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Immunoassay-based determination of phenobarbital using size-exclusion chromatography

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Abstract

The measurement of the anti-epileptic drug phenobarbital from serum samples combining immunoassay and size-exclusion chromatography is presented. The immunoreaction is based on the competitive binding of the analyte (unlabelled phenobarbital) and the fluorescent-labelled phenobarbital to anti-phenobarbital antibodies. Mixing of the reagents and the immunoreaction takes place in a flow system. The products are separated on-line on a short gel chromatographic column and the fluorescence intensity of the marker is measured. The calibration curve shows good linearity in the range 5–80 μg/ml, corresponding to therapeutically relevant serum levels. Intra-day precision values are between 7.32 and 9.48%; the accuracy is between 0.97 and 9.43%. Inter-day precision and accuracy measured on 6 different days fall between 5.38 and 10.05% and −8.27 and −4.97%, respectively. The results obtained with the proposed method show a good correlation with those of other methods (radioimmunoassay and fluorescence polarisation immunoassay) already established in clinical laboratories.

Keywords: Phenobarbital; Fluorescent immunoassay; Size-exclusion chromatography

1. Introduction

Phenobarbital in combination with other anti-epileptic drugs such as phenytoin, or alone, is a frequently used anticonvulsant. The measurement of this drug in body fluids is important in therapeutic drug monitoring and for the optimisation of pharmacotherapy, as low concentrations of phenobarbital can cause break-through seizures, while levels above the therapeutic range may result in toxic symptoms. Laboratory testing is required when either of these two cases occurs. Routine methods for the measurement of anti-epileptic drugs are based on either high-performance liquid chromatography (HPLC) or immunoanalytical techniques.

Liquid chromatographic methods are the most frequently utilised. Chromatographic methods make possible the determination of multiple analytes and their metabolites in a single run [1–4], which is not common in immunoassays. Besides traditional HPLC

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methods [1–7], micellar electrokinetic capillary chromatography [8] and micellar liquid chromatography [9] also find application. The first two groups require time-consuming sample pre-treatment to separate the drug from the serum proteins, although direct serum injections have also been reported [9–12].

Immunoanalytical methods have become widely accepted in clinical laboratories, because the analysis of complex biological samples, often with very low concentrations of analytes, requires high sensitivity and selectivity. Antibodies can ensure that both requirements are fulfilled due to their extremely high affinity and selectivity towards their respective antigen. Consequently, these methods do not need any sample pre-treatment step, which makes the total analysis much faster and simpler. Immunoanalytical methods for the measurement of phenobarbital in serum or plasma overlap a very large scale [13–21]. Simple test strips, as well as methods with very complex instrumentation, usually high sample throughput analysers, are in use. These methods are often equilibrium methods, i.e. the immunoreaction goes to completion, which might take quite a long time. This is especially true with heterogeneous methods where one of the immunoreagents is bound to a solid phase. Homogeneous methods are somewhat faster, but the application of 0.5 to 1 h incubation times are not scarce.

Flow analysis provides a tool for automation and can be adopted to accommodate different types of immunoassays [22–29]. In flow injection immunoassay the disadvantages of the slow immunoreaction can be overcome by performing the assay under non-equilibrium conditions, which can be realised by precise timing of the analysis events. This would result in a fast and reproducible assay.

In the following we demonstrate the applicability of a recently developed flow immunoassay [30] for the determination of phenobarbital in serum. The method is based on the competitive immunoreaction between the fluorescently labelled and unlabelled antigen (phenobarbital) and the antibody. The immunoreagents and the sample are introduced into a chromatograph through a specially designed injector system. The immunoreaction proceeds to a certain extent in a reaction coil, then the products are separated on a short, high-performance gel chromatographic column. Separation is achieved due to the large size difference of the free phenobarbital and the phenobarbital bound to the large antibody. The fluorescent tags of the free and bound labelled phenobarbital are detected in a fluorescence detector.

The analytical performance of the system fulfils the requirements imposed upon bioanalytical assays. Good correlation is obtained with other routinely used immunoanalytical techniques. The total analysis time of one sample is greatly reduced compared to other immunoassay methods, where long incubation times precede the detection step. Large clinical batch analysers, using homogeneous immunoassay methods, like fluorescence polarisation immunoassay can achieve a very short analysis time per sample (2–5 min). However, these systems are not cost-effective to operate with a small number of samples, which is often the case in therapeutic drug monitoring.

In comparison with chromatographic methods, here no sample preparation is needed at all, the serum sample can be injected directly into the system. Our method is simple, cost-effective, relatively rapid, and no high instrumentation level is required. These advantages make this technique a viable candidate in hospital laboratories for the rapid determination of phenobarbital from relatively small samples.

2. Experimental

2.1. Reagents and chemicals

Polyclonal anti-phenobarbital antibody (raised in rabbit), phenobarbital fluorescently labelled with fluorescein-5-isothiocyanate (FITC), lyophilised phenobarbital serum standards (0, 5, 10, 20, 40 and 80 μg/ml) and phosphate buffered saline were part of a fluorescence polarisation immunoassay kit and were kindly provided by the Institute of Isotope (Budapest, Hungary). The serum standards of the kit were used as calibration standards. Blank serum samples spiked to 5, 20 and 40 μg/ml with phenobarbital were used as quality control samples in the validation procedure. The mobile phase used in the gel

chromatographic system was 0.01 *M* phosphate buffer, pH 7.4, prepared from analytical grade KH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$ (Reanal, Budapest, Hungary). For comparison of the method with established clinical assays, two commercially available immunoassay kits were used, RIA-RK-45 and FPIA-PFK-04 (Institute of Isotope).

2.2. Chromatographic system

A schematic of the flow immunoassay system is shown in Fig. 1a. A Beckman 114 M HPLC pump (Beckman Instruments, Berkeley, CA, USA) was applied in the chromatographic system at a flow-rate of 1.0 ml/min. A BIOSEP-SEC-S 2000, 50×7.5 mm gel chromatographic pre-column (Phenomenex, Torrance, CA, USA) was used for separation. The fluorescent-labelled components of the immunoreaction were detected using a Jasco FP-920 intelligent fluorescence detector (Jasco International, Tokyo, Japan) at room temperature with excitation and emission wavelengths set at 470 and 516 nm, respectively. Data acquisition and evaluation were carried out on a 486 AT IBM compatible computer using Borwin 1.21 chromatography software (JMBS Developpements, Le Fontanil, France).

2.3. Sample injection system

The sample introduction mechanism is shown in Fig. 1b. Two six-port rotary injector valves [A: Rheodyne (Rheodyne, Cotati, CA, USA) (loop volume 2×34 μl=68 μl), and B: Upchurch (Upchurch Scientific, Oak Harbor, WA, USA) (loop volume 8 μl)] are connected in such a way that B is inserted into A's loop (nested loop design). When both injectors are in the LOAD position, injector A is filled with a 7.5:1 volume ratio mixture of labelled and unlabelled phenobarbital. Injector B is then filled with the antibody solution. When switching injector B to the INJECT position, the antibody solution plug is inserted in the middle of the antigen solution segment filling the loop of injector A. Switching injector A, the immunoreagents are streamed into a 250 μl mixing coil situated behind the sample introduction port. Here they are mixed and the flow

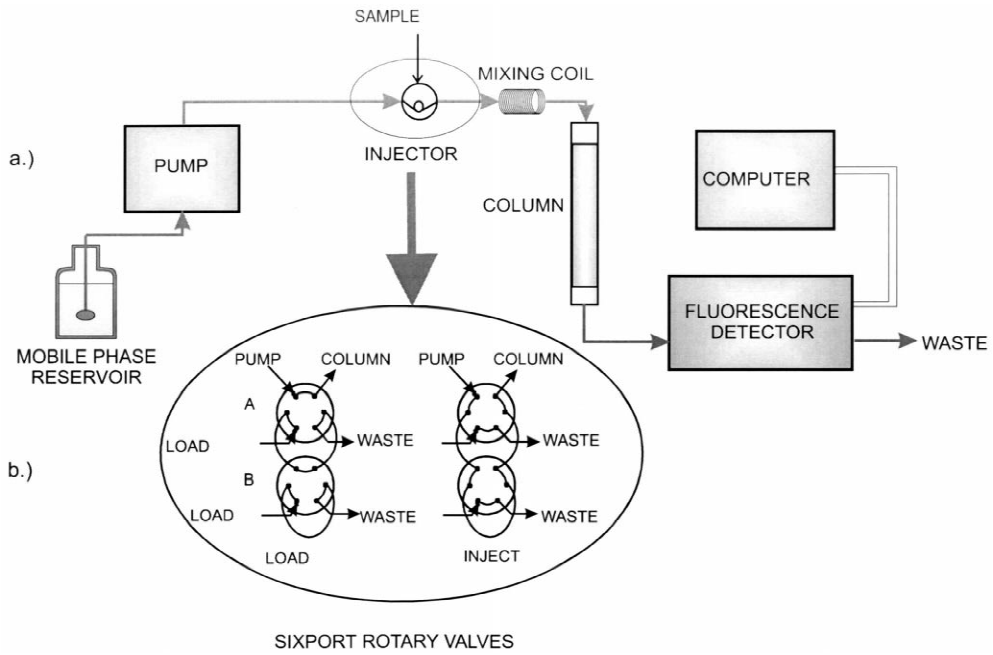


Fig. 1. (a) Schematic design of the flow injection system. (b) Sample introduction system.

is stopped to allow sufficient time for the immunoreaction to take place.

2.4. Chromatographic evaluation

A chromatogram of the 0 and 80 μg/ml calibration standards is shown in Fig. 2a and b. The first peak (at $t_R = 1.20$ min) corresponds to the antibody–fluorescent-labelled phenobarbital complex eluted with the void volume of the gel column. The second peak (at $t_R = 3.60$ min) corresponds to the unbound, fluorescent-labelled phenobarbital which is retained in the pores. The area of either peak can be used for quantitation. In this work the antibody complex peak was chosen for further calculations, since the peak width of this peak is somewhat smaller allowing for more precise peak area determination. For the first peak an inverse proportionality exists between peak area and sample concentration, while for the second peak the two quantities are proportional. Calibration curves were constructed — as is widely done in immunoanalytical practice — by plotting the ratio between the area of the antibody complex measured in each sample (B) and the area of the zero serum standard (B_0) against the logarithmic concentration of the serum standards. This representation results in a reversed S-shaped curve. The middle, linear part of the curve can be used for quantitation. The least squares method was used for linear regression without weighting.

3. Results and discussion

During immunoassay method development, generally the first step is the optimisation of the reagent concentrations, i.e. the antibody dilution, and the labelled antigen dilution to achieve good sensitivity in the desired analyte concentration range. However, as a commercial immunoassay kit was used, previously optimised by the manufacturer, this step could be omitted. As our goal was to set up a non-equilibrium assay, the sensitivity–incubation time dependency was investigated. We also determined the time taken for the immunoreactants to reach equilibrium, to be able to compare our system to traditional equilibrium assays.

3.1. Measurement of the ‘equilibrium incubation time’

The incubation time was determined both in off-line and on-line mode. In off-line mode, the immunoreactants were mixed in a glass vial and allowed to react for different time intervals. The solution was then injected into the chromatograph where the bound and free antigen fraction was separated and measured.

In on-line mode the antibody and the antigen mixture were injected separately into the system, mixed in the mixing coil and the flow was stopped for different time periods. When the flow was restarted the immunoreaction products were streamed through the gel chromatographic column and separated.

Fig. 3 shows the amount of labelled antigen-antibody complex in both on- and off-line modes as a function of the incubation time. The amount of antibody complex is expressed as a percentage relative to the amount formed in equilibrium. No increase in the amount of antibody complex could be observed after incubation times longer than 30 min. This implies that the highest sensitivity in this system, i.e. equilibrium, is achieved after 30 min incubation. It should also be noted that similar peak areas were obtained for the same incubation time in both experiments. This is an indication that there is adequate mixing of the immunoreactants in the flow system and the immunoreaction proceeds to the same extent as in batch.

3.2. Optimisation of the ‘non-equilibrium incubation time’

Since the 30 min incubation time required for equilibrium measurement would have resulted in low sample throughput, the use of a shorter incubation time seemed to be advantageous. As non-equilibrium measurements require precise timing, the overall reproducibility and sensitivity of the system were thoroughly studied. After sample injection, the flow was stopped for different time intervals. Samples were assayed with six parallels at one concentration (5 μg/ml) using 2.5, 5 and 10 min incubation times. The reproducibility calculated for different incubation times is summarised in Table 1. It can clearly be

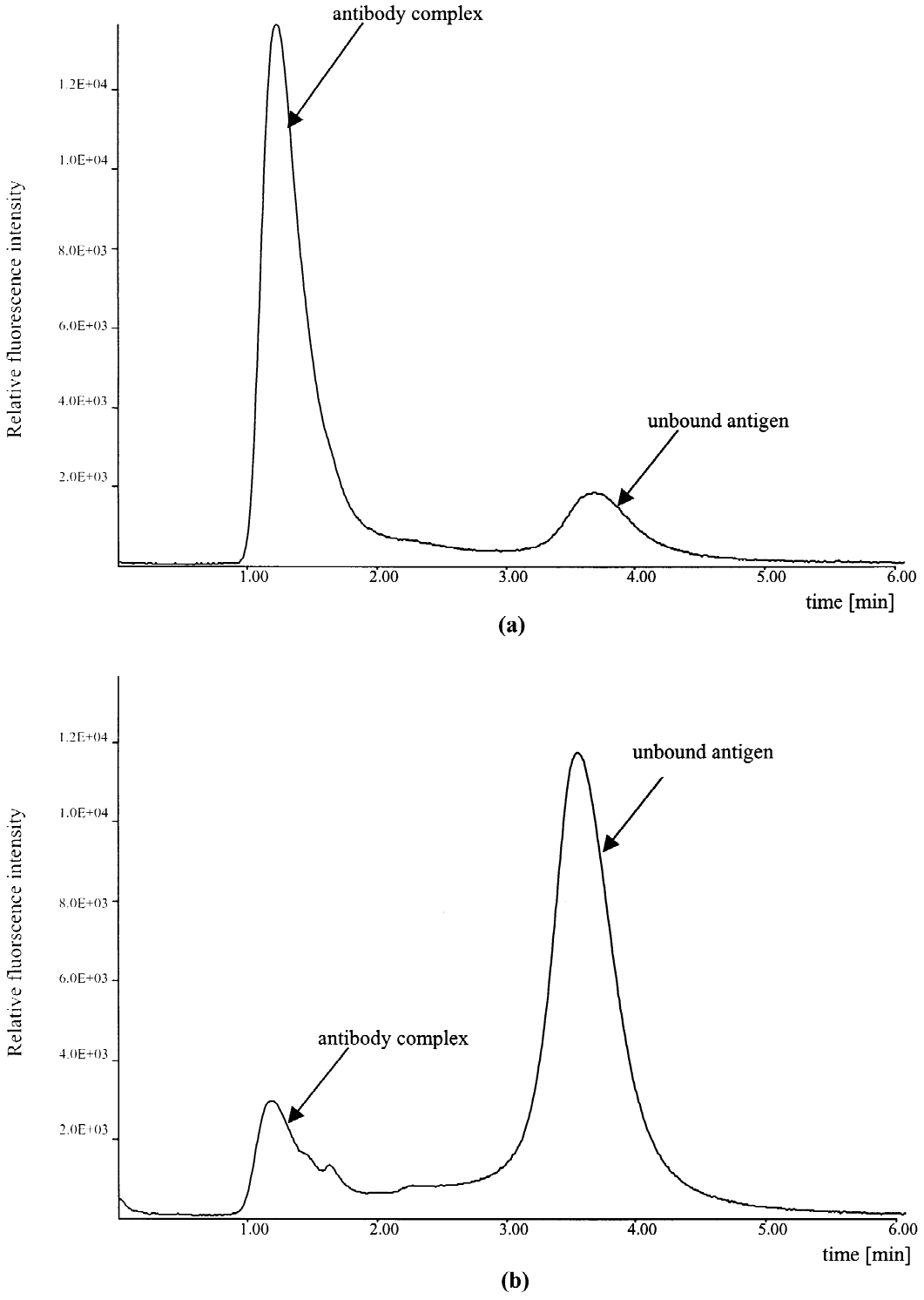


Fig. 2. (a) Chromatogram of the 0 μg/ml phenobarbital serum standard. (b) Chromatogram of the 80 μg/ml phenobarbital serum standard.

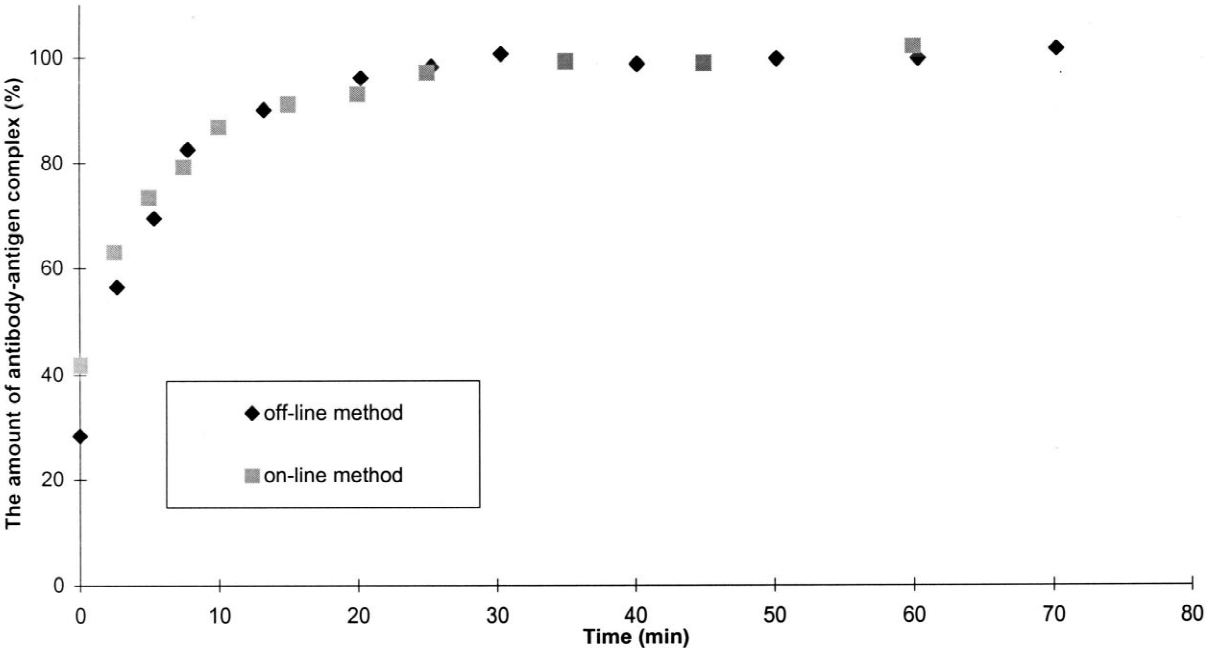


Fig. 3. Variation of the percentage of immunocomplex formed using different incubation times.

Table 1
Influence of incubation time on the reproducibility of the system

Conc. (μg/ml)	Stopped-flow time (min)	Peak area mean±SD	RSD (%)
5	10	$(2.48\pm0.01)\cdot10^5$	4.1
5	5	$(2.22\pm0.10)\cdot10^5$	4.9
5	2.5	$(1.85\pm0.07)\cdot10^5$	3.8

seen that at 2.5 min incubation time the reproducibility is still acceptable. However, further experiments showed that the sensitivity at the upper end of the calibration range (80 μg/ml) is too low, i.e. low concentration of antibody-antigen complex formed. As a reasonable compromise, 5 min incubation time (stopped-flow time) was used for further experiments.

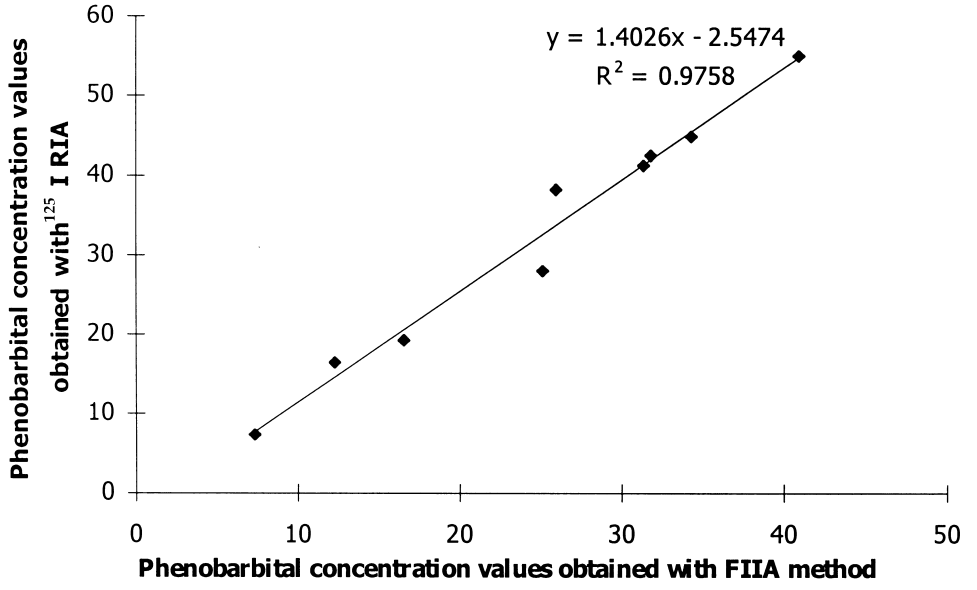
Fig. 3 shows that, after this time elapsed, the conversion of the immunoreaction was ~70%. Using this timing, the total analysis time of one sample, including separation on the gel column, was 10 min.

3.3. Validation of the flow system

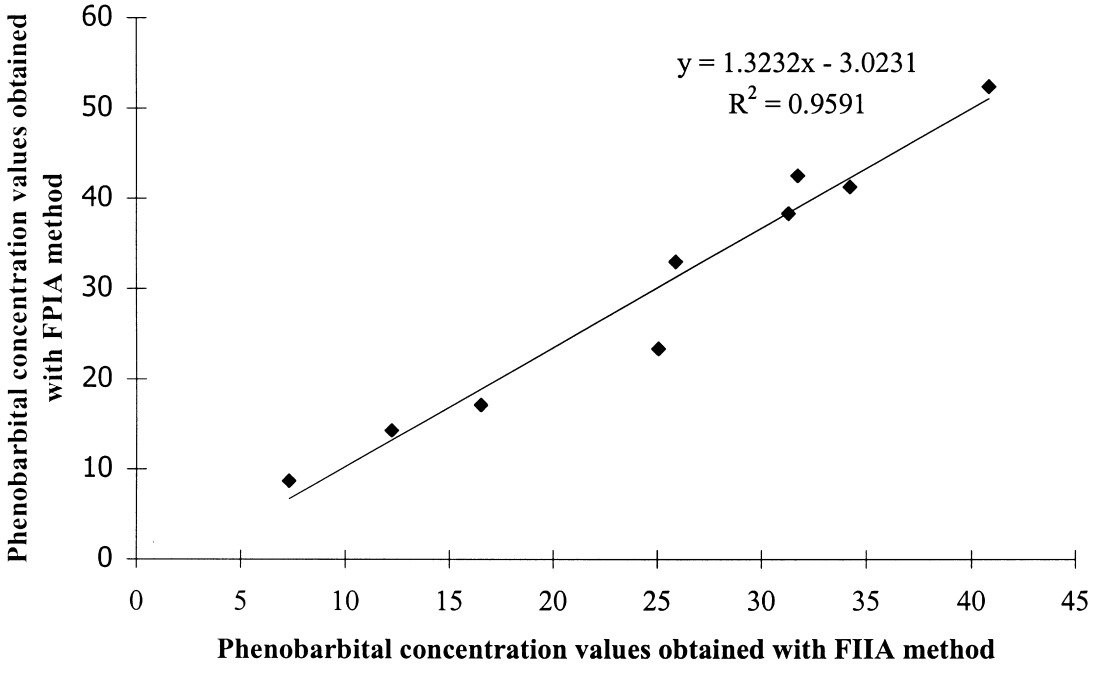
To demonstrate the analytical applicability of the system, validation of the phenobarbital determination in serum was carried out. The linearity of the method was checked by taking calibration curves with phenobarbital serum standards at concentrations of 0,

Table 2
Statistical evaluation of standard curve parameters ($n = 5$)

Slope			Intercept			Regression coefficient		
Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)
0.34	0.04	10.8	0.88	0.05	6.2	0.997	0.002	0.2



(a)



(b)

Fig. 4. (a) Correlation between the FIIA and RIA methods studied on spiked serum samples. (b) Correlation between the FIIA and FPIA methods studied on spiked serum samples.

Table 3
Intra-day precision and accuracy of the FIIA system ($n = 5$)

Nominal conc. (μg/ml)	Conc. found mean±SD (μg/ml)	RSD (%)	Accuracy (%)
5.00	5.1±0.5	9.5	1.0
20.00	21.8±1.6	7.3	9.2
40.00	43.8±3.3	7.5	9.4

Table 4
Inter-day precision and accuracy of the FIIA system ($n = 6$)

Nominal conc. (μg/ml)	Conc. found mean±SD (μg/ml)	RSD (%)	Accuracy (%)
5.00	4.6±0.5	10.1	−8.3
20.00	20.8±1.1	5.4	4.1
40.00	42.0±2.3	5.5	5.0

5, 10, 20, 40 and 80 μg/ml on 5 different days. The calibration range was set to accommodate expected serum levels after oral administration of the drug. The C.V. values of the standard curves are presented in Table 2.

Intra-day precision and accuracy were determined by measuring five parallel phenobarbital serum quality control samples at different concentrations (5, 20 and 40 μg/ml). The precision and accuracy were calculated as the relative standard deviation of the parallel results and as the percentage difference between the measured and nominal concentration relative to the nominal value, respectively. The results obtained are shown in Table 3. The intra-day precision values are between 7.32 and 9.48% and the accuracy is between 0.97 and 9.43%. These values are well below the 15% limit required in bioanalytical methods. Inter-day precision and accuracy were measured on 6 different days using phenobarbital serum quality control samples at different concentrations (5, 20 and 40 μg/ml). The inter-day reproducibility data are presented in Table 4. In this case the precision values fall between 5.38 and 10.05%, and the accuracy values are between −8.27 and 4.97%. These results are also well below the acceptable 15% limit, showing that the method is readily applicable in bioanalytical measurements.

In order to test our method in the measurement of unknown clinical samples a comparison with two well established reference methods was carried out. A drug-free human serum was spiked with various amounts of the pure substance and assayed. The serum samples were analysed with our system, a radioimmunoassay (RIA) method using ^{125}I as a label (RIA-RK-45 immunoassay kit), and a fluorescence polarisation immunoassay (FPIA) method (FPIA-PFK-04 immunoassay kit). The results of our method correlated well with those of RIA ($R = 0.9758$) and FPIA ($R = 0.9591$) (Fig. 4a and b). Table 5 shows the nominal and measured concentrations of spiked serum samples together with the accuracy for each method. From the accuracy values

Table 5
Concentrations of spiked serum samples obtained with the FIIA, RIA and FPIA methods

No. of spiked samples	Phenobarbital concentration (μg/ml)				Accuracy obtained with		
	Spiked	Obtained with			FIIA (%)	RIA (%)	FPIA (%)
		FIIA	RIA	FPIA			
1	12.5	12.3	16.5	14.3	−2.0	31.8	14.2
2	37.5	31.8	42.5	42.5	−15.3	13.3	13.4
3	7.5	7.3	7.3	8.7	−2.4	−2.3	15.7
4	15.0	16.5	19.3	17.1	10.2	28.4	14.3
5	47.5	40.9	55.0	52.4	−13.9	15.9	10.2
6	35.0	31.3	41.3	38.3	−10.6	17.9	9.5
7	37.5	34.3	44.9	41.3	−8.7	19.8	10.1
8	27.5	25.9	38.3	32.9	−5.8	39.1	19.8
9	20.0	24.8	28.0	23.5	35.2	40.1	16.8

it is clear that the two reference methods somewhat overestimate, while our method slightly underestimates, the phenobarbital concentration.

Based on the good correlation of the data sets we propose our method as a viable alternative to other techniques to determine phenobarbital levels in serum.

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