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# SIMPLIFIED QUANTITATIVE DETERMINATION OF PLASMA PHENYTOIN: ON-LINE PRE-COLUMN HIGH-PERFORMANCE LIQUID IMMUNOAFFINITY CHROMATOGRAPHY WITH SAMPLE PRE-PURIFICATION

BENNY JOHANSSON

*Toxicology Section, Department of Clinical Chemistry, General Hospital, S-214 01 Malmö (Sweden)*

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## SUMMARY

A method for on-line high-performance liquid immunoaffinity chromatographic analysis of 5,5-diphenylhydantoin (phenytoin) in human plasma is described. The technique is simple and does not require sample preparation or addition of an internal standard, and the phenytoin is eluted from the columns in 12 min. A phenytoin-specific polyclonal immunoglobulin was attached to an organic silane derivative on silica. The immunosorbent was packed into a short liquid chromatography column and installed in a modified high-performance liquid chromatography system for on-line sample pre-purification. Standard curve linearity of plasma phenytoin was obtained at concentrations up to 160 μmol/l. When compared to conventional high-performance liquid chromatography, plasma phenytoin levels gave a correlation coefficient of 0.993. The phenytoin is highly bound to plasma proteins (ca. 87%). When plasma is injected onto the pre-column, a dissociation and partition of phenytoin from plasma proteins to a complete association with on-column insolubilized antibodies results.

## INTRODUCTION

Conventional analytical purification, extraction and enrichment of low-molecular-weight drugs from body fluids such as plasma, serum or urine is complicated because of the complex nature of the sample [1]. With high-performance liquid chromatography (HPLC), various preparation techniques such as solvent extraction, protein precipitation, direct sample injection, filtration or centrifugation and chromatographic pre-column separation have been used [2, 3].

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An alternative is immunospecific purification of the drugs directly from the biological sample by enrichment on-line onto an immunoselective pre-column in connection with an analytical reversed-phase column. The immobilization method used for gentle and optimum attachment of hapten-specific antibodies to an organic silane derivative on silica is reported elsewhere [4].

This study describes quantitative high-performance liquid immunoaffinity chromatography (HPLIC) applied to the assay of the anticonvulsant phenytoin (5,5-diphenylhydantoin) [5] in human plasma by using an immobilized phenytoin-specific immunoglobulin.

## EXPERIMENTAL

### *Chemicals*

5,5-Diphenylhydantoin was obtained from AB LEO (Helsingborg, Sweden), the centrifree micropartition system from AB Lab Kemi (Gothenburg, Sweden) and LiChrosorb RP-18 (7 μm) and LiChrosorb Si 60 (10 μm) from Merck (Darmstadt, F.R.G.). All other chemicals mentioned were of analytical grade and supplied by Merck.

### *Apparatus*

Liquid chromatography was performed using a modified HPLC system composed of a Model 6000A high-pressure pump (Waters Assoc., Milford, MA, U.S.A.) and a Model Perpex peristaltic pump (LKB, Bromma, Sweden). UV detection was monitored with a Waters Model 400 UV—VIS absorbance detector and samples were injected into a Waters U6K loop injector. The immunosorbent column was connected to a pneumatic six-port valve (Rheodyne, Cotati, CA, U.S.A.) for back-flash elution.

### *Immunosorbent material*

The immunosorbent was made up according to the method described elsewhere [4] by attachment and immobilization of an affinity-purified phenytoin-specific immunoglobulin to an organic silane derivative on silica.

LiChrosorb Si 60 (10 μm) was transformed into its γ-glycidoxypropyltrimethoxy derivative [9] and oxidized first to its aldehydic form [10] and then converted to the corresponding carboxylic acid derivative [4]. The latter was then esterified with N-hydroxysuccinimide [11], and the active ester was immobilized in the last step with an affinity-purified immunoglobulin solution [12].

Storage conditions were 0.01 mol/l phosphate-buffered saline (pH 7.2) containing the preservative merthiolate (0.02%) at 4°C.

### *Liquid chromatography*

The immunosorbent was slurry-packed in a short pre-column (50 × 4 mm I.D.) with distilled water for 1.5 h at 200 bar. The pre-column was combined with modified HPLC equipment (Fig. 1). For analytical analysis, the immunosorbent column was equilibrated in 0.10 mol/l phosphate buffer (pH 7.4) from pump I, and the analytical reversed-phase column (LiChrosorb RP-18, 7 μm, 25 cm × 3.9 mm I.D.) was equilibrated in desorption buffer consisting of equilibrating buffer—ethanol (99%) (60:40, v/v) from pump II.

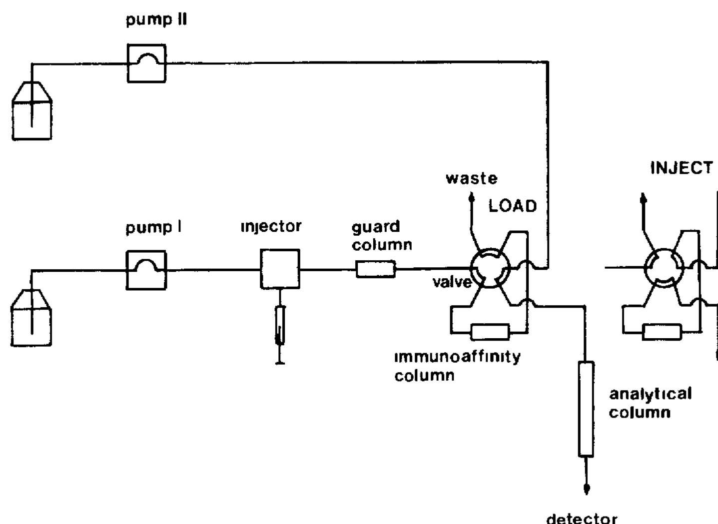


Fig. 1. HPLIC system. The immunoaffinity column was equilibrated in 0.1 mol/l phosphate buffer (pH 7.4) from pump I and the analytical column in desorption buffer consisting of equilibrating buffer—ethanol (99%) (60:40, v/v) from pump II. A 25-µl aliquot of a sample or a standard solution was injected into the loop injector. Phenytoin was enriched on the immunosorbent. After washing the column in phosphate buffer for 4 min, the phenytoin (I, II) was eluted during the next 2-min period by the desorption buffer [LOAD → INJECT (IV) → LOAD (III)], separated by the analytical column and detected at 254 nm.

After injection of the sample or plasma standard solution, the immunosorbent column was washed with equilibration buffer for 4 min and the switching valve (Fig. 1) was changed from LOAD to INJECT. Specifically retained phenytoin was then eluted from the column during the next 2-min period (IV), whereupon the column was re-equilibrated in buffer (III). Desorbed phenytoin (I, II) was separated from a cross-reacting substance by the analytical column before detection at 254 nm. Compounds without affinity for the immunosorbent were eluted in the waste fluid.

According to results reported elsewhere [4], an optimal mobile phase flow-rate of below 1.5 ml/min was found to be necessary. In these experiments, the flow-rate was set at 1.3 ml/min. The volume of the plasma sample and of the phenytoin standard solutions (20—160 µmol/l) was 25 µl. All samples were analysed at ambient temperature (21—24°C).

*Recovery, precision and selectivity*

Analytical recovery of phenytoin retained by the immunosorbent column was measured by injection of a 25-µl phenytoin standard solution (80 µmol/l) in triplicate at increasing ethanol concentrations in the mobile phase, and compared with direct reversed-phase analysis.

The precision of the method was tested in five determinations at two plasma concentration levels, 20 and 80 µmol/l, respectively. Interference from commonly used anticonvulsant drugs was investigated at a therapeutic plasma concentration level of valproic acid (450 µmol/l), carbamazepine (30 µmol/l), phenobarbital (100 µmol/l) and of ethosuximide (400 µmol/l), respectively, by

adding a known amount of each drug to plasma containing phenytoin (60 μmol/l).

*Comparison with HPLC*

The plasma samples from phenytoin-treated epileptic patients were analysed for the phenytoin content by comparing the results with those obtained by ordinary HPLC [6]. Plasma samples of 25 μl were injected onto the immunosorbent column. The content of the plasma samples was quantified using a plasma standard solution containing phenytoin in concentrations ranging from 20 to 160 μmol/l (therapeutic plasma concentrations are 40–80 μmol/l).

*Immunosorbent binding study of free/plasma-protein-bound phenytoin*

Four plasma samples from the ordinary epileptic patient pool were analysed for the free physiologically active phenytoin fraction in binding equilibrium with plasma proteins. Ultrafiltration was used for pre-separation of the free fraction [7]. The free and total phenytoin concentrations in the samples were assessed by HPLIC and HPLC [6]. A 200-μl aliquot of plasma-protein-free solution containing the free phenytoin fraction was obtained from a 500-μl plasma sample transferred to a centrifree separation tube, and centrifuged at 2500 *g*. A 25-μl aliquot of the supernatant, and of the original plasma sample, respectively, were injected onto the immunosorbent column, immobilized with affinity-purified antibodies and analysed for the phenytoin concentration.

## RESULTS

*Chromatography and standard curve*

The chromatogram in Fig. 2 shows a plasma sample from a phenytoin-treated epileptic patient (a) and a plasma standard (b), analysed and enriched on the immunosorbent column. The standard graph plot, represented by the peak area plotted against the double therapeutic plasma concentrations of phenytoin (20–160 μmol/l), was linear.

*Analytical recovery, precision and selectivity*

The average peak-area immunosorbent phenytoin recovery compared to reversed-phase analysis was estimated to be 98% at an ethanol content of 40% (v/v) in the mobile phase.

The between-run coefficients of variation were 1.0% (19.3 ± 1.9) at 20 μmol/l and 4.2% (78.8 ± 3.3) at 80 μmol/l, respectively.

The assay was shown to be completely free from interference from the anticonvulsant drugs tested.

*Analysis of phenytoin*

The relationship between the concentrations of phenytoin in ten plasma samples analysed by HPLIC compared to conventional HPLC [6] is illustrated in Fig. 3. The values between the two methods were in good agreement and had a coefficient of correlation of 0.993 ($P < 0.001$).

*Free/plasma-protein-bound phenytoin*

The influence of plasma protein binding on recovery was tested with

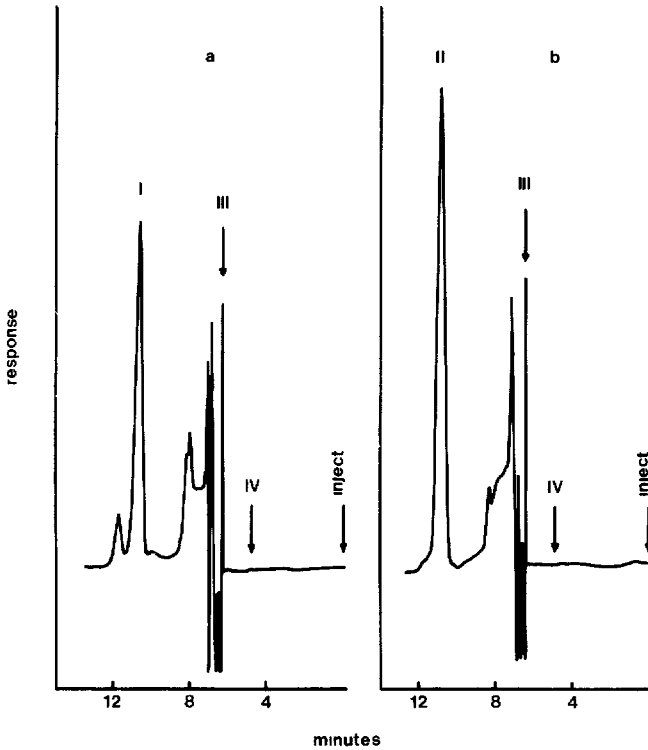


Fig. 2. HPLIC chromatogram of a human plasma sample (a) and a plasma standard solution (b) containing phenytoin 113 μmol/l (I) and 160 μmol/l (II), respectively. Chromatographic conditions as in Fig. 1.

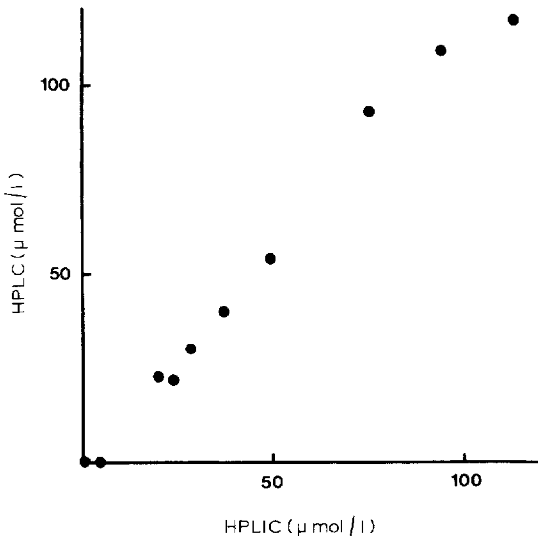


Fig. 3. The correlation between determinations of phenytoin concentrations in human plasma samples measured by HPLC and HPLIC (affinity-purified immunoglobulin); $y = 1.13x - 1.30$, $r = 0.993$.

reference to free and total amounts of phenytoin in plasma, respectively, by analysing four plasma samples after pre-separation of the free drug fraction [7].

Table I shows the results from the values of the percentile plasma-protein-bound phenytoin fractions assessed by the two methods.

From the results, it is confirmed that free and protein-bound phenytoin, respectively, are quantitatively partitioned from plasma and distributed onto the immunosorbent.

TABLE I

PLASMA-PROTEIN-BOUND 5,5-DIPHENYLHYDANTOIN ASSESSED BY HPLC AND HPLIC

All values reported are an average of two determinations.

| Human plasma sample No. | Percentage bound | |
|---|---|---|
| | HPLC | HPLIC |
| 1 | 92 | 91 |
| 2 | 86 | 90 |
| 3 | 89 | 88 |
| 4 | 82 | 81 |

*Immunosorbent column stability*

The immunosorbent column stability was checked continuously during a three-month period by analysing plasma standard solutions (20—160 μmol/l). The linearity and slope of the standard curves were found to be identical. Approximately 200 injections of plasma were made onto the immunosorbent column during this period of time. The column was stored in equilibration buffer containing merthiolate (0.02%) at 4°C when not in use.

## DISCUSSION

The present study describes a simplified on-line sample immunoselective purification HPLC assay for phenytoin in human plasma. Simultaneous on-line immunoselective enrichment of phenytoin from directly injected plasma onto the pre-column, and subsequent separation by an analytical reversed-phase column, seems to offer an alternative for quantitative low-molecular analysis. The method provides selective enrichment of phenytoin without loss of substance and without interference from metabolites or chemically and physically analogous endogenous compounds. This results in increased accuracy and sensitivity. The procedure is simple, requires no manual sample preparation or need for an additional internal standard, and phenytoin elutes from the column in 12 min.

Specifically retained phenytoin was eluted with approximately 2 ml of desorption buffer via back-flushing. Elution was performed in the reverse direction of sample application to avoid additional band broadening and tailing effects, probably caused by the heterogeneity and variability of the insolubilized polyclonal immunoglobulins and the influence of unoccupied

antibody binding sites. The affinity-purified immunoglobulin used for conjugation to the silica derivative spheres was a low avidity and high binding capacity immunoglobulin fraction. From the correlation relationship shown in Fig. 3, a selective drug—antibody interaction is demonstrated, completely without endogenous non-specific interference. Quantitatively reliable results are obtained independently of the disposition for cross-reactivity with the main metabolite of phenytoin, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin. When the described method was designed as a model chromatography method for on-line HPLIC enrichment of low-molecular-weight compounds from biological samples, it was found sufficient to use only ten samples in the comparison study.

Hydrophilic phenytoin metabolites accumulate in plasma from patients with renal failure [8]. The cross-reactivity of these metabolites with the immobilized antibodies has not been tested. However, if there exists any enrichment of metabolites in the isolation step, these are easily separated on the analytical column.

At equilibrium conditions, the total amount of drug in plasma is the sum of protein-bound drug and the free drug fraction. In the assay, both fractions are simultaneously introduced onto the column. The phenytoin—plasma-protein conjugate is dissociated, solubilized and quantitatively partitioned and associated with immobilized antibodies. The partition of phenytoin from plasma proteins onto the immunosorbent depends on different avidities, characterized by the corresponding binding constants. The quantitative redistribution of phenytoin from the proteins onto the immunosorbent indicates a higher antibody avidity ($K_a = 7.6 \cdot 10^7$ l/mol) [4] compared to the influence from plasma proteins ($K_a = 4 \cdot 10^3$ l/mol) [8].

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