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Comparison of fluorescence polarization immunoassay and high performance liquid chromatography for the quantitative determination of phenytoin, phenobarbitone and carbamazepine in serum

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The Abbott TDx fluorescence polarization immunoassay (FPIA) system has been evaluated and compared with well-established high performance liquid chromatography (HPLC) for the determination of three anticonvulsant drugs: phenytoin, phenobarbitone and carbamazepine. These assays were evaluated for precision, calibration curve stability, specificity and accuracy. Within-run precision studies using control samples (n = 15) in the subtherapeutic, therapeutic, and toxic concentrations, resulted in coefficients of variation in the range of 1·79–3·99% (FPIA) and 1·16–2·52% (HPLC), respectively. Between-run precision ranged from 2·32–6·34% for FPIA and from 2·04–3·38% for HPLC. Comparison of 122 patient samples assayed with both methods indicated an extremely good analytical correlation (r = 0·96) for all three comparisons. The FPIA method offers significant advantages in calibration curve stability while maintaining accuracy and precision comparable with those of established HPLC procedures.

The value of therapeutic drug monitoring as an aid to rational drug therapy in patients with various diseases has been firmly established. Monitoring of the plasma concentrations of antiepileptic drugs such as phenytoin, phenobarbitone and carbamazepine have undoubtedly proved useful in the clinical management of epileptic patients.

A variety of analytical techniques have been employed for the analysis of anticonvulsant drugs in biological fluids. Many of the initial studies on the therapeutic or toxic effects of these drugs were facilitated by the use of ultraviolet spectroscopy and thin layer chromatography (TLC). The former procedure is not very practical because it requires a large volume of sample, complex extraction techniques, is time consuming, and the assay is subjected to potential interference of other compounds (Fellenberg & Pollard 1976; Spiehler 1976; Kabra et al 1981). TLC, on the other hand, possesses adequate resolution for identifying many of the anticonvulsant drugs, but it is difficult to quantify these drugs accurately and precisely (Breyer & Villumsen 1975; Kabra et al 1981). However, Wilson et al (1983) have shown that TLC has high precision for measurement of carbamazepine in serum.

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Modern analytical technology has provided new insights and approaches to therapy. With the advent of such techniques as gas liquid chromatography (GLC) (Abraham & Gresham 1977; Ayers et al 1977; Heipertz & Eickhoff 1977), radioimmunoassay (RIA) (Paxton et al 1977; Wilson et al 1983), enzyme multiplied immunoassay technique (EMIT) (Brunk et al 1976; Finley et al 1976; Turri 1977; Vanlente et al 1977) and high performance liquid chromatography (HPLC) (Adams & Vandemark 1976; Kabra et al 1977, 1978, 1983; Adams et al 1978; Szabo & Browne 1982; Ching-Nan & Rognerud 1984; El-Sayed et al 1984), plasma levels of anticonvulsant drugs can be measured routinely, resulting in improved patient care.

GLC analysis of anticonvulsant drugs requires a relatively large sample and a significant amount of time for sample preparation and derivatization.

Advantages of immunoassays include rapid analysis, no need for derivatization, and minimal sample volume. Disadvantages include inability to determine multiple drugs and active metabolites simultaneously without using separate calibrators, and potential interference with the antibody used in a specific analysis.

HPLC offers several advantages over the other techniques, sample manipulation before chromato-

graphy is minimal, and several classes of compounds and their metabolites can be analysed simultaneously with good specificity, precision, and accuracy (El-Sayed et al 1984).

Recently, another simplified rapid method using the principle of fluorescence polarization immunoassay (FPIA) has been introduced by Abbott for determination of anticonvulsant drugs in patients' serum (Apple et al 1983; Vasiliades et al 1983; Oeltgen et al 1984; Wang & Peter 1985). FPIA is an homogenous immunoassay for drug measurement in serum or plasma, based upon the competitive binding between patients' drug and tracer (drug labelled with fluorescein) for the antibody. After reaching equilibrium, the patient's drug molecules displace the tracers resulting in lowering the fluorescence polarization. Thus, the extent of the fluorescence polarization is inversely proportional to the patient's drug concentration.

The purpose of this study was to evaluate the recovery, precision and accuracy of fluorescence polarization immunoassay for the determination of phenytoin, phenobarbitone and carbamazepine and to compare the results obtained on patients' serum samples by this technique with those obtained by an HPLC procedure developed in our laboratory (El-Sayed et al 1984).

MATERIALS AND METHODS

High performance liquid chromatography

Apparatus. HPLC equipment comprised a single piston pump (model 112; Beckman Instruments Int., Geneva, Switzerland), a fixed wavelength detector (model 160; Beckman), and an autosampler injector WISP (Waters Intelligent Sample Processor) model 710B (Waters Associates, Milford, Mass., USA). Chromatographic separations were performed using Ultrasphere ODS 5 μm column (4·6 × 250 mm), protected by a guard column (4·6 × 45 mm) of the same material (Beckman).

Reagents. All solvents used were of HPLC-grade. All other chemicals and reagents were of spectroquality or analytical grade. Phenytoin, phenobarbitone, carbamazepine, and 5-(4-methylphenyl)-5-phenylhydantoin were obtained from Sigma Chemicals Co. (St Louis, Missouri, USA). The HPLC mobile phase comprised 10 mM potassium phosphate pH 4·8 (adjusted with 0·9 M phosphoric acid)–methanol–acetonitrile (50:28:22% v/v). The solution was filtered and degassed with helium before use.

Procedure. Fifty μl of serum, aqueous standard or blank was added to a 50 μl solution of acetonitrile containing 0·5 μg of 5-(4-methylphenyl)-5-phenylhydantoin (internal standard) in a 1·5 ml microcentrifuge tube. The sample was vortex-mixed vigorously for 30 s and centrifuged for 2 min at 9500*g* in a microcentrifuge (Abbott No. 9527-15). The supernatant was transferred to a similar tube and centrifuged for 30 s to ensure that no particulate matter would be injected into the column. Supernatant (5–10 μl) was automatically injected onto the column using the WISP and eluted with the mobile phase at a flow rate of 1 ml min^{-1}. The column temperature was 40 °C and the detector wavelength 214 nm.

Quantitation was achieved by measuring peak heights of phenytoin, phenobarbitone and carbamazepine relative to the internal standard and comparing values with a standard curve prepared from blank serum samples spiked with standard drug at five different concentrations. The retention times for phenobarbitone, phenytoin, carbamazepine and internal standard were 5·0, 8·1, 9·3 and 12·0 min, respectively.

Fluorescence polarization immunoassay

Apparatus. The Abbott TDx fluorescence polarization analyzer (Abbott Diagnostics, North Chicago, Illinois, USA) was used for FPIA procedure.

Reagents. All reagents (antisera, pretreatment solution and fluorescein-labelled analytes), calibrators and controls were components specifically designed for use with the Abbott TDx fluorescence polarization analyzer. These reagents were supplied by the manufacturer and were used according to the manufacturer's instructions.

Patients' specimens. Patients' samples for method evaluation were obtained from outpatient clinics at King Abdulaziz University Hospital. Phenytoin concentrations in 61 samples, carbamazepine in 32 samples, and phenobarbitone in 29 samples were compared by the two techniques. Blood samples were taken from patients with normal renal and liver functions who had been on long-term anticonvulsant therapy before dose administration.

RESULTS

Calibration of the anticonvulsant assays

With the HPLC assay, the calibration curves for phenytoin, phenobarbitone and carbamazepine were linear between 2 and 80 μg ml^{-1}. Each point on the curve was based on at least seven determinations.

For phenytoin, r = 0·9999, slope = 0·1374, y-intercept = −0·0206; for phenobarbitone, r = 0·9998, slope = 0·1995, y-intercept = −0·1577, and for carbamazepine, r = 0·9993, slope = 0·2524, y-intercept = 0·0419. A typical chromatogram showing the separation of the three anticonvulsant drugs is shown in Fig. 1.

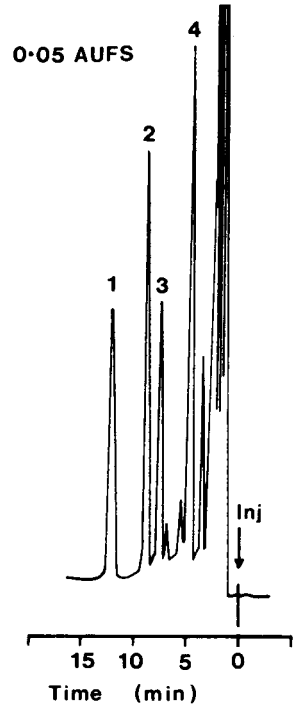

FIG. 1. HPLC chromatogram from serum of a patient with 8·9 μg ml^{-1} phenobarbitone, 6·8 μg ml^{-1} phenytoin, and 4·7 μg ml^{-1} carbamazepine: 1, internal standard; 2, carbamazepine; 3, phenytoin; 4, phenobarbitone.

Calibration curve stability of the FPIA assay was assessed over 4 weeks for all three drugs by examining the drift in control values of a pooled serum prepared in-house containing each of these drugs at three different concentrations (subtherapeutic, therapeutic and toxic). Maximum drift for any of the assays was 6·34% (coefficient of variation, CV). In all instances, the concentration of the three drugs was determined from the initial calibration curve derived from the assays done on day 1 and subsequently stored in the TDx computer memory.

Precision

The within-run precision (random analytical variation) of both HPLC and FPIA was obtained by replicate analysis of a pooled serum spiked with each of these drugs at three different concentrations (subtherapeutic, therapeutic and toxic). All the specimens used to study precision and bias were interspersed with clinical specimens during analysis. The within-run precision of the HPLC assay showed a coefficient of variation of 1·16–2·31, 1·98–2·16 and 1·95–2·52 for phenytoin, phenobarbitone and carbamazepine, respectively. The FPIA assay showed within-run variation of 1·79–3·17, 2·29–2·35 and 2·15–4·0 for phenytoin, phenobarbitone and carbamazepine, respectively. The results of the within-run precision study are presented in Table 1.

The between-run precision (total analytical variation) of both methods was similarly evaluated on several days up to 4 weeks. The HPLC assay showed coefficient of variation of 2·16–3·16, 2·04–2·89 and 2·61–3·38 for phenytoin, phenobarbitone and carbamazepine, respectively, whereas for the FPIA assay,

Table 1. Within-run precision of HPLC and FPIA methods in the measurement of phenytoin, phenobarbitone and carbamazepine in serum-based standards.

		HPLC			FPIA		
Drug	Target level μg ml^{-1}	Mean measured level* μg ml^{-1} ± s.d.	CV, %	Bias, %**	Mean measured level* μg ml^{-1} ± s.d.	CV, %	Bias, %**
Phenytoin	7·5	7·79 ± 0·18	2·31	3·87	7·88 ± 0·25	3·17	5·10
	15	15·46 ± 0·18	1·16	3·07	15·51 ± 0·31	2·00	3·40
	30	29·70 ± 0·50	1·68	−1·00	30·10 ± 0·54	1·79	0·33
Phenobarbitone	15	15·12 ± 0·30	1·98	0·80	15·31 ± 0·35	2·29	2·10
	30	30·65 ± 0·66	2·15	2·17	30·70 ± 0·72	2·35	2·33
	60	60·10 ± 1·30	2·16	0·17	60·44 ± 1·39	2·30	0·73
Carbamazepine	3	3·17 ± 0·08	2·52	5·67	3·26 ± 0·13	3·99	8·70
	6	6·15 ± 0 12	1·95	2·50	6·20 ± 0·16	2·58	3·33
	16	16·27 ± 0·34	2·10	1·69	16·30 ± 0·35	2·15	1·88

s.d., Standard deviation; CV, coefficient of variation.
* Mean values represent 15 different serum samples for each concentration.
** Bias = 100 × (measured concentration–target concentration)/target concentration.

Table 2. Between-run precision of HPLC and FPIA methods in the measurement of phenytoin, phenobarbitone and carbamazepine in serum-based standards.

		HPLC			FPIA		
Drug	Target level μg ml^{-1}	Mean measured level* μg ml^{-1} ± s.d.	CV, %	Bias, %**	Mean measured level* μg ml^{-1} + s.d.	CV, %	Bias, %**
Phenytoin	7·5	7·60 ± 0·24	3·16	1·33	7·97 ± 0·36	4·52	6·27
	15	15·38 ± 0·40	2·60	2·53	15·56 ± 0·41	2·63	3·73
	30	30·57 ± 0·66	2·16	1·90	30·44 ± 0·83	2·73	1·47
Phenobarbitone	15	14·79 ± 0·35	2·37	−1·40	15·20 ± 0·57	3·75	1·33
	30	30·43 ± 0·88	2·89	1·43	31·51 ± 1·48	4·70	5·03
	60	61·22 ± 1·25	2·04	2·03	61·65 ± 1·77	2·87	2·75
Carbamazepine	3	2·96 ± 0·10	3·38	−1·33	3·31 ± 0·21	6·34	10·33
	6	6·17 ± 0·17	2·76	2·83	6·29 ± 0·21	3·34	4·83
	16	16·10 ± 0·42	2·61	0·63	16·37 ± 0·38	2·32	2·31

s.d., Standard deviation: CV, coefficient of variation.
* Mean values represent 15 different serum samples for each concentration analysed on different days over a 4-week period.
** Bias = 100 × (measured concentration–target concentration)/target concentration.

the coefficients of variation were 2·63–4·52, 2·87–4·7 and 2·32–6·34 for phenytoin, phenobarbitone and carbamazepine, respectively (Table 2).

Recovery

To determine the ability of the assay procedures to recover phenytoin, phenobarbitone and carbamazepine, known amounts of these drugs at three different concentrations (subtherapeutic, therapeutic and toxic), were added to drug-free, pooled serum. These sera were then assayed in replicates of fifteen by using FPIA and HPLC assay and the percentage of recovery of the added amount was determined (Table 3). The mean recovery of added drugs at the three different concentrations by FPIA and HPLC were 102·9, 100·8 for phenytoin, 101·7, 100·8 for phenobarbitone and 104·6, 102·7 for carbamazepine, respectively.

Table 3. Analytical recovery of phenytoin, phenobarbitone and carbamazepine in serum.*

Drug	Concentration μg ml^{-1}	Mean recovery, % FPIA	HPLC
Phenytoin	7·5	105·1	100·3
	15	103·4	103·1
	30	100·3	99·0
Phenobarbitone	15	102·1	100·8
	30	102·3	100·8
	60	100·75	100·2
Carbamazepine	3	108·7	104·0
	6	103·3	102·5
	16	101·9	101·7

* 15 replicate determinations for each concentration.

Correlation

To compare the results from FPIA with those of the HPLC in patients' serum, samples from 61 patients receiving phenytoin, 32 patients receiving carbamazepine, and 29 patients receiving phenobarbitone were analysed by both procedures. Linear regression analysis relating the anticonvulsant patients' values obtained by HPLC (x-axis) to the values obtained by FPIA (y-axis) are presented in Table 4. The phenytoin assay (Fig. 2) gave a correlation coefficient, r = 0·9897, the phenobarbitone assay (Fig. 3) gave r = 0·9975, and the carbamazepine assay (Fig. 4) gave r = 0·9692. The significance of difference between the two methods was tested by the paired Student's *t*-test. There is no significant difference (95% confidence level) between the two methods as determined by the paired *t*-test, $t = 0·586$, 1·196 and 1·112 for phenytoin, phenobarbitone and carbamazepine, respectively.

Table 4. Least-squares linear regression analysis of anticonvulsant drugs.

Drug	Slope	Intercept	Correlation coefficient (r)
Phenytoin	0·9510	0·3932	0·98974
Phenobarbitone	0·9997	0·1419	0·99748
Carbamazepine	0·9756	0·2422	0·96924

DISCUSSION

The measurement of serum drug concentration is of increasing significance for the clinical management of ambulatory and hospitalized patients. Ideally,

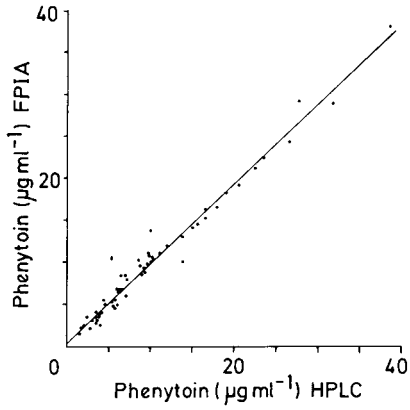

FIG. 2. Estimation of phenytoin concentration in 61 patients by FPIA (y) and HPLC (x).

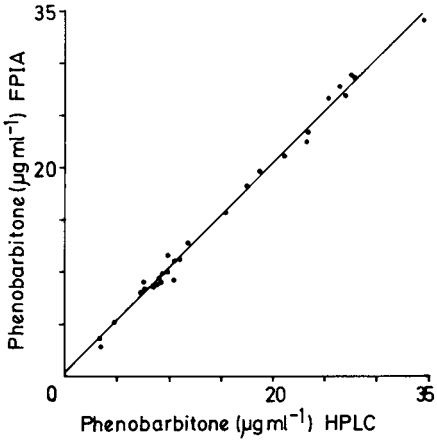

FIG. 3. Estimation of phenobarbitone concentration in 29 patients by FPIA (y) and HPLC (x).

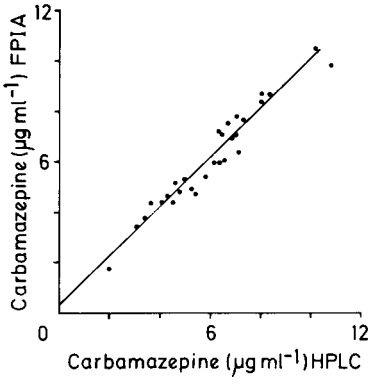

FIG. 4. Estimation of carbamazepine concentration in 32 patients by FPIA (y) and HPLC (x).

therapeutic drug monitoring for phenytoin, phenobarbitone and carbamazepine should be simple, accurate, precise, and available 24 h a day, with a short turn-around time. The within-run and between-run precisions for both methods are excellent with an average CV of less than 7% for the three anticonvulsant drugs. This compares well with a recent study for which a CV of 4·7% has been reported using FPIA for the determination of five anticonvulsant drugs in serum (Vasiliades et al 1983). From our precision studies we conclude that calibration curves for the three anticonvulsant drugs can be used for at least 4 weeks. Because of the stability of the fluorescein-labelled tracer and other reagents, daily calibration of assays is unnecessary as long as controls representing the subtherapeutic, therapeutic and toxic concentrations are run on a daily basis and are within control limits.

The precision is further reflected by the correlation studies. Samples from 122 patients with normal kidney function were measured in duplicate by FPIA and no sample appeared to be different from the HPLC value to a clinically significant degree. The correlation coefficients (r) were: 0·9897 (n = 61) for phenytoin, 0·9975 (n = 29) for phenobarbitone, and 0·9692 (n = 32) for carbamazepine. Figs 2–4 show a very acceptable degree of correlation over the entire range of samples assayed. Statistical evaluation of the duplicate determinations in the FPIA procedure showed that a single assay would be sufficient for clinical purposes. Our results are in agreement with recently published data on anticonvulsant assay by FPIA (Apple et al 1983; Vasiliades et al 1983; Oeltgen et al 1984; Wang & Peter 1985). We conclude that the FPIA method for monitoring phenytoin, phenobarbitone and carbamazepine concentrations in patients' serum is simple, precise, accurate, and correlates well with the HPLC method.

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