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Simultaneous determination of methylphenobarbital enantiomers and phenobarbital in human plasma by on-line coupling of an achiral precolumn to a chiral liquid chromatographic column

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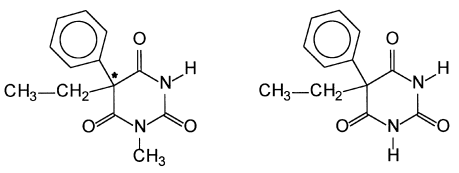
Abstract

A fully automated liquid chromatographic (LC) method for the simultaneous determination of methylphenobarbital enantiomers and phenobarbital in human plasma has been developed. The method is based on the use of a precolumn packed with an internal-surface reversed-phase packing material (LiChrospher ADS) for sample clean-up coupled to LC analysis on a cellulose tris(4-methylbenzoate) based chiral stationary phase (Chiralcel OJ-R). A 100-μl plasma sample was injected directly on the precolumn packed with LiChrospher RP-18 ADS using a mixture of pH 5.0 phosphate buffer–methanol (97:3, v/v) as washing liquid. The analytes were then eluted in the back-flush mode with the LC mobile phase. The enantiomeric separation of methylphenobarbital was achieved on Chiralcel OJ-R. The retention times were modelled using a D-optimal design with ten experimental points in order to optimise the LC mobile phase for the separation of phenobarbital from the enantiomers of mephobarbital. The factors selected were the acetonitrile content, the pH and the sodium perchlorate concentration in the mobile phase. A Derringer's desirability function was used to find an optimal and robust solution within the experimental domain. The mobile phase selected consisted of a mixture of pH 7.0 phosphate buffer–acetonitrile (60:40, v/v). The elution profiles of phenobarbital, methylphenobarbital and blank plasma samples on the precolumn and the time needed for analyte transfer from the precolumn to the analytical column were then determined. Finally, the method developed was validated.

Keywords: Enantiomer separation; Methylphenobarbital; Phenobarbital

1. Introduction

Methylphenobarbital (MPB) is an antiepileptic chiral drug (cf. Fig. 1) that is clinically administered as a racemate. Different studies have shown that MPB is stereoselectively metabolised in the body [1–4] and that the (−)-*R*-enantiomer of MPB is narcotically active while the (+)-*S*-MPB has convulsant properties, an effect which is not apparent when the racemate is used [5].



Methylphenobarbital (pKa = 8.0) Phenobarbital (pKa = 7.4)

Fig. 1. Structures of methylphenobarbital and phenobarbital. The stereogenic centre is marked by an asterisk.

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On the other hand, it is interesting to note that the metabolisation of methylphenobarbital mainly leads to the formation of a non-chiral compound, phenobarbital (PB) (cf. Fig. 1) [6].

A number of methods have been developed for the quantitative analysis of barbiturates and particularly MPB and PB. They are based on polarography, potentiometric titration, UV spectrophotometry, gas chromatography (GC) and especially liquid chromatography (LC) [7–15]. Several LC methods have been applied to the determination of MPB or PB in biological fluids [10–15].

The stereoselective determination of methylphenobarbital has been achieved by chiral GC [16,17] or LC [18–29], but very few results about the analysis of MPB enantiomers in plasma have been reported. The chiral stationary phases (CSPs) used for the LC enantioseparation of MPB involve different classes of chiral selectors such as β-cyclodextrins [18] and β-cyclodextrin derivatives [19,20], proteins [21–23] and cellulose derivatives [24–29].

Chiralcel OJ-R is a CSP based on the use of cellulose tris (4-methylbenzoate) as chiral selector which can be used with aqueous mobile phases [29–31], the reversed-phase mode being particularly interesting for bioanalytical applications.

The column switching technique is an interesting alternative to liquid–liquid or off-line solid-phase extraction for the preparation of biological samples. This automated technique which involves the use of two columns connected by a switching valve is particularly useful when a large number of assays has to be performed. The role of the first column (precolumn) is to retain selectively the analytes. After rotation of the switching valve, the latter are eluted and transferred to the analytical column and individually quantitated [32].

One of the most drawbacks of column switching systems is the risk of protein adsorption in the precolumn when it is filled with conventional reversed-phase material. This results in a decrease in performance, an increase in back pressure and a limited lifetime of the precolumn. Recently, Boos et al. introduced a new kind of restricted access materials called alkyl–diol silica (ADS) which can be packed in small precolumns used for the clean-up of protein-rich samples in column switching systems [12,33–35].

This kind of internal-surface reversed-phase packing material presents two different surfaces: hydrophilic diol groups are bound to the external surface of the particles (25 μm) and prevent the adsorption of proteins while the internal surface consists of a hydrophobic octadecyl bonded phase accessible to low-molecular-mass compounds such as drugs or metabolites. The LiChrospher ADS column has a pore diameter of about 6 nm and a molecular mass cut-off of 15 000.

The aim of this work is to develop a fully automated coupled-column LC method for the simultaneous determination of MPB enantiomers and PB in plasma.

The first part includes the selection of a chiral stationary phase and the optimisation of the LC conditions in order to obtain an adequate separation and quantitation of the enantiomers of MPB and PB. Retention factors (k') are modelled to predict chromatographic parameters in the experimental domain and a multicriteria decision method using the Derringer's desirability function is applied to find an optimum and robust solution. The second part is focused on the development of the sample preparation procedure and in particular on the selection of appropriate washing liquid and valve switching time. Finally, the method developed is validated.

2. Experimental

2.1. Chemical and reagents

RS-Methylphenobarbital was purchased from Siegfried (Zofingen, Switzerland) and phenobarbital from Sigma (St. Louis, MO, USA). *R*-Methylphenobarbital and *S*-methyl phenobarbital were prepared using the simulated moving bed (SMB) technology [36–38] and were then purified by crystallization. Each enantiomer was identified by polarimetric measurements.

Sodium dihydrogenphosphate dihydrate, sodium hydroxide, phosphoric acid (85%) and sodium perchlorate monohydrate were all of analytical grade from Merck (Darmstadt, Germany). Methanol and acetonitrile were of HPLC grade from Fisher Scientific (Loughborough, UK). Water used in all

experiments was of Milli-Q quality (Millipore, Bedford, MA, USA).

The chiral stationary phase used for the enantioseparation of methylphenobarbital was Chiralcel OJ-R (5 μm) packed in a column (150×4.6 mm I.D.) from Daicel (Tokyo, Japan). The latter was preceded by a LiChroCart guard column (4×4 mm I.D.), packed with LiChrospher 100 DIOL (5 μm) from Merck.

The precolumn was a LiChroCart (25×4 mm I.D.) column packed with the LiChrospher RP- 18 ADS (25 μm), from Merck.

2.2. Apparatus

The chromatographic instrumentation consisted of the following units: a model 422 HPLC pump from Kontron Instruments (Schliereu, Switzerland) (pump 1), a model L-6200 A (pump 2), a model AS-2000 A autosampler equipped with a 100 μl loop, a L-5025 programmable column oven and a L-4250 UV–Vis detector, all from Merck–Hitachi. The switching valve was a universal valve switching module from Anachem (Luton, UK).

An IBM compatible computer equipped with the D-7000 HPLC Manager software was used to control the LC system and to collect the data that were printed on a HP 500 Deskjet printer (Hewlett–Packard, Palo Alto, CA, USA). The 422 HPLC pump (pump 1) was controlled manually.

2.3. Chromatographic technique

All chromatographic experiments were carried out in the isocratic mode. Preliminary studies for obtaining the enantioseparation of MPB on Chiralcel OJ-R were performed with mobile phases consisting of mixtures of 50 m*M* phosphate buffer and acetonitrile. For the optimisation of the LC conditions for the separation of methylphenobarbital enantiomers and phenobarbital, the mobile phases consisted of mixtures of acetonitrile and 10 m*M* phosphate buffers containing sodium perchlorate.

The mobile phase used for method validation consisted of a mixture of a 10 m*M* phosphate buffer (pH 7.0)–acetonitrile (60:40, v/v).

The liquids used for sample loading and clean-up were mixtures of 50 m*M* phosphate buffer and methanol.

The flow-rate was 0.8 ml min^{-1} for the sample clean-up (pump 1) and 0.6 ml min^{-1} for the chiral LC analysis (pump 2). The analytical column and the LC mobile phase were kept at 27°C in the programmable oven and the UV detection was performed at 225 nm.

2.4. Solutions

2.4.1. Solutions used for the optimisation of chiral LC conditions

Two stock solutions of *RS*-methylphenobarbital (*RS*-MPB) and phenobarbital (PB) were prepared by dissolving 50 mg of each compound in 50 ml of methanol. A mixed solution of *RS*-MPB and PB was obtained by diluting with water 1.0 ml of each stock solution to a final volume of 50 ml (20 μg ml^{-1} for each barbiturate).

2.4.2. Solutions used for development of the sample preparation procedure

The methanolic stock solutions of *RS*-MPB and PB were diluted with water to obtain two solutions containing either 100 μg ml^{-1} of *RS*-MPB or 100 μg ml^{-1} of PB. A mixed solution containing 100 μg ml^{-1} of the two barbiturates was also prepared.

2.4.3. Solutions used for method validation

A stock solution containing *RS*-methylphenobarbital and phenobarbital was prepared by dissolving 125 mg of each compound in 50 ml of methanol. This solution was then diluted with water to obtain concentrations of 500 μg ml^{-1} and 125 μg ml^{-1} for both analytes. These two solutions were used to spike plasma samples (2 ml) for calibration curves (from 1.25 to 50 μg ml^{-1} for PB and from 0.625 to 25 μg ml^{-1} for each enantiomer of MPB).

2.5. Sample preparation procedure

The plasma sample was first centrifuged at 4500 rpm for 10 min and a 1.0 ml volume of plasma was transferred manually into a vial on the appropriate rack of the autosampler. All the other operations were then executed automatically. The sample preparation was performed in two steps.

(1) Injection and washing of sample: a 100-μl plasma sample was injected, the column-switching system being set according to Fig. 2A. The sample was washed for 8 min with a mixture of phosphate buffer (pH 5.0) and methanol (97:3, v/v). The flow-rate was 0.8 ml min^{-1}. Proteins and hydrophilic endogenous compounds were eliminated from the precolumn while the analytes were retained. Mean-

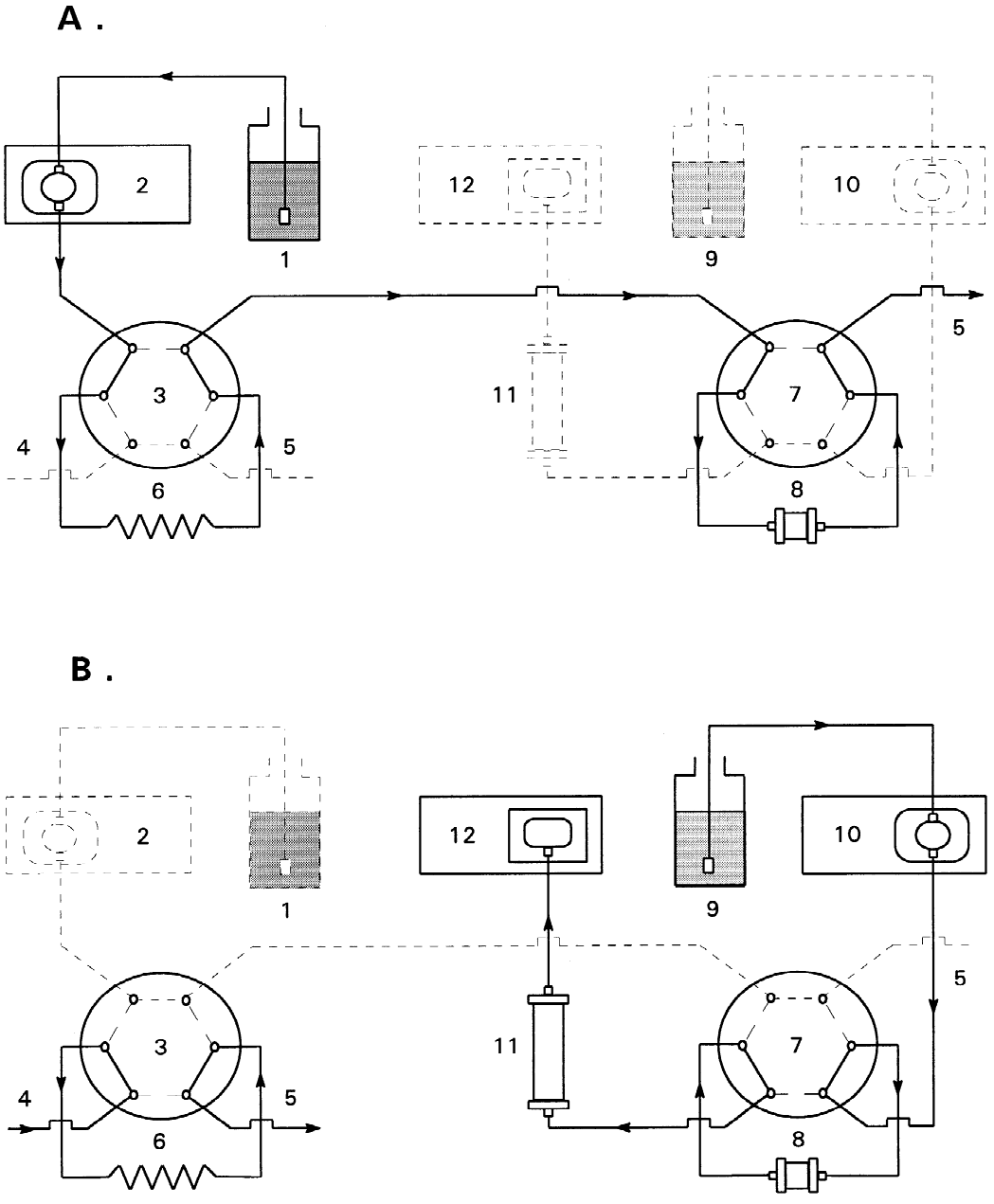

Fig. 2. Schematic representation of a column-switching system. 1. Washing liquid; 2. HPLC pump 1; 3. injection valve; 4. sample loading; 5. waste; 6. injection loop; 7. switching valve; 8. ADS precolumn; 9. HPLC mobile phase; 10. HPLC pump 2; 11. analytical column; 12. detector.

while, the analytical column was re-equilibrated with the LC mobile phase.

(2) Elution of the analytes from the precolumn and chiral LC analysis: After 8 min, the valve was switched in order to connect the precolumn with the analytical column. The analytes were eluted in the back-flush mode by the LC mobile phase (cf. Fig. 2B). Five min later, the switching valve was returned to its initial position and the precolumn was re-equilibrated with the washing liquid. In the meantime, the LC analysis was performed on the chiral column.

3. Results and discussion

3.1. Optimisation of LC conditions

3.1.1. Enantioseparation of methylphenobarbital

The first step in the development of the chiral LC method was the selection of the chiral stationary phase. The Chiralcel OD-R CSP, on which a wide range of chiral separations were performed, was first tested but did not lead to enantiomeric resolution for methylphenobarbital. Another cellulose based CSP, also used in the reversed-phase mode, Chiralcel OJ-R, was then selected, since the enantioseparation of MPB was previously reported on this CSP using mobile phases consisted of mixtures of methanol and water, acetonitrile or methanol and aqueous perchlorate solution [29].

The influence of the mobile phase pH on the separation of MPB enantiomers was studied with a mobile phase consisting of a 50 m*M* phosphate buffer–acetonitrile (50:50, v/v). As expected (cf. Table 1), changes in retention, enantioselectivity and resolution for MPB enantiomers were limited in the pH range from 3 to 7. A slight decrease in resolution was observed at pH 7, which is obviously related to the dissociation of the compound (pK_a=8.0). However, owing to the very high stereoselectivity obtained with this CSP, an impressive resolution value (7.2) could be obtained at that pH for MPB enantiomers.

Acetonitrile was the only organic modifier tested in this study. The mobile phases consisted of mixtures of pH 5.0 phosphate buffer and acetonitrile. The concentration of the latter was varied from 30 to 65%. As can be seen in Table 2, an increase in the acetonitrile concentration causes the retention times for both enantiomers and resolution to decrease. However, enantioselectivity was less influenced by these changes in acetonitrile concentration. It should be noted that even in the higher concentration range, complete enantiomeric resolution (R_s>4) was obtained for MPB.

Table 1
Influence of mobile phase pH on retention and enantioseparation of methylphenobarbital enantiomers

	Mobile phase pH		
	3.0	5.0	7.0
k'_S	1.07	1.06	0.95
k'_R	2.27	2.24	1.97
α	2.11	2.11	2.09
R_s	8.18	8.25	7.20

Chromatographic conditions: Chiralcel OJ-R, phosphate buffer (50 m*M*)–acetonitrile (50:50, v/v), flow-rate: 0.6 ml min^{-1}, detection UV at 225 nm, aqueous solution of MPB (20 μg ml^{-1}).

3.1.2. LC separation of methylphenobarbital enantiomers and phenobarbital

In order to optimise the analytical method for the separation of methylphenobarbital enantiomers and phenobarbital (PB), a D-optimal design with ten experimental points (Table 3) was selected. The mobile phase pH, the organic modifier (acetonitrile) content and the sodium perchlorate concentration were the three factors selected in this experimental design [29,30].

Taking into account the pK_a values of PB and MPB (7.4 and 8.0, respectively), the mobile phase pH range was comprised between 5 and 7 while the

Table 2
Influence of acetonitrile concentration on retention and enantioseparation of methylphenobarbital. Chromatographic conditions: Chiralcel OJ-R, pH 5.0 phosphate buffer (50 mM)–acetonitrile, flow-rate: 0.6 ml/min, detection UV at 225 nm, aqueous solution of MPB (20 μg/ml)

	Acetonitrile (%)							
	30	35	40	45	50	55	60	65
k'_S	4.77	3.05	2.00	1.47	1.06	0.79	0.59	0.40
k'_R	10.62	6.66	4.29	3.15	2.24	1.65	1.23	0.87
α	2.23	2.18	2.14	2.14	2.11	2.10	2.11	2.16
R_s	13.81	11.65	8.72	9.80	8.25	6.92	5.65	4.43

Table 3
Experiments required for the D-optimal design used in the optimisation of chromatographic conditions for the separation of methylphenobarbital enantiomers and phenobarbital

Experiment	pH	Acetonitrile (%)	$NaClO_4$ (*M*)
1	5.0	30	0
2	5.0	30	0.25
3	5.0	65	0
4	5.0	65	0.125
5	5.0	65	0.25
6	7.0	30	0
7	7.0	30	0.125
8	7.0	30	0.25
9	7.0	65	0
10	7.0	65	0.25

percentage of acetonitrile ranged from 30 to 65% and the concentration of $NaClO_4$ was varied from 0 to 0.25 *M*.

The D-optimality [39] was obtained assuming that in the experimental domain the logarithm of the retention factor (k') can be modelled as a linear function of pH (X_1) and percentage of acetonitrile (X_2) but as a quadratic function of $NaClO_4$ concentration (X_3) [40,41]. The two-factor interactions were also considered in the model:

$$\ln k' = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_3^2 + \beta_5 X_{12} + \beta_6 X_{13} + \beta_7 X_{23} \quad (1)$$

where X_1, X_2 and X_3 are the variables (factors) in the model and X_{12}, X_{13} and X_{23} refer to the corresponding interactions between these factors two by two. The β coefficients correspond to the parameters of the model to be estimated.

After having performed the ten experiments, nine hyper-surfaces for the logarithms of the retention factors of all key times for the three peaks, i.e. times at the beginning, at apex and at the end of the peaks as well as three hyper-surfaces for the heights of the peaks at apex were then fitted using the selected model (cf. Eq. (1)). No assessment of the quality of fit was performed.

The critical chromatographic parameters, i.e. the minimum resolution ($R_{s_{min}}$), the minimum retention time (t_{rmin}), the maximum retention time (t_{rmax}) and the peak asymmetry, were numerically derived from the fitted hyper-surfaces over the whole experimental domain using a 10^3-points treillis digitizing technique. The local derivative of $R_{s_{min}}$ was also calculated at each point in the experimental domain by taking the average difference with its neighbours on the 10^3-points treillis. The local derivative of $R_{s_{min}}$ is here considered as a robustness criterion [42] since it quantifies the local rate of change of the separation with respect to small changes in the operational factors, i.e. the pH, the percentage of acetonitrile and the concentration of sodium perchlorate.

The $R_{s_{min}}$, its local derivative, the t_{rmin}, and the t_{rmax} as well as the asymmetry factor were combined in a Derringer's desirability function [43,44] to find an optimal and robust solution within the experimental domain, i.e. practically within the set of 10^3 points. The Derringer desirability Function allows to find efficiently a trade off between several criteria or responses to be optimised jointly.

The lower and the upper limits used for $R_{s_{min}}$, its local derivative and the asymmetry factor were the minimum and maximum observed values, respectively, over the set of 10^3 values obtained as indicated above. For the maximum retention time, the lower limit was the minimum value observed while the upper limit was 20 min. For the minimum retention time, the lower limit was fixed to 6 min while the upper limit was fixed at the maximal value observed over the set of 10^3 values. These limits were defined to obtain a reasonable analysis time (20 min) and to avoid interferences of endogenous compounds eluted just after the solvent peak.

The mobile phase corresponding to the optimal solution obtained by this technique consisted of a mixture of pH 7.0 phosphate buffer–acetonitrile (59.5:40.5, v/v), the sodium perchlorate concentration being equal to 0 (cf. Fig. 3).

3.2. Development of the preparation procedure

In order to develop a method for the simultaneous determination of methylphenobarbital enantiomers and phenobarbital in plasma using a column-switching system with a precolumn packed with restricted access material, it was necessary to determine the most appropriate valve-switching times.

3.2.1. Determination of the elution profiles of the analytes and selection of the washing liquid

The first step consisted in the determination of the

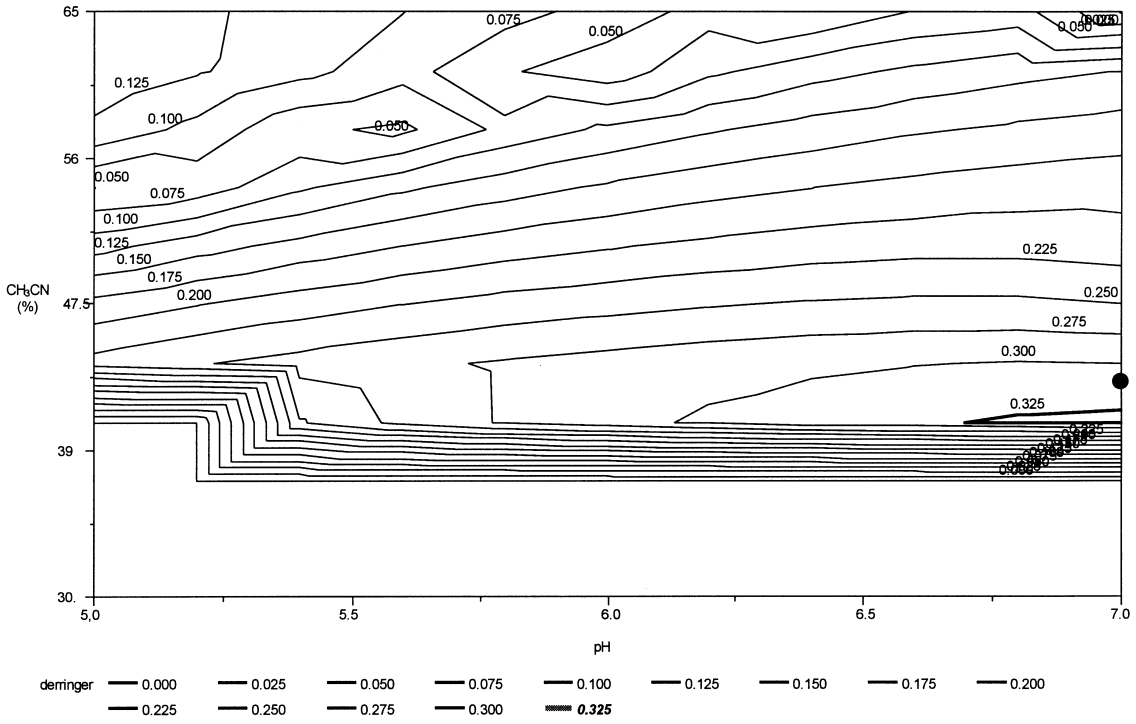

Fig. 3. Predicted contour plot of the Derringer's global desirability as a function of pH and acetonitrile when sodium perchlorate concentration is set to 0. The filled circle indicates the predicted optimal solution.

elution profiles of the analytes in order to deduce the time from which a beginning of elution of the analytes would take place. This time obviously depends on the composition of the washing liquid, its flow-rate and also on the type of solid-phase in the precolumn and the dimensions of the latter.

The elution profiles of MPB and PB on a C_{18}-ADS precolumn with a washing liquid consisting of a 50 m*M* phosphate buffer containing a small percentage of methanol were determined by monitoring the UV absorbance of aqueous solutions of MPB and PB injected on the precolumn directly connected to the UV detector.

Three different phosphate buffers with pH values of 3, 5 and 7 and containing 10% of methanol were tested as washing liquids. An adequate retention of the analytes on the precolumn was only obtained at pH 3 and 5, PB being insufficiently retained at pH 7, due to its lower pK_a value (7.4). A pH value of 5 was then selected for the washing liquid as the clean-up of plasma samples was found to be more efficient at this pH than at pH 3, giving chromatograms devoid of interfering peaks from plasma components in the elution zones of PB and MPB (cf. Fig. 4)

The elution profiles of MPB and PB were then determined on the C_{18}-ADS precolumn with washing liquids of pH 5 containing different concentrations of methanol (cf. Table 4). As expected, a decrease of the methanol content in the washing liquid led to a significant increase in the retention of the analytes.

Finally, a washing liquid consisting of a pH 5 buffer containing 3% of methanol was selected since an adequate retention for phenobarbital on the precolumn was obtained in this case (cf. Table 4). Under these conditions, the valve-switching time should be lower than 12 min, which corresponds to the beginning of elution of PB.

3.2.2. Determination of the elution profile of blank plasma

To determine the valve-switching time, it was also necessary to measure the time needed for a complete elution of the proteins present in plasma samples

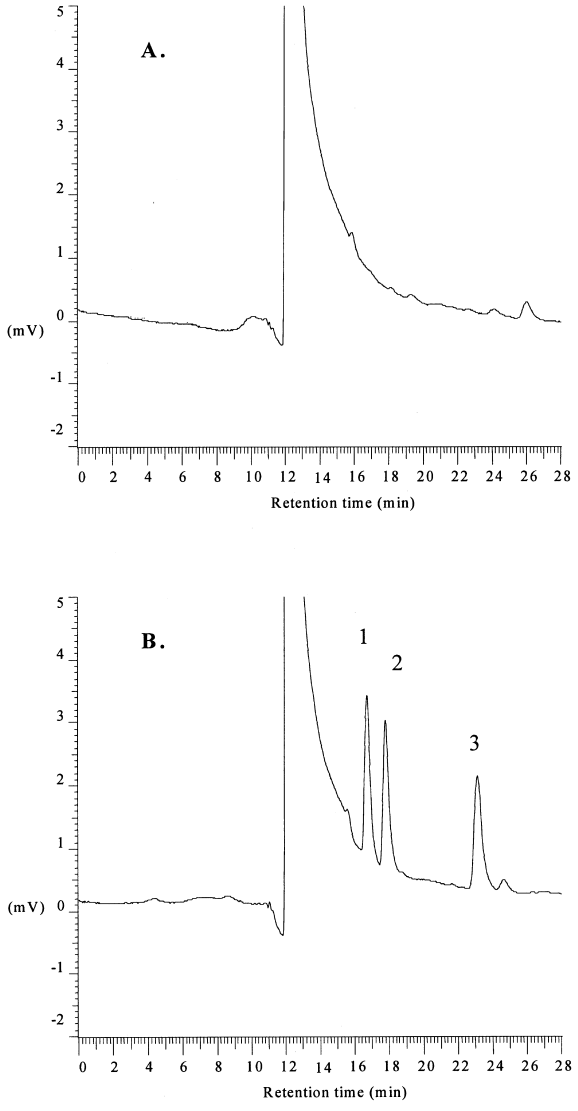

Fig. 4. Chromatograms illustrating the separation of methylphenobarbital enantiomers and phenobarbital in plasma. Precolumn: LiChrospher RP-18 ADS (25×4.0 mm I.D.), washing liquid: phosphate buffer (pH 5.0)–methanol (97:3, v/v), flow-rate: 0.8 ml min^{-1}, loading step: 8 min, transfer step: 5 min. Column: Chiralcel OJ-R (150×4.6 mm I.D.), mobile phase: phosphate buffer (pH 7.0)–acetonitrile (60:40, v/v), flow-rate: 0.6 ml min^{-1}, detection UV at 225 nm, injection: 100 μl of blank plasma (A) and plasma containing 0.625 μg ml^{-1} of each enantiomer of MPB and 1.25 μg ml^{-1} of PB(B). 1=Phenobarbital, 2=*S*-(+)-Methylphenobarbital, 3=*R*-(−)-Methylphenobarbital.

Table 4
Times corresponding to the beginning of elution of methylphenobarbital and phenobarbital on the precolumn

Methanol (%)	PB (min)	MPB (min)
3%	12	45
4%	10	40
5%	9	30
10%	6	18

Precolumn: LiChrospher ADS RP-18 (25×4.0 mm I.D.); washing liquid: 50 m*M* phosphate buffer (pH 5.0) containing methanol; flow-rate: 0.8 ml min^{-1}; detection: 220 nm; sample: aqueous solution of methylphenobarbital (100 μg ml^{-1}) or phenobarbital (100 μg ml^{-1}); injection: 50 μl.

from the precolumn. Such experiments were achieved by connecting directly the precolumn to the UV detector set at 280 nm and by injecting blank plasma samples (100 μl). The flow-rate was the same as that used for the determination of the elution profiles of the analytes.

With a washing liquid consisting of phosphate buffer (pH 5.0)–methanol (97:3, v/v), a complete elution of proteins was achieved within 4 min. The valve switching time was therefore set at 8 min. This time is two-fold higher than the elution time of the proteins and lower than to the time corresponding to the beginning of elution of PB (12 min).

3.2.3. Determination of the period of time needed for analyte transfer

In order to perform the chromatographic separation on the Chiralcel OJ-R column, the analytes retained on the precolumn must be transferred quantitatively to the analytical column. This is achieved by rotation of the switching valve.

The time period for analyte transfer is that required to backflush the analytes from the precolumn to the analytical column. It allows to deduce the time from which the valve can be switched back to its original position. The determination of this time was performed with the detector directly connected to the switching valve.

PB and MPB were eluted rapidly from the C_{18} ADS precolumn because of the much stronger eluting strength of the LC mobile phase (pH 7.0 buffer–acetonitrile, 60:40, v/v). It took about 3 min to transfer quantitatively MPB and PB from the precolumn to the analytical column.

Finally, a period of time of 5 min was selected for analyte transfer to the chiral column. Thus, 13 min after sample injection, the switching valve was returned to its initial position and the C_{18}-ADS column was then re-equilibrated with the washing liquid before the next injection. The total analysis time, including sample preparation and chiral LC analysis, was 28 min.

3.3. Method validation

3.3.1. Selectivity

Fig. 4 shows a typical chromatographic trace of a plasma extract containing racemic methylphenobarbital and phenobarbital. Under the conditions selected for the LC separation of MPB enantiomers and PB, the mean retention times were 10.8 and 16.9 min for the (+)-*S*-MPB and (−)-*R*-MPB enantiomers, respectively and 9.3 min for PB ($n=20$). The absence of interfering endogenous components at the retention times of the three compounds of interest is clearly demonstrated in the figure. The order of elution of MPB enantiomers was determined by injecting separately solutions of each enantiomer.

3.3.2. Absolute recovery

The absolute recovery was determined by comparing peak areas obtained from freshly prepared sample extracts and those found by direct injection of an aqueous standard solution at the same concentration, using the same autosampler equipped with the same loop of 100 μl [45]. The absolute recovery for both enantiomers of MPB was found to be of about 95% and close to 70% for PB (cf. Table 5).

3.3.3. Linearity

The linear regression analysis for MPB enantiomers and for PB was made by plotting peak area (y) versus analyte concentration (x) in μg ml^{-1}. The concentration ranges were 1.25 to 50 μg ml^{-1} for PB and 0.625 to 25 μg ml^{-1} for each enantiomer of MPB. The following equations were obtained:

Phenobarbital:	$y=34870.44x-3672.16$
	$r^2=0.9991$
(+)-*S*-Methylphenobarbital:	$y=79819.03x-8239.18$
	$r^2=0.9992$
(−)-*R*-Methylphenobarbital:	$y=81296.81x-5590.79$
	$r^2=0.9992$

The linearity of the relationship between peak area and concentration is demonstrated by the determination coefficients (r^2) obtained for the regression lines in the case of the three analytes. Moreover, an analysis of variance (ANOVA) was carried out on calibration curves in order to confirm the linearity (F_1) and to test the quality of the fitting (F_2) [46]. The linearity was assessed for PB, (+)-*S*-MPB and (−)-*R*-MPB with $F_{calc} \gg F_{(0.95;\ 1,\ 16)}$ (4.49), as well as the fitting, with a $F_{calc} < F_{(0.95;\ 4,\ 12)}$ (3.26) (cf. Table 5).

3.3.4. Detectability

The limits of detection (LODs) and quantitation (LOQs) were determined as analyte concentrations giving rise to signal-to-noise ratios of 3 and 10, respectively. The LODs and LOQs for the enantiomers of MPB were found to be 52 and 173 ng ml^{-1} for (+)-*S*-MPB, 67 and 223 ng ml^{-1} for (−)-*R*-MPB, respectively. The LOD and LOQ for PB were 64 and 213 ng ml^{-1}, respectively.

3.3.5. Precision

The precision of the automated bioanalytical method was determined by measuring the repeatability and intermediate precision for the three compounds at three concentration levels, ranging from 2.5 to 100 ng ml^{-1}. The mean values for repeatability and intermediate precision were 1.7% and 5.5% for PB, 2.2 and 5.7 for the (+)-*S*-MPB enantiomer and 2.1% and 5.7% for the (−)-*R*-MPB enantiomer, respectively.

3.3.6. Accuracy

The overall accuracy of the procedure was assessed by plotting the analyte amount found versus the amount spiked in the plasma sample at three concentration levels ($n=6$) ranging from 5 to 200 μg ml^{-1} for PB ($r^2=0.9993$) and from 2.5 to 100 μg ml^{-1} for (+) and (−) MPB ($r^2=0.9992$ and 0.9995, respectively). *t*-Tests indicated that the slopes of the regression lines were not significantly different from unity (calculated t values were 1.12, 1.58 and 0.04 for PB, (+)-MPB and (−)-MPB, respectively) and that intercepts were not signifi-

Table 5
Validation of the automated method for the determination of MPB and PB in plasma

Validation Criterion	PB	(+)-*S*-MPB	(−)-*R*-MPB
Absolute recovery			
(mean±S.D., $n=3$)	67.0±1.2%	93.2±4.1%	92.1±5.0%
Linearity ($n=6$, $k=3$)			
Concentration range	1.25–50 μg ml^{-1}	0.625–25 μg ml^{-1}	0.625–25 μg ml^{-1}
	$y=34870x-3672$	$y=79819x-8239$	$y=81296x-5590$
	$r^2=0.9991$	$r^2=0.9992$	$r^2=0.9992$
F-test for the slope	$F_1=18615$	$F_1=20514$	$F_1=19587$
F-test for fitting	$F_2=1.81$	$F_2=2.52$	$F_2=1.54$
LOD	64 ng ml^{-1}	52 ng ml^{-1}	67 ng ml^{-1}
LOQ	213 ng ml^{-1}	173 ng ml^{-1}	223 ng ml^{-1}
Repeatability			
($n=6$; 1 day)			
1.25 or 0.625 μg ml^{-1}	3.1	3.8	3.2
6.25 or 12.5 μg ml^{-1}	1.2	1.8	2.0
25 or 50 μg ml^{-1}	0.78	1.0	1.1
mean	1.7	2.2	2.1
Intermediate precision			
($n=18$; *3* days)			
1.25 or 0.625 μg ml^{-1}	6.4	4.7	4.9
6.25 or 12.5 μg ml^{-1}	4.8	7.1	7.1
25 or 50 μg ml^{-1}	5.4	5.4	5.2
mean	5.5	5.7	5.7
Overall Accuracy ($n=18$)			
t-test for the slope	1.12	1.58	0.04
t-test for the origin	0.02	0.21	0.72

cantly different from zero (calculated *t* values were 0.02, 0.21 and 0.72 for PB, (+)-MPB and (−)-MPB, respectively). The critical *t* value was 2.12 ($P=0.05$).

The automated LC procedure developed for the simultaneous determination of MPB enantiomers and PB in human plasma can therefore be considered as accurate within the concentration range investigated.

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