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Separation of the stereoisomers of the main metabolite of a non-steroidal anti-inflammatory drug, flobufen, by chiral high-performance liquid chromatography

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Abstract

The major metabolite of a novel non-steroidal anti-inflammatory drug, DL-4-(2′,4′-difluorobiphenyl-4-yl)-4-oxo-2-methylbutanoic acid (flobufen, **I**), namely 4-(2′,4′-difluorobiphenyl-4-yl)-2-methyl-γ-butyrolactone (4-dihydroflobufen lactone, **III**), has four stereoisomers consisting of two racemic pairs of enantiomers. Of three chiral stationary phases tested, Cyclobond I β-RSP (Astec) (β-cylodextrin derivatized with *R,S*-hydroxypropyl) was best able to separate the (++)(−−) racemate, with a liquid phase containing acetonitrile as modifier and triethylamine acetate as buffer. Using the Box–Wilson Central Composite Design for three factors, an optimum combination of pH and concentrations of the modifier and buffer was eventually obtained. A chromatographic response function based on a combination of the Kaiser peak separation function, P_i, and retention time of the second eluting enantiomer, t_{R_L}, served as a response criterion for the process of optimization. The optimum conditions developed for the (++)(−−) racemate were also found to be suitable for separating the (+−)(−+) racemate, for which earlier studies had shown the separation to be more facile. Separation of the four stereoisomers of **III**, for which the chiral chromatographic system optimized in this study is proposed as the second stage, is targeted at a biochemical study of the stereoisomeric metabolism of **I**.

Keywords: Flobufen; Enantiomer separation

1. Introduction

1.1. Flobufen pharmacology and biotransformation to stereoisomers

During studies on non-steroidal anti-inflammatory arylalkanoic acids [1], DL-4-(2′,4′-difluorobiphenyl-4-yl)-4-oxo-2-methylbutanoic acid (flobufen) was synthesized in the Research Institute of Pharmacy and Biochemistry in Prague and found to exhibit favourable pharmacological properties [2,3]. Biochemical tests indicate that flobufen has inhibitory activity against 5-lipoxygenase and cyclooxygenase, and that it shows affinity to the LT B_4 receptors.

The metabolism of flobufen was studied in vivo and in vitro. In tissues from most experimental animals the metabolites included the 4-dihydroderivative **II** and its lactone **III** (in vitro studies), while in vivo studies revealed 2-(2′,4′-difluorobiphenyl-4-yl)-acetic acid **IV** [4]. In man, however, **IV** was not detected and the main metabolite was found to be **II**/**III** [5], see Fig. 1.

It is clear from the formulae that compound **I** has 2 enantiomeric forms and that **III** and **IV**, with two

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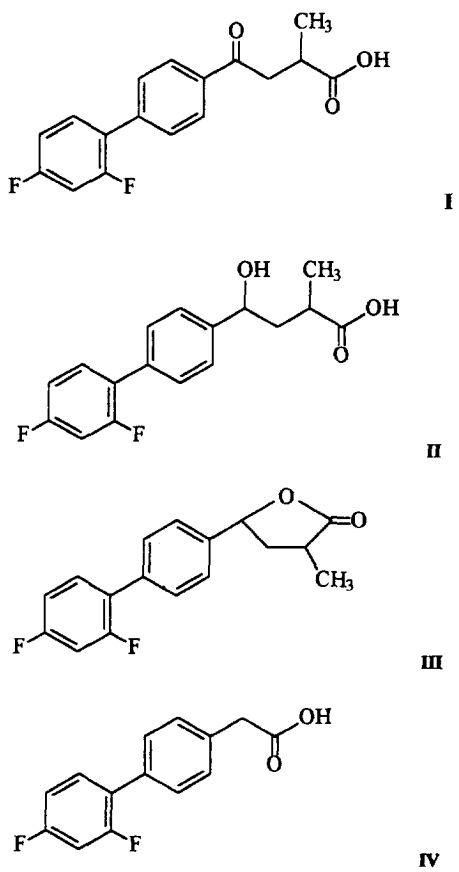

Fig. 1. Chemical structures of flobufen (**I**), 4-dihydroflobufen (**II**), 4-dihydroflobufen lactone (**III**) and 2-(2′,4′-difluorobiphenyl-4-yl)-acetic acid (**IV**).

stereogenic centres, have four stereoisomeric forms. The enantiomeric forms of flobufen **I** were obtained by Kuchař and Poppová (unpublished) and from each of them a mixture of diastereomeric pairs of **II** and, after acidification, of **III** was prepared by reduction (Kuchař and Poppová, unpublished). These diastereomeric pairs, namely the mixture of (+−) with (−−) deriving from (−)-flobufen, and that of (++) with (−+) deriving from (+)-flobufen, could be resolved using an achiral normal-phase chromatographic system (Poppová and Kuchař, unpublished). The four individual stereoisomers thus obtained were kindly made available to us for these studies [6].

The ratio of **II** to **III** depends on the lactonization equilibrium and rate and, thus, on operating conditions. For the sake of simplicity, biochemical analysis should concentrate on one of them, so product **III** was chosen for separation on the basis that the added rigidity of the lactonized side chain may conceivably assist chiral separation.

The aim of the present study was to develop a method capable of separating the individual enantiomers of **III**, which had been already preseparated on an achiral normal-phase column as the diastereomers, (++)(−−) and (+−)(−+). Of the three chiral stationary phases (CSPs) tested in preliminary experiments (see below), all separated the (+−)(−+) pair, whereas the (++)(−−) pair could only be separated on Cyclobond I β-RSP. Therefore, the main thrust of this study was directed at the more difficult separation of the (++)(−−) enantiomers, assuming, as later proved to be correct, that the optimum conditions for (++)(−−) would also be appropriate for the (+−)(−+) pair.

1.2. Selection of the CSP and mobile phase

Three types of CSP were explored in preliminary experiments, namely Cyclobond I β-RSP (β-cyclodextrin derivatized with *R,S*-hydroxypropyl), Ultron ES-PhCD (β-cyclodextrin derivatized with phenylcarbamate) and Chiralcel ODR [cellulose derivatized with tris(3,5-dimethylphenylcarbamate)] with a number of mobile phase compositions (using one of methanol, ethanol, 2-propanol, acetonitrile or tetrahydrofuran in several concentrations as organic modifiers) together with one of phosphate, citrate, perchlorate or triethylamine as buffer constituents at various pH values. Thus, acetonitrile was selected as organic modifier and triethylamine acetate as buffer (report on this aspect in preparation). As already stated, both the (++)(−−) and (+−)(−+) enantiomeric pairs could be separated on Cyclobond I β-RSP, whereas, with the two other CSPs, the (−−)(++) pair could not be separated.

1.3. Principles of a central composite design (CCD)

There are several dozens of optimization designs in the literature. The Box–Wilson design (CCD) is practically useful as it does not require an excessive number of runs [7,8]. Let f denote the number of factors (independent variables of the analytical procedure). The CCD used in the present work was

based on a two-level factorial design ($N_c=2^f$ runs) augmented with N_o runs (at least one) at the centre of the design, together with $2f=N_a$ extra star points. These are located at the upper and lower extreme of each factor, and also at the centre of all other factors (cf. Fig. 2 for the present authors' design, which involves three factors). Whereas, for $f=3$ a three-level factorial design would require $3^f=27$ runs, a CCD based on the central composite design requires only $2^f+2f+1=15$ runs. The difference becomes more striking with a higher number of factors; thus for $f=6$, the full factorial design for three factors, $3^f=729$, compared with 77 for the CCD.

To construct a CCD for a specific number of factors, the number of replicate runs at the central point, N_o, must be selected. This then defines the geometric distance between the centre point and each star point, a. For each factor, these distances are expressed in terms of a unit interval (or distance), defined in terms of the distance from the centre point of the cube and the centre of the corresponding wall for that factor. The star point distance a is then calculated [8] from Eq. (1):

$$a^2 = \frac{\sqrt{(N_c + N_a + N_o)N_c} - N_c}{2} \quad (1)$$

where a is the axial spacing, N_c the number of the factor points, N_a the number of the axial star points and N_o the number of replicate experiments at the centre point. For $f=3$, $N_c=8$, $N_a=6$, N_o was chosen as 9 giving $a=1.668$ using Eq. (1).

Central composite designs provide sufficient data for a linear polynomial model to be fitted to a data set. Such models are amenable to regression analysis. For three factors the polynomial takes the form [8]:

$$y = b_0 + b_1X_1 + b_2X_1^2 + b_3X_2 + b_4X_2^2 + b_5X_3 + b_6X_3^2 + b_7X_{12} + b_8X_{13} + b_9X_{23} \quad (2)$$

where y is the dependent variable or response (chromatographic response function, CRF, see below), and X_1, X_2 and X_3 are the variables (i.e. factors) in the model; X_{12}, X_{13} and X_{23} refer to the corresponding interactions betweeen these factors. The b coefficients represent the parameters of the model which are iteratively optimised.

1.4. Chromatographic response criteria

The chromatographic parameters used to establish appropriate response criteria were the Kaiser peak separation function, P_i, and the retention time of the second eluting enantiomer t_{RL}. The Kaiser peak separation function is defined as the average valley depth expressed as a percentage of the average peak height of two adjacent peaks. Selection of the appropriate combination of these two parameters to give a CRF which permits the development of a satisfactory separation in a reasonable time is, of

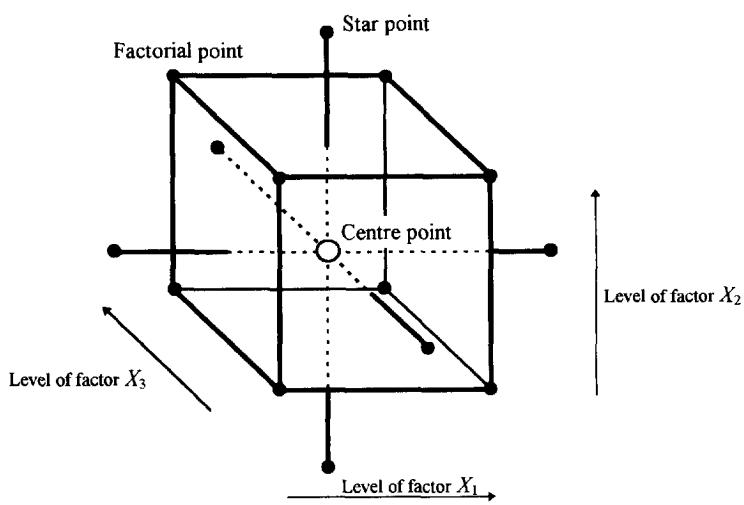

Fig. 2. Factor combinations for a central composite design in three-dimensional factor space.

course, to a large extent arbitrary. The present authors explored a number of chromatographic response functions, including CRF_1 [9] and a new empirical response function CRF_2 proposed in the present work:

$$CRF_1 = P_i + (60 - t_{RL}) \quad (3)$$

$$CRF_2 = \frac{P_i^3}{t_{RL}} \quad (4)$$

The CRF_2 function takes account of the retention time, while giving more weight to the Kaiser function than in CRF_1. In effect this represents an attempt to normalise the Kaiser function.

1.5. Statistical software package

The statistical software package produced by SAS Institute (release version 6.04, SAS Software, Cary, NC, USA) comprises a number of "procedures" – graphical, statistical, reporting, processing and tabulating procedures – that enable simple and rapid data evaluation. A bench-top Elonex 486 DX33 PC system with 16 Mb RAM and 200 Mb disc was adequate to evaluate the data acquired for the present experiments.

2. Materials and equipment

2.1. Chemicals and reagents

rac-Flobufen (**I**), its enantiomers and all four stereoisomers of the 4-dihydro derivative of flobufen (the main metabolite) in the lactone form **III** were obtained from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic).

Acetonitrile (ACN) (AnalaR grade) was purchased from Fisons (Loughborough, UK). Triethylamine (TEA) (HPLC grade) was purchased from Sigma (Poole, UK) and pH was adjusted within 0.01 unit with glacial acetic acid from May and Baker (Dagenham, UK). All aqueous mobile phases were filtered using a 0.45-μm filter (Anachem, UK) and degassed by sonication under vacuum.

2.2. High-performance liquid chromatography

Liquid chromatography was carried out using a Hewlett-Packard Series II 1090 liquid chromatograph with diode-array detector and autosampler. Flobufen and its metabolites were monitored at a detection wavelength of 240 nm. In the later part of the work, the column system was thermostatted using a "column chiller", Model 7950 from Jones Chromatography (Hengoed, UK).

Most work was performed using a 25×0.46 cm I.D. Cyclobond I β-RSP (*R,S*-hydroxypropyl ether) column (Astec, Advanced Separation Technologies, Whippany, NJ, USA). Preliminary tests were also made using Ultron ES-PhCD columns (β-cyclodextrin derivatized with phenylcarbamate, kindly provided by Shinwa Chemical Industries, Kyoto, Japan) and Chiralcel ODR (Daicel Chemical Industries, Tokyo, Japan) [cellulose derivatized with tris(2,5-dimethylphenyl carbamate)].

As with most chiral phases, it was important to monitor the column performance using a system suitability test (SST), based on a suitable analyte and a standard set of operating conditions for that column. In the present work, the SST used as a control system for the Cyclobond I β-RSP column was based on tioconazole (0.1 mg/ml in methanol), with mobile phase triethylamine (4.37% v/v) adjusted to pH 4.3 with acetic acid, plus methanol in the ratio 72.5:27.5 (v/v). The resolution of enantiomers should be ca. 90% according to the Kaiser peak separation function.

3. Results

3.1. Selection of the centre point and of the unit dimension in CCD

Preliminary experiments showed that the following coordinates were suitable for the centre point: concentration of the modifier (acetonitrile) in the mobile phase, 11% (v/v); concentration of triethylamine in the buffer, 6% (v/v); buffer pH, 3.8 (before addition of acetonitrile).

Further preliminary experiments indicated that the unit distance for each coordinate (factor) in the CCD

was: concentration of acetonitrile in the mobile phase, 6% (v/v); concentration of the buffer, 3% (v/v); and an interval corresponding to 0.4 for the buffer pH.

3.2. Computer treatment of experimental results

Samples of 1:1 (v/v) mixtures of (− −) and (+ +) **III** were examined with the mobile phases described in Table 1. The values for Kaiser peak separation function, P_i, and of the retention time for the second eluting isomer, t_{RL}, are shown in Table 2, together with the two alternative CRF models, CRF_1 and CRF_2 .

The data shown in Table 3 were then entered into the appropriate SAS programme to calculate the combination of factors corresponding to the highest (optimum) CRF. As the results fell outside the usable factor space for the column system, and did not converge to give a satisfactory development solution, they were used as the basis for developing an empirical chromatographic response function (CRF_2). This could be shown to converge to a practicable optimum.

Table 1
Experiments required for a three-factor central composite design for *rac*-metabolite of flobufen, (+ +) and (− −) enantiomers

Experiment No.	pH	ACN (% v/v)	TEA (% v/v)
1	3.4	5	3
2	3.4	5	9
3	3.4	17	3
4	3.4	17	9
5	4.2	5	3
6	4.2	5	9
7	4.2	17	3
8	4.2	17	9
9	3.8	11	1
10	3.8	11	11
11	3.8	1	6
12	3.8	21	6
13	3.13	11	6
14	4.47	11	6
15-1	3.8	11	6
15-2	3.8	11	6
15-3	3.8	11	6
15-4	3.8	11	6
15-5	3.8	11	6
15-6	3.8	11	6
15-7	3.8	11	6
15-8	3.8	11	6
15-9	3.8	11	6

Table 2
Calculated data from experiments required for a 3-factor central composite design for *rac*-metabolite of flobufen, (+ +) and (− −) enantiomers

Experiment No.	P_i (%)	t_{RL} (min)	CRF_1	CRF_2
1	66.30	39.279	87.021	7420
2	0.5	5.876	54.624	0
3	20.79	10.519	70.271	854
4	0	3.904	56.096	0
5	77.40	258.100	−120.7	1796
6	61.46	58.174	63.286	3991
7	59.94	33.233	86.707	6480
8	25.35	12.590	72.76	1294
9	75.92	121.426	14.494	3604
10	4	6.957	57.043	9
11	58.45	56.475	61.975	3536
12	5	6.766	58.234	18
13	0	4.299	55.701	0
14	59.94	66.081	53.859	3259
15-1	35.62	18.075	77.545	2500
15-2	36.30	18.073	78.227	2647
15-3	35.86	18.083	77.777	2550
15-4	35.00	18.098	76.902	2369
15-5	34.48	18.025	76.455	2274
15-6	35.42	18.030	77.39	2465
15-7	35.46	18.049	77.411	2470
15-8	34.48	17.989	76.491	2279
15-9	35.86	17.979	77.881	2565

3.3. Graphical representation of the chromatographic response function

In the next stage a series of three-dimensional graphs was constructed, where the CRF was plotted as the third (vertical) coordinate, and the other coordinates corresponded to two of the factors, the third factor being held constant at a predetermined value based on the preliminary results presented by the SAS computation discussed in Section 3.2.

In this way a set of six graphs (three for CRF_1 and three for CRF_2) was obtained (Figs. 3 and 4),

Table 3
Determination of experimental values for the CCD with a specified axial spacing[a]

	Axial star point (lower)	Factor point (lower)	Centre point	Factor point (upper)	Axial star point (upper)
pH	3.13	3.4	3.8	4.2	4.47
% ACN	1	5	11	17	21
% TEA	1	3	6	9	11

[a] For $N_0=9$, $N_c=8$, $N_a=6$: $a=1.668$ [cf. Eq. (1)]

illustrating the location of the optimal values of the CRF in each case.

3.4. Examples of chromatograms

Fig. 5 shows a chromatogram run with X_1, X_2 and X_3 chosen on the basis of an empirical, trial and error (intuitive) approach, for which P_i was 36% at t_{RL} 24 min. Fig. 6 shows the improvement obtained when X_1, X_2 and X_3 were optimized according to CRF_2 (cf. Fig. 4), where P_i is 73% at t_{RL} 60 min.

As anticipated, the factors optimized for the separation of the difficult pair of **III** (++)(−−) enantiomers on the basis of CRF_2, proved suitable for the separation of the **III** (−+)(+−) pair (cf. Fig. 7).

4. Discussion and conclusions

The major metabolite of the non-steroidal anti-inflammatory drug, flobufen, namely 4-dihydroflobufen, contains two stereogenic centres. This can be conveniently studied in the lactone form, **III**. It is proposed that a first step be developed, based on normal-phase chromatographic separation of the two diastereoisomers, i.e. the enantiomeric pairs (++)(−−) and (−+)(+−). As noted in Section 1.1 above, this is not the subject of this paper. Thus, the chiral resolution of the (++)(−−) enantiomers has been studied.

According to preliminary experiments, Cyclobond I β-RSP can be used as a chiral stationary phase for this pair, with acetonitrile as modifier and triethylamine acetate as buffer. The optimum values for organic modifier and buffer concentrations, and for pH, were derived from the CCD, to give satisfactory resolution of the enantiomeric pair. The quality of separation was expressed in terms of two chromatographic response functions, each incorporating P_i (Kaiser peak separation function) and the retention time of the second eluting component t_{RL}. The intrinsic merit of the Box–Wilson CCD is that it requires fewer experiments than alternate designs.

The optimum of the three variables calculated initially using CRF_1 did not yield experimental conditions within the usable factor space for this column system, for reasons that remain to be clarified. On the basis of iterative treatment of the chromatographic data using the SAS optimisation software, a second was developed, CRF_2, that yielded optimum factor values lying within the permitted factor space for this column. Thus, six three-dimensional graphs showing the dependence of the CRF on pairs of factors (the third being held constant) were constructed to illustrate the optimum zones and the regions of maximum ruggedness of the method, i.e. where the gradient of the response surface in the region of acceptable separation was at its lowest value [10].

The coordinates of the apparent maxima of this CRF_2 were then recommended for the routine separation of the pairs of enantiomers. As expected, it was found that this combination of factors was also suitable for the separation of the more easily separated (+−)(−+) **III** pair.

It is envisaged that an off-line or on-line separation will be developed, using, as the first step, a normal-phase separation to resolve the (++)(−−) and (+−)(−+) enantiomeric pairs as their diastereoisomers, followed by a second chiral resolution step applied separately to each diastereoisomer, as outlined in this paper.

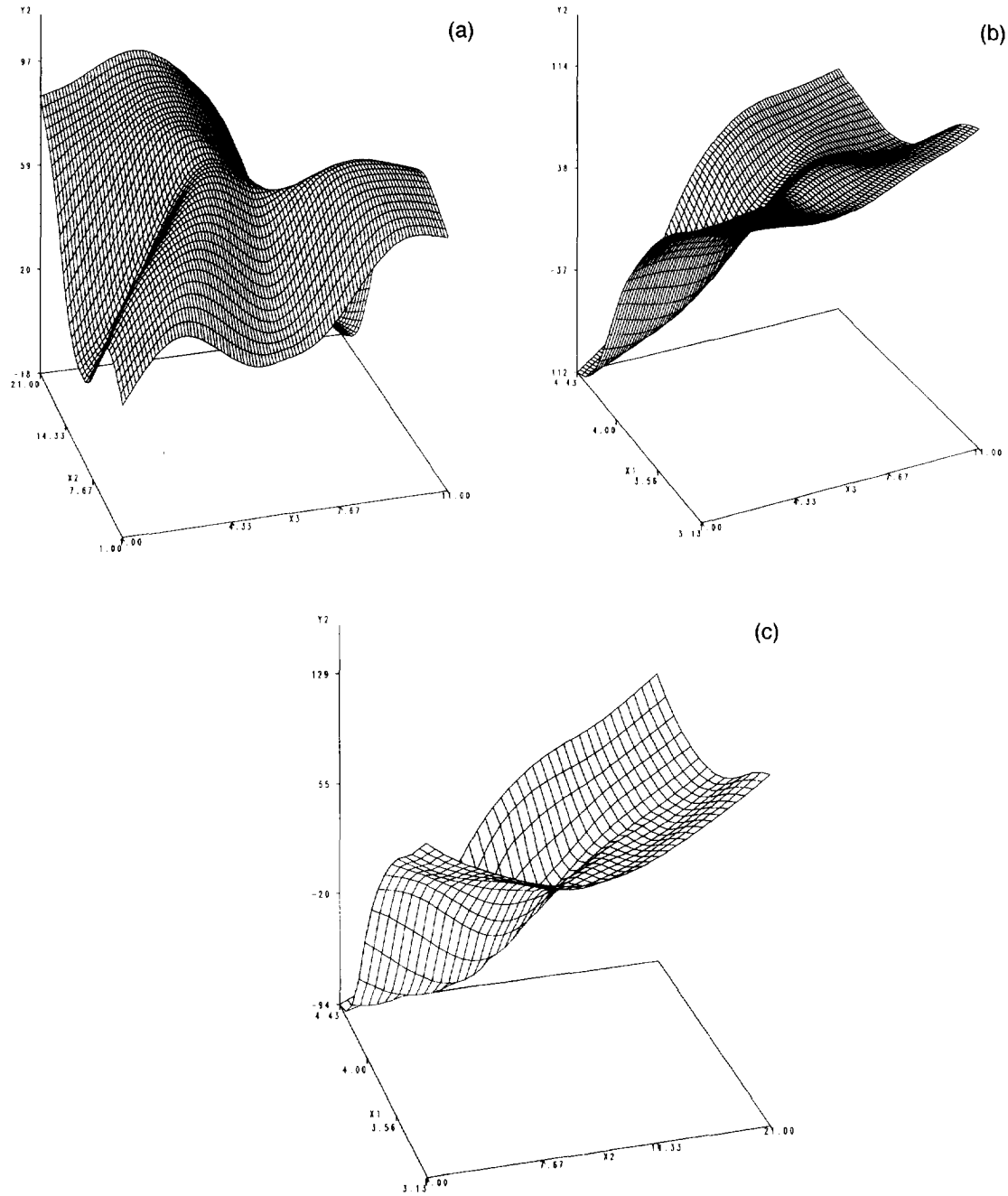

Fig. 3. Response surface for CRF_1 response model of (++)(−−) pair of enantiomers of 4-dihydroflobufen lactone: (a) pH 4.2; ACN (X_2% v/v) vs. TEA (X_3% v/v); (b) ACN 13.45% (v/v), pH (X_1) vs. TEA (X_3% v/v); (c) TEA 4.99% (v/v), pH (X_1) vs. ACN (X_2% v/v).

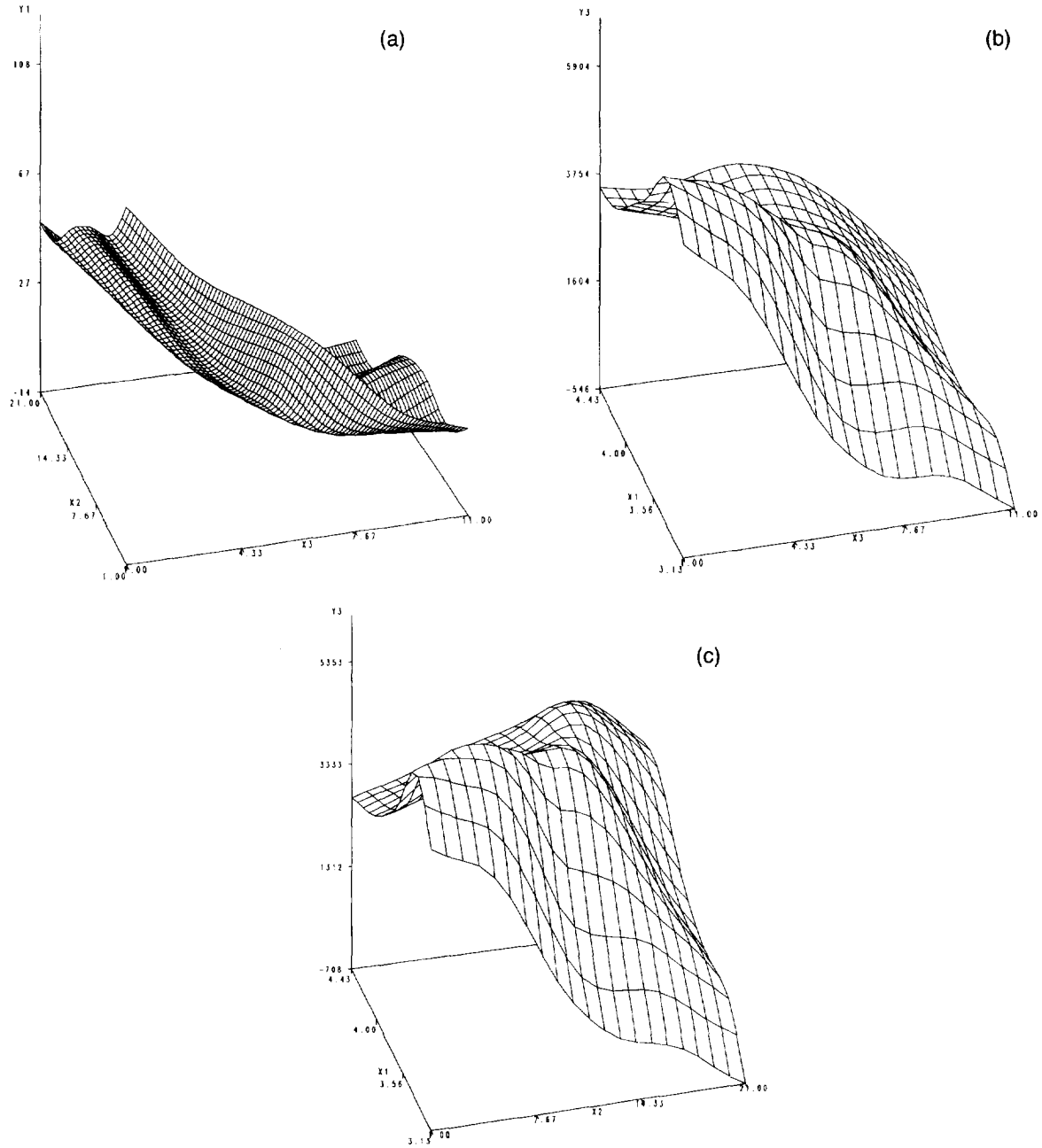

Fig. 4. Response surface for CRF_2 response model of (++)(−−) pair of enantiomers of 4-dihydroflobufen lactone: (a) pH 4.35; ACN (X_2% v/v) vs. TEA (X_3% v/v); (b) ACN 15% (v/v), pH (X_1) vs. TEA (X_3% v/v); (c) TEA 4.35% (v/v), pH (X_1) vs. ACN (X_2% v/v).

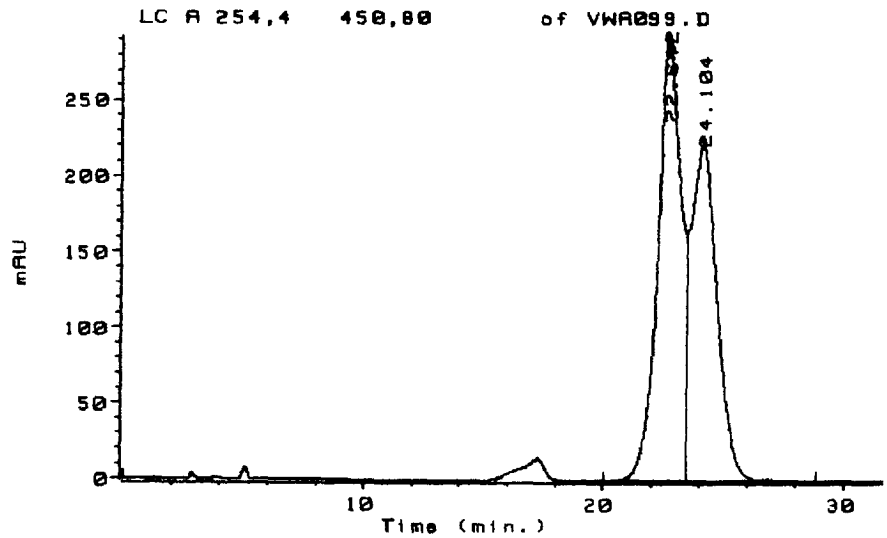

Fig. 5. HPLC chromatogram of initial separation of (+ +)(− −) pair of enantiomers of 4-dihydroflobufen lactone. TEA 1% (v/v), pH=4.2, ACN 21% (v/v) at a flow-rate of 1.0 ml/min and 240 nm.

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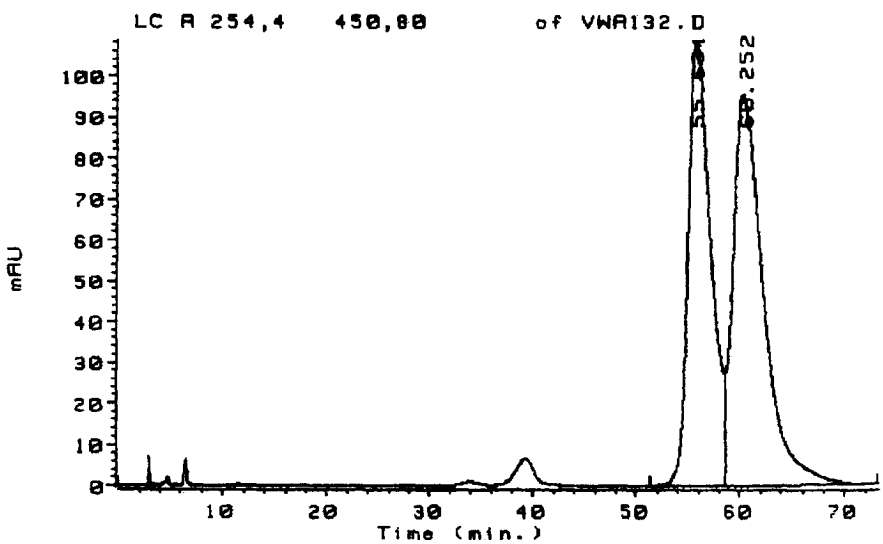

Fig. 6. HPLC chromatogram of final (CRF_2 optimized) separation of (+ +)(− −) pair of enantiomers of 4-dihydroflobufen lactone. TEA 3.5% (v/v), pH=4.29, ACN 15% (v/v) at a flow-rate of 1.0 ml/min and 240 nm.

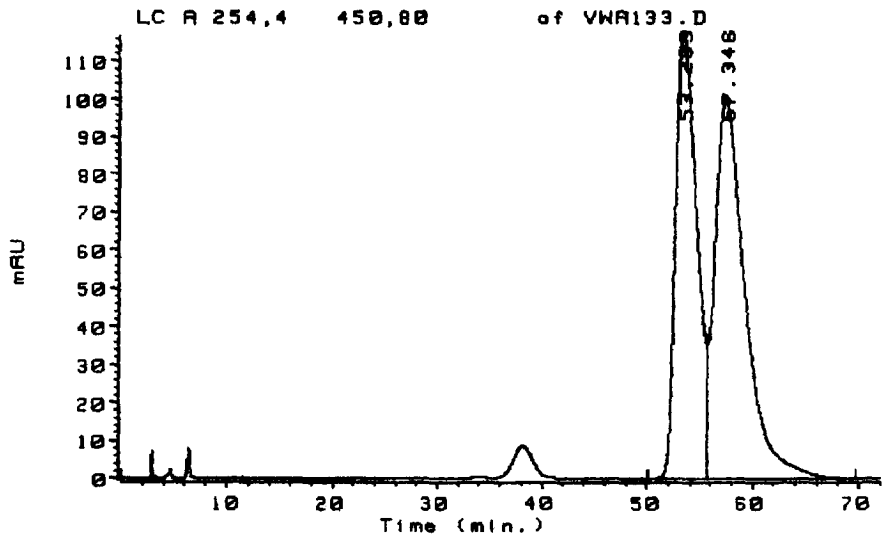

Fig. 7. HPLC chromatogram of final separation of (+−)(−+) pair of enantiomers of 4-dihydroflobufen lactone. TEA 3.5% (v/v), pH=4.29, ACN 15% (v/v) at a flow-rate of 1.0 ml/min and 240 nm.

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