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Development and optimisation of a flow injection assay for fluticasone propionate using an asymmetrical design and the variable-size simplex algorithm

C. Vannecke [a], A. Nguyen Minh Nguyet [a], M.S. Bloomfield [b], A.J. Staple [b], Y. Vander Heyden [a], D.L. Massart [a,*]

[a] *ChemoAc, Department of Pharmaceutical and Biomedical Analysis, Vrije Universiteit Brussel, Pharmaceutical Institute, Laarbeeklaan 103, 1090 Brussels, Belgium*
[b] *Glaxo–Wellcome, Pharmaceutical Development Sciences 2, Temple Hill, Dartford DA1 5AH, UK*

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Abstract

A flow injection analysis method is described to determine fluticasone propionate, based upon a novel adaptation of the reaction of *o*-phthalaldehyde with a thiol and a primary amine. The method, which allows both UV and fluorescence detection, has been optimised using experimental design. First a screening is executed to select the significant factors and in a second step these factors are optimised with the variable-size simplex algorithm. In the screening step, a two-level fractional factorial design is compared with an asymmetrical design containing the same number of experiments, but in which one factor is at three levels. It was found that in both designs the same significant variables are detected for the two-level factors, but that for the three-level factor the asymmetrical design confirms an expectation of having a (local) optimum in the examined domain, whilst from the two-level design this is not at all apparent. Complete optimisation was carried out for both UV and fluorescence detection. The two detection methods did not have the same significant variables. For the UV detection, the temperature and the pH adjustment on-line (concentration of sodium hydroxide and amount of boric acid) were the most critical parameters. For the fluorimetric detection the temperature and the fraction of methanol were critical. Moreover the conditions found to be optimal are different for both detection methods.

Keywords: Flow injection analysis; Fluticasone propionate; Fractional factorial design; Asymmetrical design; Variable-size simplex

1. Introduction

Fluticasone propionate (FP) is a fluorated corticosteroid with a potent anti-inflammatory action, used in the treatment of asthma [1,2]. It is usually administered as an inhalation formula-

* Corresponding author. Tel.: +32-2-4774737; fax: +32-2-4774735.
E-mail address: fabi@vub.vub.ac.be (D.L. Massart).

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tion. The European Pharmacopoeia [3] provides a monograph to which inhalation preparations should conform. These include, along with other tests, requirements for 'the uniformity of dose test', intended to control the dose delivered by the device, and 'the aerodynamic assessment of fine particles test', to determine the fine-particle fraction and confirm that a significant fraction is deposited in the lower respiratory tract. For the latter test a more sensitive analytical method for fluticasone propionate is necessary because small fractions of a dose need to be quantified. The delivery of FP in inhalation formulations is tightly controlled through routine analytical testing. The use of flow injection analysis (FIA) [4,5] is an excellent approach for the analysis of the large number of samples involved because it is fast, sensitive, reproducible and can be automated.

Table 1
Construction of an asymmetrical design according to Addelman [16]

	Factors						
	1 X_1	2 X_2	3 X_3	4 X_1X_2	5 X_1X_3	6 X_2X_3	7 $X_1X_2X_3$
(a) Saturated two-level design for seven factors							
1	0	0	0	0	0	0	0
2	0	0	1	0	1	1	1
3	0	1	0	1	0	1	1
4	0	1	1	1	1	0	0
5	1	0	0	1	1	0	1
6	1	0	1	1	0	1	0
7	1	1	0	0	1	1	0
8	1	1	1	0	0	0	1

	Factors				
	1 A	2 X_3	3 X_1X_3	4 X_2X_3	5 $X_1X_2X_3$
(b) 4×2^4 design					
1	0	0	0	0	0
2	0	1	1	1	1
3	1	0	0	1	1
4	1	1	1	0	0
5	2	0	1	0	1
6	2	1	0	1	0
7	3	0	1	1	0
8	3	1	0	0	1
(c) 3×2^4 design					
1	0	0	0	0	0
2	0	1	1	1	1
3	1	0	0	1	1
4	1	1	1	0	0
5	2	0	1	0	1
6	2	1	0	1	0
7	1	0	1	1	0
8	1	1	0	0	1

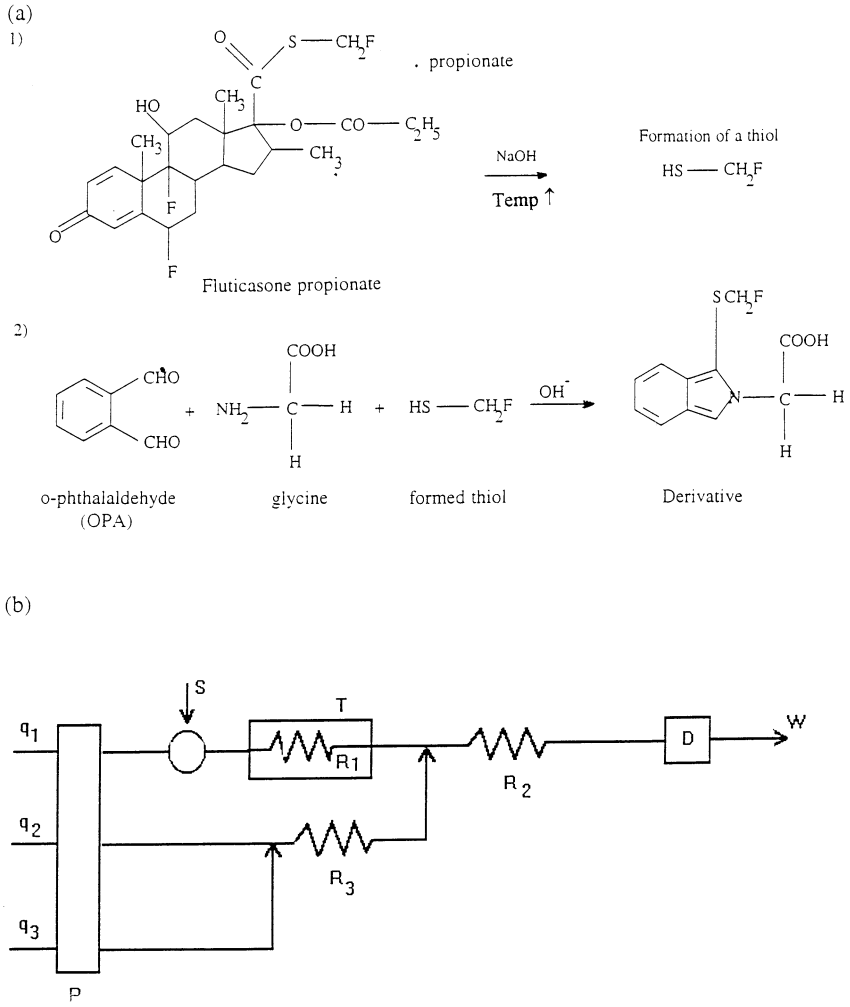

Fig. 1. Chemical reaction (a) and FIA-configuration (b) in the determination of FP.

Table 2
Factors examined in the screening and their levels

		Level −1	Level 0	Level 1
A	Concentration OPA[a] (mg l^{-1})	150		750
	Concentration OPA[b] (mg l^{-1})	100		300
B	Concentration glycine[a,b] (mg l^{-1})	100		500
C	Temperature[a,b] (°C)	50		60
D	Total flow rate[a,b] (ml min^{-1})	1.75		2.25
E	Ratio MeOH/H_2O[a,b] (v/v)	60:40		70:30
F	Length of R_2[a,b] (m)	0.75		1.25
G	Concentration NaOH[a,b] (M)	0.5		1
H	Amount of boric acid[a,b] (g l^{-1})	48	60	72

[a] UV detection.
[b] Fluorescence detection.

Table 3
3×2^7 asymmetrical design for the screening of factors with UV and fluorescence detection

	Factors													Peak height	
	A	B	C	D	E	F	G	H	9	10	11	12	13	UV detection (50 μg ml^{-1})	Fluorescence detection (5 μg ml^{-1})
1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	2689	58 397
2	−1	1	1	−1	1	1	1	−1	−1	1	1	1	−1	5948	131 271
3	1	−1	1	1	−1	1	1	−1	1	−1	−1	1	1	8137	155 486
4	1	1	−1	1	1	−1	−1	−1	1	1	1	−1	1	2490	39 152
5	−1	−1	−1	−1	1	1	−1	0	1	1	−1	1	1	2709	39 944
6	−1	1	1	−1	−1	−1	1	0	1	−1	1	−1	1	11 276	244 579
7	1	−1	1	1	1	−1	1	0	−1	1	−1	−1	−1	9541	131 349
8	1	1	−1	1	−1	1	−1	0	−1	−1	1	1	−1	2886	54 488
9	−1	−1	−1	1	−1	1	1	1	−1	1	1	−1	1	4515	96 617
10	−1	1	1	1	1	−1	−1	1	−1	−1	−1	1	1	5797	116 870
11	1	−1	1	−1	−1	−1	−1	1	1	1	1	1	−1	6617	164 543
12	1	1	−1	−1	1	1	1	1	1	−1	−1	−1	−1	5796	90 735
13	−1	−1	−1	1	1	−1	1	0	1	−1	1	1	−1	3372	46 645
14	−1	1	1	1	−1	1	−1	0	1	1	−1	−1	−1	6496	148 985
15	1	−1	1	−1	1	1	−1	0	−1	−1	1	−1	1	6186	136 991
16	1	1	−1	−1	−1	−1	1	0	−1	1	−1	1	1	6005	74 174

FIA methods have been previously reported for the determination of steroids by colourimetry [6] and chemiluminescence [7,8].

For the routine application of these tests to fluticasone propionate in industry, a relatively simple approach, using readily available equipment, is required which could employ either UV or fluorescence detection and hence should offer a wide sensitivity range. The *o*-phthalaldehyde (OPA) derivatisation reaction for primary amines in the presence of a thiol function [9] is a possibility, in that a UV/fluorescence absorbing derivative is formed very quickly. This approach has recently been applied and optimised for glycine [10] and a primary amine drug, L-*N*-monomethylarginine [11].

In this work a novel adaptation of this reaction has been employed to determine the thiol function released by the on-line hydrolysis of the FP corticosteroid thio-ester group. The sample is injected into a stream of sodium hydroxide where the FP hydrolysis is facilitated by the use of a heating stage. This stream is then partially-neutralised/buffered by a second stream of boric acid containing OPA. Glycine, the primary-amine reagent, is introduced via a third stream. The resulting reaction product is formed rapidly and can be quantified by either UV or fluorescence detection depending upon the FP concentration and thus the required sensitivity.

The resulting three-stream method requires the optimisation of several variables. Experimental design [12,13] was therefore used to produce optimal experimental conditions for the routine determination of fluticasone propionate.

In a first step a selection of the most important factors was carried out with screening designs, more specifically two-level factorial designs and asymmetrical designs. Factors found to be important were in a second step further optimised using the variable-size simplex algorithm [12,14,15]. Since both the dose delivered by the device and the fine particle fraction have to be assayed, the proposed strategy is first applied for an FIA method with UV detection. It was checked whether both concentration ranges could be determined, or whether it is necessary to additionally optimise the more sensitive fluorescence detection method. If fluorescence detection is necessary, it will be verified whether both detection methods indicate the same critical variables and whether the same optimal conditions are found.

Table 4
2^{8-4} design for the screening of factors with UV-detection[a]

	Factors								
	A	B	C	D	E	F	G	H	Peak height (50 μg ml^{-1})
1	−1	−1	−1	−1	−1	−1	−1	−1	2830
2	1	−1	−1	−1	1	1	−1	1	2551
3	−1	1	−1	−1	1	−1	1	1	5195
4	1	1	−1	−1	−1	1	1	−1	5868
5	−1	−1	1	−1	1	1	1	−1	4544
6	1	−1	1	−1	−1	−1	1	1	11 354
7	−1	1	1	−1	−1	1	−1	1	7292
8	1	1	1	−1	1	−1	−1	−1	7464
9	−1	−1	−1	1	−1	1	1	1	4018
10	1	−1	−1	1	1	−1	1	−1	3764
11	−1	1	−1	1	1	1	−1	−1	2512
12	1	1	−1	1	−1	−1	−1	1	2756
13	−1	−1	1	1	1	−1	−1	1	5065
14	1	−1	1	1	−1	1	−1	−1	6537
15	−1	1	1	1	−1	−1	1	−1	8120
16	1	1	1	1	1	1	1	1	12 316

[a] Generators: E = ABC; F = ACD; G = BCD; H = ABD.

Table 5
Effects of the factors on the peak height with UV detection using (a) a 3×2^7 design and (b) a 2^{8-4} design

Factors	Effect	Normalised effect (%)
(a) 3×2^7 design		
Concentration OPA	607	10.7
Concentration glycine	366	6.5
Temperature	3692	65.3
Total flow rate	−499	−8.8
Ratio MeOH/H_2O	−848	−15.0
Length of R_2	−639	−11.3
Concentration NaOH	2340	41.4
Amount of boric acid		
(0, −1)	1243	22.0
(1, 0)	−378	−6.7
(1, −1)	865	15.3
9	415	7.3
10	−227	−4.0
11	−485	−8.6
12	−940	−16.6
13	471	8.3
E_{crit}	1438	25.4
$E_{crit(ME)Lenth}$	1924	34.0
$E_{crit(ME)Dong}$	1453	25.7
(b) 2^{8-4} design		
Concentration OPA	1629	28.3
Concentration glycine	1357	23.6
Temperature	4150	72.0
Total flow rate	−251	−4.6
Ratio MeOH/H_2O	−670	−11.6
Length of R_2	−114	−2.0
Concentration NaOH	2271	39.4
Amount of boric acid	1113	19.3
Interactions		
AB+CE+DH+FG	−308	−5.3
AC+BE+DF+GH	1533	26.6
AD+CF+BH+EG	−215	−3.7
BC+AE+DG+FH	565	9.8
BD+CG+AH+EF	222	3.9
CD+AF+BG+EH	597	10.4
ABCD+DE+BF	1227	21.3
+AG+CH		
E_{crit}	1943	33.7
$E_{crit(ME)Lenth}$	2444	42.4
$E_{crit(ME)Dong}$	2303	40.0

2. Theory

2.1. Construction of an asymmetrical design

For screening purposes, factorial designs are mostly used, in which all factors occur with the same number of levels (normally two). In some cases however, some factors should be examined at more levels and then the much less well known asymmetrical designs can be used. To construct an asymmetrical design, a number of fractional plans are usually combined [16]. This means for instance that when a three-level factor (levels −1, 0 and 1) and three two-level factors have to be examined, a fractional factorial design at two levels for three factors is replicated with the three level factor once at each level. This requires 12 experiments. Addelman [16] proposed orthogonal main-effect plans for asymmetrical factorial experiments which require less trials. For the given example only eight experiments have to be performed. Identical plans were described by Wei Guang Lan et al. [17] as mixed-level orthogonal array designs.

The way these asymmetrical designs are constructed is explained briefly here with an example. For more details, we refer to Ref. [16]. Suppose that a design is needed where one factor has three levels and four factors have two. First a two-level full factorial design for three factors (X_1, X_2, X_3) is constructed, for which the contrast coefficients for all interactions are shown (Table 1a). From this design three columns are selected of which the third is the column of contrast coefficients of the first two. Suppose that X_1, X_2 are selected. This leads to X_1X_2 as third column. These three two-level factors can then be replaced by one four-level factor according to the correspondence scheme $0\,0\,0\rightarrow0$; $0\,1\,1\rightarrow1$; $1\,0\,1\rightarrow2$; $1\,1\,0\rightarrow3$. This results in a design where one factor is examined at four levels (A) and four factors at two levels, represented as a 4×2^4 design (Table 1b). From this design, a 3×2^4 design, where one factor is at three levels and four are at two levels, can be obtained by collapsing the four-level factor to a three-level factor by the correspondence $0\rightarrow0$; $1\rightarrow1$; $2\rightarrow2$; $3\rightarrow1$. One obtains in this way a 3×2^4 design (Table 1c).

Using these asymmetrical designs permits to estimate uncorrelated main effects.

2.2. Calculation of effects and interpretation of significance

Effects were calculated according to the equation

$$E_x = \frac{\sum Y(i)}{n_i} - \frac{\sum Y(j)}{n_j}$$

with $\Sigma Y(i)$ and $\Sigma Y(j)$ the sums of the responses where factor x is at higher (i) and lower (j) level, respectively, and n_i or n_j the number of times each factor x is at (i) or (j) level, respectively [12,13]. In this way, three effects can be calculated for a three-level factor namely $E(0,-1)$, $E(1,0)$ and $E(1,-1)$. Normalised effects were calculated as

$$\%E_x = \frac{E_x}{\overline{Y}} \times 100$$

where $\overline{Y}$ was the average response of the design results [18].

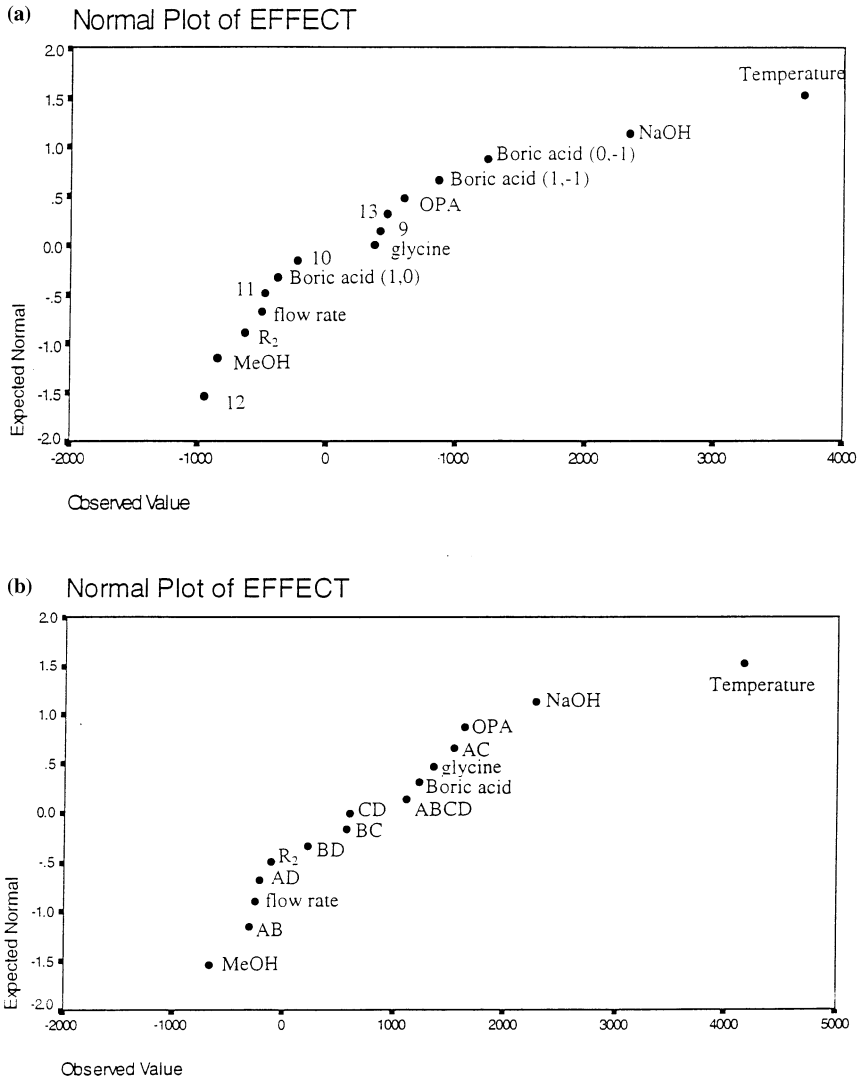

Fig. 2. Normal probability plot for UV response of (a) a 3×2^7 design and (b) a 2^{8-4} design.

Table 6
Variable-size simplex search [14] for the optimisation of temperature, concentration of NaOH and amount of boric acid with UV detection[a]

Vertex	Exp+step	Factors			Response
		Temperature (°C)	NaOH (M)	Boric acid (g l^{-1})	Peak height (100 μg ml^{-1})
	1	55.0	0.750	54.00	13 067
	2	64.4	0.868	55.88	41 030
	3	57.4	1.221	55.88	17 300
	4	57.4	0.868	61.54	17 932
1,2,3,4	5(R:1)	64.5	1.221	61.54	46 382
1,2,3,4	6(E:5)	69.2	1.457	65.31	49 097
2,3,4,6	7(R:3)	69.9	0.907	65.94	50 218
2,3,4,6	8(E:7)	76.2	0.751	71.20	0
2,4,6,7	9(R:4)	78.3	1.287	63.55	0
2,4,6,7	10(Cw:4)	62.6	0.973	61.96	40 509
2,6,7,10	11(R:10)	73.1	1.182	62.80	65 796
2,6,7,10	12(E:10)	78.3	1.287	63.55	0
2,6,7,10	13(R:2)	77.0	1.497	73.87	0
2,6,7,11	14(Cw:2)	67.6	1.025	60.28	54 469
6,7,11,14	15(R:6)	71.2	0.619	60.70	49 555
6,7,11,14	16(Cr:6)	70.7	0.828	61.86	57 057
7,11,14,16	17(R:7)	70.9	1.117	57.35	62 526
11,14,16,17	18(R:14)	75.6	1.060	61.05	0
11,14,16,17	19(Cw:14)	69.6	1.034	60.45	60 220
11,16,17,19	20(R:16)	71.7	1.393	58.56	60 708
11,16,17,19	21(Cr:16)	71.4	1.252	59.36	61 307

[a] R, reflection; E, expansion; Cw, contraction on the R (reflection) side; Cr, contraction on the W (worse) side [14].

Statistical significance of the effects was checked by applying a t-test [18] where a critical effect (E_{crit}) is calculated as

$$E_{crit} = t_{crit} \times (SE)_e$$

with t_{crit} a tabulated critical t-value and $(SE)_e$ the standard error on the effect, which can be estimated in different ways as is explained below. If the absolute value of a factor effect is larger than this critical effect, then the factor is statistically significant.

A first estimation for $(SE)_e$ can be calculated using multiple-factor interaction effects (E_{xyz}) which are not confounded with main-factors, as

$$(SE)_e = \sqrt{\frac{\sum E_{xyz}^2}{n_{xyz}}}$$

where n_{xyz} is the number of these interaction effects and also the number of degrees of freedom for t_{crit}.

Another way to estimate the SE on the effect is to use the distribution of the non-significant effects as described by the algorithms of Lenth [19] and Dong [20], which are based on the use of the median of effects.

Lenth estimates in a first step s_0, the standard deviation (S.D.) of the effects, as

$$s_0 = 1.5 \times \underset{x}{\text{median}} |E_x|$$

A second estimate for the S.D., which Lenth calls the pseudo standard error (PSE) is derived as

$$PSE = 1.5 \times \underset{|E_x| < 2.5 s_0}{\text{median}} |E_x|$$

where the effects with a value larger than $2.5s_0$ are excluded when selecting the median. The PSE is then used to calculate two critical effects called 'margin of error'

$$E_{crit(ME)} = t_{(0.975;m/3)} \times PSE$$

with m the total number of effects from the performed design, and 'simultaneous margin of

error', $E_{crit(SME)}$ using the same formula but with the t-value corrected for multiple comparisons of t-tests, i.e. working at smaller α-values. For more theoretical background we refer to the original paper of Lenth [19].

Dong [20] estimates the SE of the effect by calculating

$$s_1 = \sqrt{\frac{1}{n} \times \sum_{|E_x| < 2.5 s_0} E_x^2}$$

with n the number of effects with a value smaller than $2.5s_0$, which replaces in fact the PSE of Lenth's method. The number of degrees of freedom for the critical effect is n.

According to Nijhuis et al. [21], an effect is considered significant when the critical limit ME is exceeded while ignoring the more conservative SME-limit. We therefore calculated only ME-values.

Normal probability plots [18,22] were also drawn to study graphically the significance of effects.

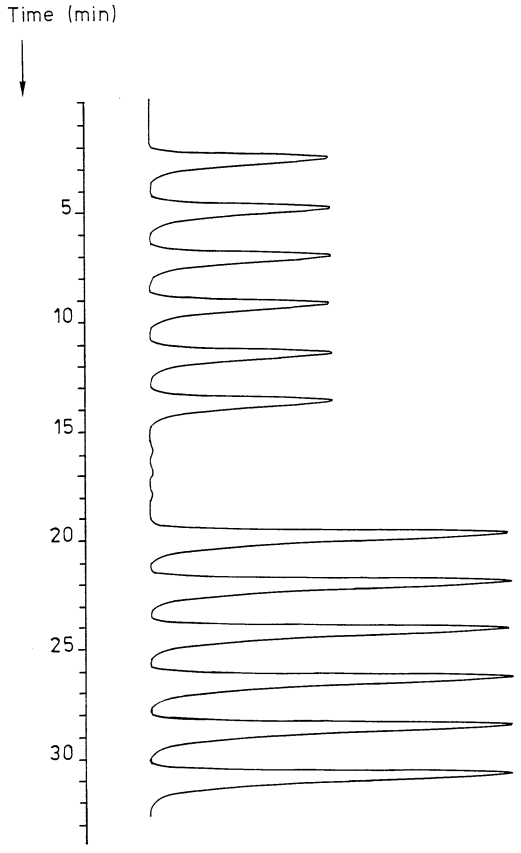

Fig. 3. Optimal conditions for the determination of FP with UV detection.

Table 7
Effect of the factors on the peak height with fluorescence detection using a 3×2^7 design

Factors	Effect	Normalised effect (%)
Concentration OPA	−4549	−4.2
Concentration glycine	8785	8.1
Temperature	91 240	84.4
Total flow rate	−18 880	−17.4
Ratio MeOH/H_2O	−33 039	−30.5
Length of R_2	−2649	−2.4
Concentration NaOH	26 436	24.4
Amount of boric acid		
(−1, 0)	13 568	12.5
(0, 1)	7547	7.0
(−1, 1)	21 115	19.5
9	16 239	15.0
10	−9770	−9.0
11	12 293	11.4
12	−20 423	−18.9
13	9675	8.9
E_{crit}	36 738	33.9
$E_{crit(ME)Lenth}$	49 866	46.1
$E_{crit(ME)Dong}$	36 074	33.3

2.3. The modified-size simplex algorithm

The modified-size simplex algorithm is a sequential optimisation method which is based on reflection, contraction and expansion of the vertices of a geometric figure with one vertex more than the number of variables to be optimised [14,15]. For the optimisation of two variables, the simplex is therefore a triangle. Each vertex corresponds to a set of experimental conditions. A regular starting simplex is constructed by defining for each variable (1) a starting value, which acts as a reference point for the generation of the other vertices, and (2) a step length, which is a measure of the span of the initial simplex in each factor dimension [14]. After performing the exper-

iments of the initial simplex, the values of the responses are ranked from worst (W), next to worst (N) and best (B). The vertex with the worst result is then reflected over the centroid of the remaining vertices to generate a new experiment (R). Depending on the response of R, different paths can be followed [14,15]. This procedure is continued until (1) the reflections do not give any further improvement, (2) all responses of the vertices of the last simplex are similar or (3) until one vertex is retained a number of times.

3. Experimental

3.1. Equipment

The flow injection analysis was performed on a Burkard (Burkard Scientific, Uxbridge, UK) FIA-flo flow injection system equipped with PTFE six-port valves. PTFE tubing (0.5 mm i.d.) was used for all connections, except for the heating unit which had a fixed i.d. of 0.8 mm. A schematic representation of the chemical reaction and the

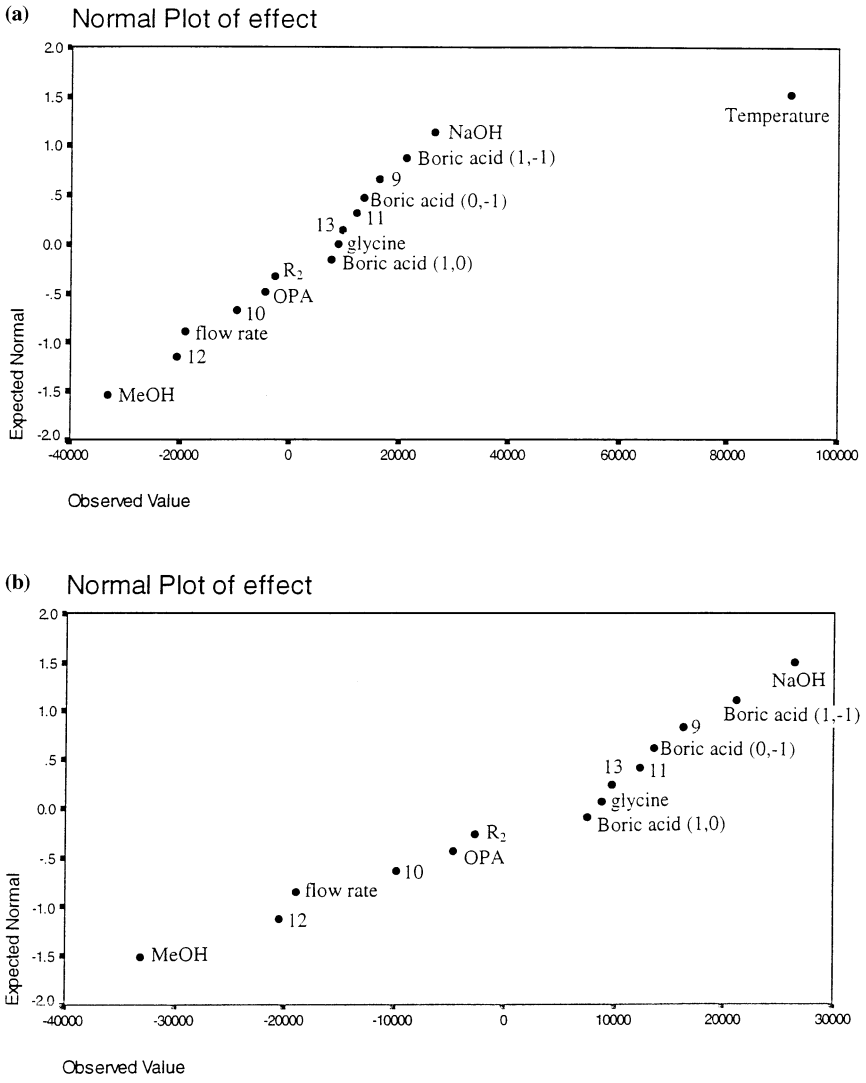

Fig. 4. Normal probability plot of fluorescence detection with (a) all effects and (b) without the effect of the temperature.

Table 8
Variable-size simplex search [14] for the optimisation of the temperature and the fraction of MeOH with fluorescence detection

Vertex	Exp+step	Factors		Response
		Temperature (°C)	Fraction MeOH	Peak height (5 μg ml^{-1})
	1	62.0	0.580	391 066
	2	66.7	0.586	498 882
	3	63.2	0.604	358 817
1,2,3	4(R:3)	65.5	0.562	531 687
1,2,3	5(E:3)	66.7	0.542	0
1,2,4	6(R:1)	70.2	0.568	642 704
1,2,4	7(E:1)	74.4	0.562	0
2,4,6	8(R:2)	69.1	0.545	0
2,4,6	9(Cw:2)	67.3	0.576	498 807
2,4,6	10(R:4)	71.4	0.592	592 610
2,6,10	11(R:2)	75.0	0.574	0
2,6,10	12(Cw:2)	68.8	0.583	570 506
6,10,12	13(R:12)	72.9	0.577	638 893
6,10,13	14(R:10)	71.7	0.553	630 364

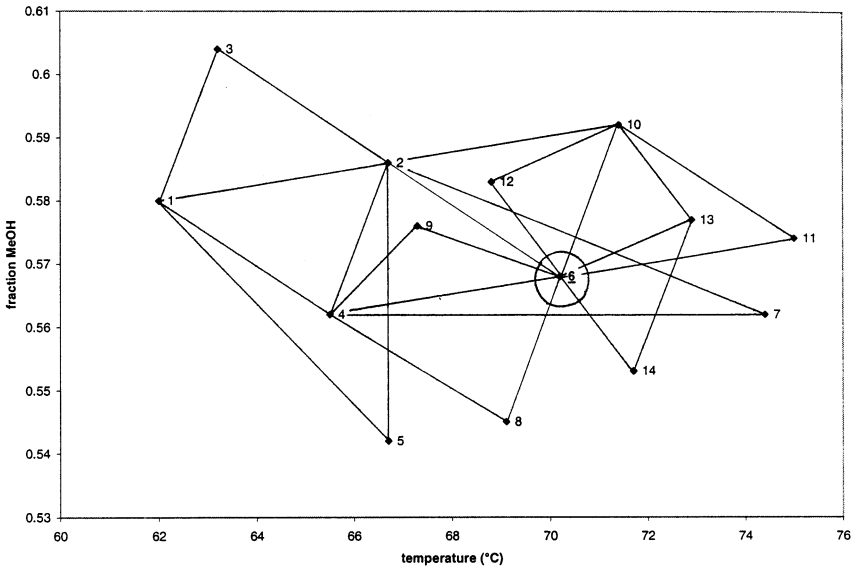

Fig. 5. Evolution of the simplex during the optimisation of FP with fluorescence detection.

three-stream FIA-system used is shown in Fig. 1. Fluticasone propionate (FP), dissolved in a mixture of methanol/water, is injected (S) in a sodium hydroxide line (q_1) and heated (T) to hydrolyse the drug on-line (R_1). The thiol-group of the obtained compound then reacts with a second line (R_2) containing o-phthalaldehyde (q_2) and a primary amine, glycine (q_3) [9] which are mixed on-line (R_3) and which are both dissolved in a mixture methanol/water containing boric acid. The reaction product is measured spectrophotometrically (D) and the peak height recorded. The stream emerging from the sensing system is wasted (W).

For the UV-detection, a Merck–Hitachi L-4200 variable wavelength UV–Vis Detector, equipped

with a 11.3-μl flow-cell, was applied to monitor the reaction product. The detection wavelength was 336 nm. A Merck–Hitachi F-1050 Fluorescence Spectrophotometer with a 12-μl flow-cell (excitation wavelength 335 nm and emission wavelength 425 nm) was used for the fluorescence detection. Peak heights were measured with a Merck–Hitachi D-7500 integrator.

3.2. Reagents and solutions

Fluticasone propionate was provided by Glaxo–Wellcome (Dartford, UK). A 100 μg ml^{-1} stock solution of FP was prepared in a mixture of methanol (BDH, Poole, UK) and Milli-Q Water (Milli-Q water purification system, Millipore, Bedford, MA), of which the ratio varied according to the experimental design experiments and which was sonicated to release possible air-bubbles. Depending on the detection used, working solutions of the sample were injected, prepared in the same methanol–water mixture in concentrations of 50 and 100 μg ml^{-1} for UV-detection and of 2 and 5 μg ml^{-1} for fluorescence detection.

Sodium hydroxide pellets (Merck, Darmstadt, Germany) were dissolved in the methanol–water mixture to obtain a concentration necessary for the design experiments and the same was done with boric acid (Merck, Darmstadt, Germany). *O*-Phthaldehyde 97% (OPA) (Sigma Aldrich, Steinheim, Germany) was dissolved in 5 ml methanol and adjusted to 100 ml with the boric acid solution. Glycine (Merck, Darmstadt, Germany) was dissolved directly in the boric acid solution. OPA and glycine varied according to the experimental design requirements. All solutions were sonicated before use and kept in dark bottles.

4. Results and discussion

4.1. Preliminary investigations

Initially some experiments were executed to select an FIA-configuration and to gain information about the solubility of the reagents. These experiments revealed that a solvent of methanol/water was required to ensure solution of both FP, the reagents and boric acid. A heating stage with a sodium hydroxide concentration of at least 0.5 M was also necessary to ensure adequate hydrolysis of the FP thio-ester function. Use of a mixed reagent of OPA and glycine in boric acid was investigated but this was found to be unstable after a period of a few hours. A three-line manifold was hence developed employing a sodium hydroxide stream for hydrolysis and separate OPA and glycine reagent streams; the latter two both prepared in boric acid solution. The FIA-configuration is shown in Fig. 1b.

4.2. UV-detection

The reaction was first monitored using UV-detection. The volume injected, the length of the reaction coil in the heating unit (R_1), the length of R_3 and the ratio of the flow rate of the NaOH-line (q_1) and of the mixture OPA and glycine ($q_2 + q_3$), were set at a constant value based on the residence time, i.e. the time from injection of the sample to the detection of the maximum of the peak, which was required to be as short as possible. The volume injected was 120 μl, since it was difficult to change this volume during the design experiments. The length of the reaction coil in the heating unit (R_1) was selected as 2 m, since the other possible options performed less adequately. With 3 m, the residence time was too long and with 1 m too short for reaction. The ratio of the flow rate of the NaOH-line (q_1) and of the mixture OPA and glycine ($q_2 + q_3$), was kept constant (1:1) during all experiments, since the concentration of NaOH, OPA and glycine were examined as factors in the design. Entering the ratio of the flow rate as a factor is not useful because it cannot be varied independently of the other three factors (concentrations). The length of R_3 was set at 0.5 m since this tubing is only necessary to insure a proper mixing of q_2 and q_3. Eight factors were eventually selected to be optimised further (Table 2). From preliminary experiments, executed to gain an idea about the solubility of the reagents and about the pH installed on-line, it was suspected that the amount of boric acid might have an intermediate optimal value in the selected domain. For this reason a 3×2^7 asymmetrical screening

design with seven factors at two levels and one factor (boric acid) at three levels was constructed according to Addelman's rules [16]. Sixteen experiments (Table 3) were executed and compared with the results of a two-level fractional factorial design for eight factors, 2^{8-4}, of resolution IV (Table 4), with the same number of experiments to evaluate which design delivers the most informative results. It should be mentioned that the notations of the levels for the asymmetrical design (-1, 0, 1) have been changed compared to Addelman's notations (0, 1, 2) to be able to compare more easily with the two-level fractional factorial design. For each factor an upper ($+1$) and a lower (-1) level and for boric acid also an intermediate level (0) (Table 2) were selected based on the preliminary experiments. First the asymmetrical design was executed and effects of the factors on the peak height were calculated (Table 5a). The temperature has clearly a very large influence on the response. Drawing a normal probability plot (Fig. 2a) also shows that the temperature is important as well as the amount of sodium hydroxide. The effect of boric acid in the region -1 level to 0 is probably on the limit of significance. These results are confirmed when calculating a critical effect using columns 9–13, which are combinations of interaction terms, to estimate the SE on the effect (Table 5a). Applying the methods of Lenth and Dong (Table 5a) to select significant effects confirms the previous conclusions. For the three level factor boric acid, the results of the asymmetrical design confirm that there is a (local) optimum value in the selected region since the effect of (0, -1) is important, while the effect of boric acid (1, 0) is less so. The total effect of boric acid over the range (1, -1), which is the sum of the two effects (0, -1) and (1, 0), is non-significant, which shows that two-level designs can lead to incorrect results and that chemical knowledge should be applied wherever possible in the planning of experimental design.

The experiments of the 2^{8-4} design were then executed (Table 4) and the effects of the factors on the peak height calculated (Table 5b). Drawing a normal probability plot (Fig. 2b) indicates the temperature and the concentration of sodium hydroxide to be important factors. This conclusion is confirmed by the E_{crit} (Table 5b), calculated using all interaction effects to estimate the SE of the effect. The method of Lenth and the method of Dong (Table 5b) indicate only the temperature as significant. As was shown before [10], the ME value from Lenth's method depends very much on the value of the median so that one has to be careful with its interpretation. It can also be remarked that the value of the effect of the interaction (AC + BE + DF + GH) is larger than all other interaction effects. This could be explained as a result of the interaction GH (amount of sodium hydroxide–boric acid) since this interaction controls the pH. The pH has to be established on-line and the reaction performs well only at a suitable pH. Consequently one has to be careful with the use and interpretation of screening designs where the pH is indirectly incorporated, which confirms again the importance of chemical knowledge when factors have to be selected or defined.

When comparing the asymmetrical and the fractional factorial design, it can be remarked that in both designs the factors temperature and the amount of NaOH are significant. For the three level factor boric acid, the results of the asymmetrical design confirm that there is a (local) optimum value in the selected region. The effect of boric acid (1, -1) has the same importance in both designs. From the fractional factorial design it would not be assumed that an optimum is present for boric acid and neither would this factor be further examined since its effect (1, -1) is considered as non-significant. Thus, the asymmetrical design provides more information about this factor. It is however not always obvious to decide beforehand on using an asymmetrical design for screening quantitative variables. If it is suspected that one or more variables could have a maximum (or minimum) in the examined region, the use of this type of design should be taken into consideration.

Although the effect of the concentration of OPA and glycine is somewhat higher in the fractional factorial design, the conclusions drawn for the two-level factors of the asymmetrical design

confirm those obtained by the fractional factorial design. It can thus be said that the asymmetrical design provides in this case more information with the same number of experiments. The significant factors, the sodium hydroxide and the temperature perform the hydrolysis of FP, while the amount of boric acid establishes a good pH to perform the reaction of OPA afterwards.

The factors found important (temperature, concentration sodium hydroxide) were further optimised in a second step using the variable-size simplex algorithm [14,15]. Although the amount of boric acid in the interval (0, −1) was on the limit of significance, it was also further optimised. The factors that had no significant influence on the response were set at their nominal level, being the average of their upper and lower level, except for the total flow rate and the length of R_2 which were set at their low level. Although a lower flow rate results in a longer residence time, the lower value was preferred because the baseline was more stable under these conditions. This results in the following levels: concentration of OPA 450 mg l^{-1}, glycine 300 mg l^{-1}, the ratio MeOH/water 65:35 v/v, total flow rate 1.75 ml min^{-1} and the length of R_2 0.75 m. At these levels the optimisation of the three other factors was executed. Some constraints had to be taken into account for the area in which the optimum was searched. The temperature could not exceed 74°C because for higher values, spikes were observed probably due to boiling of the methanol/water mixture. A concentration of 72 g l^{-1} and of 1.5 M for boric acid and sodium hydroxide, respectively, was the limit to be used because of precipitation of the compounds in the solvent.

The starting values for the variable-size simplex were selected based on the results of the screening. The effects of all three factors were positive, which means that the response was higher at a high level. The first geometrical figure was therefore built around the high levels of the factors used in the screening design (Table 6). This leads to starting values of 55°C, 0.75 M and 54 g l^{-1} for the temperature, the concentration of NaOH and the amount of boric acid, respectively and initial step lengths of 10°C, 0.5 M and 8 g l^{-1}. The experiments performed in the optimisation are shown in Table 6. When an experiment was found outside the constraints, a peak height of 0 was allocated to it. After 21 experiments, the optimisation was stopped because the peak height did not vary much anymore and experiment 11 was retained with each new simplex. Moreover the differences between the levels for each factor between new consecutive experiments became too small for practical execution.

The best conditions to determine the amount of FP with UV-detection were found to be the ones of experiment 11. Fig. 3 shows the results for injections of 50 and 100 μg ml^{-1} of FP using the conditions of experiment 11. The time from injection to detection was 2.40 min and a repeatability of 0.66% RSD was obtained for a concentration of 50 μg ml^{-1} FP injected. UV detection can be used to determine the 'uniformity of dose test' for FP since the concentration range was linear up to 300 μg ml^{-1}. The concentration of the fine particle fraction (2–5 μg ml^{-1}) however cannot be detected accurately. Therefore the use of fluorescence detection will be examined and optimised.

4.3. Fluorescence detection

The same factors were screened as for the UV-detection, although the level settings for OPA had to be adapted because of a high background fluorescence due to the fluorescence of OPA itself (Table 2). The asymmetrical design (3×2^7) was again executed (Table 3) since this design provides more information about the influence of boric acid than the 2^{8-4} design in the UV-optimisation. Effects of the factors on the peak height were calculated (Table 7) and a normal probability plot was obtained (Fig. 4a). It is obvious that the temperature has a very large influence on the peak height. When omitting the temperature and drawing a new normal probability plot (Fig. 4b), the fraction of methanol in the mixture also seems to influence the response. Calculating a critical effect using columns 9–13 to estimate the SE on the effect, and using the method of Dong (ME = 36 074) indicates the temperature to be significant and the fraction of MeOH at the limit of significance. Lenth's method (ME = 49 866) indicates only the temperature as significant.

The conclusions drawn for the UV detection are not the same as the ones for the fluorimetric detection (Table 5a, Table 7). In both cases the temperature is the most significant factor, but the concentration of NaOH, important in UV-detection, is not important using fluorimetric detection, while the fraction of methanol has an effect, probably a quenching effect. It was also noted that the noise on the baseline increases with higher concentrations of methanol. The effect of the concentration of OPA is completely different for both detection methods but this can be a result of the difference in level settings.

The temperature and the fraction of methanol were further optimised with the variable-size simplex algorithm. The factors that had no significant influence on the response were, as for the UV-detection, set at their nominal level, except for the total flow rate and the length of R_2 which were set at their low level for the reasons explained before. The optimisation was thus executed with 200 mg l^{-1} OPA, 300 mg l^{-1} glycine, 0.75 M NaOH, 60 g l^{-1} of boric acid, a total flow rate of 1.75 ml min^{-1} and a length of R_2 of 0.75 m. Some constraints had to be taken into account for the temperature, which could not exceed 74°C, and for the fraction of MeOH, which could not decrease below 0.55 (this notation will further be used to express the ratio MeOH/water 55:45 v/v) as the limiting solubility level for FP. The starting values for the variable-size simplex were again selected based on the results of the screening. The first geometrical figure was constructed around the high level for the temperature, since this effect was positive, while for the ratio of MeOH/water around the low level (Table 8). This results in starting values of 62°C and 0.58 for the temperature and the fraction of methanol, respectively and step lengths of 5°C and 0.025. The experiments performed in the optimisation and the evolution of the simplex are shown in Table 8 and in Fig. 5. When an experiment was outside the constraints of a factor, a peak height of 0 was allocated to it. After 14 experiments, the optimisation was stopped because the peak height did not vary much anymore and the differences for the factor levels between new consecutive experiments became too small for practical execution. Optimal conditions to determine FP with fluorescence detection were selected to be 200 mg l^{-1} OPA, 300 mg l^{-1} glycine, 0.75 M NaOH, 60 g l^{-1} boric acid, temperature 70°C, methanol/water ratio 57:43 v/v, a total flow rate of 1.75 ml min^{-1} and a length of R_2 of 0.75 m. The residence time was again 2.4 min and a %RSD of 0.87% was obtained for 5 μg ml^{-1} FP injected.

When comparing the best conditions to determine FP with UV or fluorescence detection, it can be observed that only the concentration of glycine, the total flow rate and the length of R_2 have the same values in both methods. All other factors examined have different values resulting in different optima.

5. Conclusions

Fluticasone propionate can be determined by the three-stream FIA-system after hydrolysis followed by reaction of the liberated thiol with OPA and a primary amine. Depending on the concentration of FP, UV or fluorescence detection can be chosen. The aim was not to select the best detection method, but to offer optimal conditions to determine FP with both UV and fluorescence detection, depending on the availability of instruments and the concentrations of FP that have to be detected.

The optimisation procedure followed was designed to first select the factors having an important influence on the response and then to optimise these factors in an acceptable number of experiments. The asymmetrical designs of Addelman used for screening of quantitative factors are useful in cases where it is suspected that one or more factors can have a (local) optimum in the examined domain. In this case these factors can be examined at more than two levels in the same number of experiments as when examining them all at two levels. It has to be remarked however that with the asymmetrical designs only main factors are checked and no interaction terms. Since in optimisation, interaction factors can be important, one has to be careful in the interpretation of the results of these designs.

The two detection methods examined, UV and fluorescence, did not have the same important variables. For the UV-detection the temperature and the pH establishment on-line are the most critical parameters, while for the fluorescence the temperature and the fraction of methanol in the mixture are critical. Moreover the conditions found to be the best are different for both detection methods.

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