water was used for HPLC.

Stock standard solutions consisted of 1 mg of PB or DPH dissolved in 1 ml of methanol, and 0.5 mg of CBZ dissolved in 1 ml of methanol. The working standard was prepared by combining 10µ l of each stock standard in the mobile phase to total volume of 1 ml. The internal standard was 1 mg of dihydrocarbamazepine (DHCBZ) dissolved in 50 ml of acetonitrile.

Plasma or serum (200µ l) containing the drug to be estimated was added to a 1.5 ml of centrifuge tube, followed by 200µ l of acetonitrile containing DHCBZ as internal standard. The contents were thoroughly mixed using a vortex mixer and centrifuged for 5 mins at 7000 rpm. A suitable aliquot (10-15µ l) was injected into the chromatograph and eluted with the mobile phase at a flow rate of 1 ml/min and the effluent was monitored at 215 nm, using the variable wavelength detector.

Results

A representative chromatogram of the standards is illustrated in figure 1. The chromatogram is well resolved, and the internal standard is eluted out after the elution of the three drugs, PB, DBH and CBZ, thus ensuring that there is no interference between the internal standards and the three drugs. A linear relationship was observed between the concentration of the drug and the peak area ratio (figure 2). The concentration of the drug in serum or plasma was calculated using this standard curve and the recovery was monitored by the use of internal standard.

- ***Chromatogram of antiepileptic drug standards***
- ***Standard curves for phenobarbital, phenytoin and carbamazepine : Peak area ratios vs concentration of drugs***

The relative and absolute recoveries of various concentrations of the drug are given in Table 1. Drugs indicated concentrations were added to drug free serum and mixed with an equal volume of the solution of internal standards, and processed as given in figure 2. The peak areas were compared with peak areas of standard solution and absolute analytical recovery was calculated. Relative recoveries were calculated by comparing the values obtained for drug supplemented serum with actual added concentrations in standard curve. The recovery ranged from 103-106%. The representative chromatogram of extracted serum sample from a patient on DPH and CBZ is illustrated in figure 3. No interference from metabolites is noted. Figure 4 illustrates the chromatogram of the extracted serum from a patient on PB and DPH. Thus, both drugs in the samples could be assayed simultaneously. The serum from a patient on the three AED namely PB, DPH, and CBZ was analysed by this method (figure 5). This sample contained very low levels of DPH (3.23µ g/ml of serum), which could not be

assayed by EMIT. By injecting varying amounts of sample (figures 5a and 5b), all the three drugs could be analysed.

Table 1 - Recovery Studies

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Values are expressed as mean ± SEM for 5 experiments each performed in duplicate

- ***Chromatogram of serum extract of a patient on DPH and CBZ (a) DPH, (b) CBZ and (c) internal standard Concentration of DPH is 4.9μg/ml and CBZ is 3.9μg/ml***
- ***Chromotagram of serum extract of a patient on PB and DPH. (a) PB (24.9μg/ml), (b) DPH (41.3μg/ml) and (c) internal standard***
- ***Chromatogram of serum extract of a patient on PB, DPH and CBZ. Injection volume (A) 50μl of serum extract (B) 100μl of serum extract. Concentration of drugs are - (a) PB (9.3μg.ml), (b) DPH (3.2μg/ml), (c) CBZ (3.9μg/ml) and (d) internal standard***

A comparison of the concentration as determined by two methods namely HPLC and EMIT was carried out (table 2). Sub-therapeutic levels of the drugs could be assayed by HPLC, which was not possible by EMIT. A slightly higher recovery was observed as compared to the EMIT, especially in sub-therapeutic and toxic range. This may be due to the fact that EMIT involves an indirect assay of drug concentration and is linear only in the therapeutic range, while HPLC method quantities the drug levels directly.

Table 2 - Comparison of serum AED levels as analysed by HPLC and by EMIT

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Each sample was analysed by HPLC and EMIT. HPLC assays were performed in duplicate and the average value is indicated

Discussion

This paper presents a rapid method for determination of AED using HPLC. Although several methods have been described for the quantification of AED using HPLC, there have been drawbacks in the methods. For example, the metabolite of DPH coelutes with PB [5], [6], poor recovery due to multiple step extraction [7], a rapid deterioration of the column packing due to basic nature (pH-8) of the mobile phase [8]. The present method has successfully overcome these short-comings. Analyses has been performed on sub-therapeutic and toxic range and no interference from metabolites is observed. The chromatographic run time is less than 15 minutes, thus ensuring that a large number of samples can be analysed in a single day.

The HPLC method for determining AED has the following advantages over the EMIT system [9].

(a) EMIT performs poorly outside the therapeutic range, while HPLC method can assay the drug in sub-therapeutic and toxic levels.

(b) Simulataneous assay of drug is possible by HPLC, accidental ingestion of other AED can be detected easily.

(c) In the EMIT system, lack of specificity may pose a problem as some metabolites cross-react with the [2].

(d) Analysis of AED by EMIT is very expensive as it involves the import of kits with short shelf lives. Chromatographic methods are very economical and involve use of chemicals available locally.
(e) In the hands of an experienced analyst, more information can be gained from a chromatogram than from a system such as EMIT.

Thus, the HPLC method offers a viable alternative to the EMIT for assay of AED. The precision of the chromatographic method can be improved considerably by the use of automated injection systems by the use of automated injection system which also require limited man-power to run the assays. The use of HPLC with microbore capabilities would lessen the cost of analysis and shorten the time of analysis.

Acknowledgement

The author thanks Mr. C P Narayanan for analysis of drug concentration in serum by EMIT.

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