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Liquid chromatographic analysis of carboxylic acids using N-(4-aminobutyl)-N-ethylisoluminol as chemiluminescent label: determination of ibuprofen in saliva

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ABSTRACT

N-(4-Aminobutyl)-N-ethylisoluminol was used for labelling of carboxylic acids. The derivatization reaction was carried out with 1-hydroxybenzotriazole as pre-activator of the carboxylic acid function and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide as the coupling reagent. Optimum conditions for the derivatization were determined by using factorial design analysis, with ibuprofen as the test compound. Chemiluminescence detection was carried out using a post-column on-line electrochemical hydrogen peroxide generation system and the addition of microperoxidase as the catalyst. The detection limit of derivatized ibuprofen in human saliva was 0.7 ng per 0.5 ml of saliva, with a recovery of 96.1 ± 1.3%. The method was linear over at least three decades (2.5 ng to 2.5 μg) and the repeatability was satisfactory (R.S.D. = 5.2% at the 25 ng level; n = 4).

INTRODUCTION

In recent years, there has been an increasing interest in the development of sensitive and selective liquid chromatographic (LC) methods. Chemiluminescence (CL) detection offers a possibility to improve detection limits, since CL is often 10–100 times more sensitive than fluorescence. For both detection modes derivatization is often required, because the number of compounds showing native fluorescence or CL is limited.

The three most important CL detection systems for LC are the peroxyoxalate, luminol and lucigenin systems. In the luminol system, which was the detection system used in this study, hydrogen peroxide reacts with luminol in a basic medium in the presence of a catalyst. Luminol itself is not suitable as label and, therefore, modified luminol derivatives are used. Isoluminol isothiocyanate has been used for the derivatization of amino acids [1], 4,5-diaminophthalhydrazide for the derivatization of α-keto acids [2,3]

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and N-(4-aminobutyl)-N-ethylisoluminol (ABEI) for the labelling of fatty acids and several amines [4], eicosapentanoic acid, a prostaglandin precursor [5], methamphetamine [6,7] and amphetamine [7].

The CL detection unit used in these studies consists of a two-pump post-column system for the addition of hydrogen peroxide and the catalyst. In our previous work [8] we used an electrochemical flow cell to generate hydrogen peroxide on-line, instead of adding it by a pump. This turned out to be a good alternative for the post-column reagent addition. An advantage of on-line electrochemical reagent generation is easier handling of the CL detection system.

In the present study ibuprofen, the test compound, was derivatized with ABEI via a carbodiimide method optimized by factorial design analysis, and detected with the electrochemical reagent generation method and post-column addition of microperoxidase as the catalyst. Ibuprofen was determined in human saliva after oral administration. This requires a sensitive analytical method, because ibuprofen is strongly bound to plasma proteins, which means that the concentration of the analyte in saliva is rather low.

EXPERIMENTAL

Reagents

ABEI and the drugs ibuprofen and flurbiprofen (internal standard) and the catalyst microperoxidase were purchased from Sigma (St. Louis, MO, USA). The derivatization reagents 1-hydroxybenzotriazole (HOBT) and N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Janssen Chimica (Beerse, Belgium) and Fluka (Buchs, Switzerland), respectively. Solutions of HOBT and EDC were prepared fresh daily, whereas solutions of ABEI could be stored for at least one month at −20°C. A 10 m*M* sodium hydrogencarbonate buffer of pH 10.5 (Brocacef, Maarssen, Netherlands) and acetonitrile of chromatography grade from J.T. Baker (Deventer, Netherlands) were used in the mobile phase. The same grade acetonitrile, dried over a 0.4-nm molecular sieve, was used to prepare the solutions of HOBT and EDC. All other reagents and solvents were of analytical-reagent grade and came from various sources.

Chromatographic conditions

A schematic diagram of the LC system with CL detection is shown in Fig. 1. The mobile phase, acetonitrile–carbonate buffer (pH 10.5), was delivered by a Spectroflow (Applied Biosystems, Ramsey, NJ, USA) Model 400 or a Gilson (Villiers-le-Bel, France) Model 302 R pump, equipped with a laboratory-made pulse damper, at a flow-rate of 0.8 ml/min. Microperoxidase, dissolved in the carbonate buffer at a concentration of 5.0 μM, was added post-column by an LKB Model 2150 pump (Pharmacia LKB Biotechnology, Uppsala, Sweden) at a flow-rate of

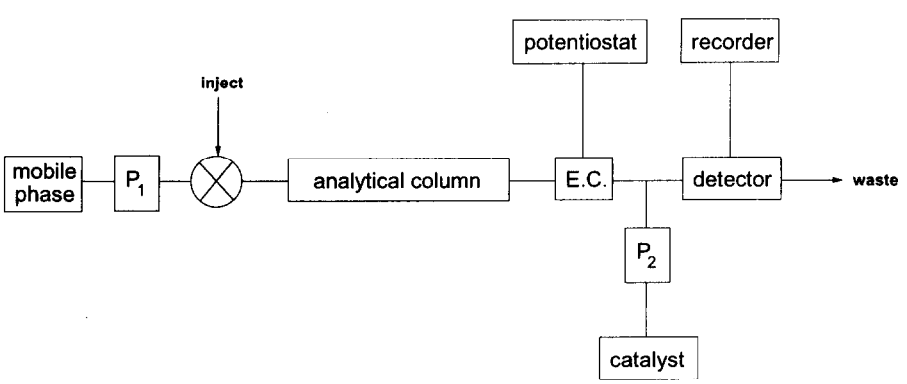

Fig. 1. Schematic diagram of the LC–CL system. E.C. = electrochemical flow-cell, operated at −600 mV; P_1 = pump for the mobile phase: acetonitrile–10 m*M* carbonate buffer (pH 10.5); P_2 = pump for the catalyst: 5 μM microperoxidase in 10 m*M* carbonate buffer (pH 10.5).

0.4 ml/min. The on-line electrochemical generation of hydrogen peroxide was carried out as described previously [8].

The derivatization mixtures (10 μl) were injected by a PROMIS injection system (Spark Holland, Emmen, Netherlands) equipped with a Rheodyne (Cotati, CA, USA) Model 7010 injection valve using a 20-μl sample loop. A polymer PLRP-S analytical column (150 mm × 4.6 mm I.D., 5 μm particle size; Polymer Labs., Church Stretton, UK) was used in combination with a laboratory-made guard column (10 mm × 2.0 mm I.D.) filled with 10-μm PLRP-S particles. The LC separation was carried out at 40°C. Detection was performed with a Kratos Spectroflow Model 980 fluorescence detector (Applied Biosystems), operating at 900 V and equipped with a 25-μl flow cell and a cut-off filter of 389 nm.

The derivatization reaction conditions were optimized by replacing the CL detection system with a Kratos Spectroflow Model 757 UV–VIS absorbance detector (Applied Biosystems) operated at 280 nm.

Derivatization procedure

A 50-μl volume of a solution of ibuprofen in methanol (0.05 mg/ml) was evaporated to dryness under a stream of dry nitrogen at ambient temperature in a 0.7-ml reaction vial (Type 3814, Eppendorf, Hamburg, Germany). The derivatization reaction was carried out as determined by factorial design analysis; to the residue were added, successively, 200 μl of a solution of HOBT (0.13 mg/ml) and 200 μl of a solution of EDC (1.5 mg/ml) in dry acetonitrile, and 10 μl of an ABEI solution in 0.04 *M* methanolic potassium hydroxide (4.0 mg/ml). After 10 s of vortex-mixing, the mixture was allowed to react for 35 min at 53°C (water-bath). Subsequently, 200 μl of the carbonate buffer were added to stop the reaction, and 10 μl of the final solution were injected into the LC system using a mobile phase of acetonitrile–10 m*M* carbonate buffer pH 10.5 (30:70, v/v).

Prior to the optimization of the derivatization reaction, the influence of the type of organic solvent used as reaction medium was studied. To this end, 50 μl of the methanolic ibuprofen solution (0.05 mg/ml) were evaporated to dryness; next, 10 μl of the methanolic solutions of HOBT (1 mg/ml) and EDC (10 mg/ml), and 300 μl of dry organic solvent were added. Then, 10 μl of the ABEI solution (5 mg/ml) were added and the reaction was allowed to proceed for 10 min at 50°C. To stop the reaction, 50 μl of methanol were added, and the sample was evaporated to dryness under nitrogen. Finally, 300 μl of the mobile phase were added and 10 μl of the resulting solution were injected into the LC system.

Determination of ibuprofen in saliva

To a 0.5-ml aliquot of saliva, 50 μl of 4 *M* hydrochloric acid and 50 μl of a methanolic solution of the internal standard flurbiprofen were added. After 20 s of vortex-mixing, extraction was performed with two 1-ml volumes of hexane–2-propanol (90:10, v/v). After centrifugation for 1 min at 2800 *g*, the combined organic layers were transferred to a 2-ml reaction vial (Eppendorf, Type Safe-Lock), and the derivatization reaction was carried out as described above. Instead of 200 μl of the carbonate buffer, 1 ml of a 0.1 *M* acetate buffer pH 4 (adjusted with 6 *M* NaOH) was added.

To remove the excess of the label ABEI, a solid-phase extraction (SPE) on C_2 cartridges (Type 7273, capacity 3 ml, J.T. Baker) was performed. The cartridge was conditioned with 2 ml of acetonitrile, 2 ml of the acetate buffer and 2 ml of acetonitrile–acetate buffer (2:5, v/v). The sample and 1 ml of the rinsing solution of the reaction vial (acetonitrile–acetate buffer) were transferred to the cartridge, which was then washed with 1 ml of buffer and 2 ml of water. The cartridge was dried by purging with air, and the analyte was eluted with 1 ml of acetonitrile. The solvents were passed through the cartridge using a Baker-10 SPE processing station, applying a pressure of 13–17 kPa. Acetonitrile was evaporated under a stream of dry nitrogen at 40°C, and the residue was dissolved in 400 μl of methanol by vortex-mixing. A 10-μl volume was injected into the LC–CL system, in a mobile phase of acetonitrile–10 m*M* carbonate buffer pH 10.5 (25:75, v/v).

To investigate the analyte recovery after SPE, an external standard of phenol (50 μl of 0.1 mg/ml acetonitrile) was added to the eluate. At high levels (2.5 μg of ibuprofen derivatized with ABEI) analysis was carried out by the LC system with UV detection at 280 nm; at lower levels (25 ng of ibuprofen derivatized with ABEI) the phenol was detected by the UV detector, and the derivatized ibuprofen was detected by CL.

Identification of derivatization product

Ibuprofen (two 25-μg amounts) was derivatized with ABEI as described before. The combined reaction mixture was applied to a pre-coated silica gel 60-F preparative TLC plate (20 cm × 20 cm) with a layer thickness of 2 mm (Merck, Darmstadt, Germany). The plate was developed over a distance of *ca.* 18 cm with ethyl acetate–chloroform–formic acid (6:4:2, v/v/v) as the eluent. The chromatographic band of the derivatization product (R_F = 0.72) was isolated by scraping the silica gel off the TLC plate and subsequently extracted with two 1-ml volumes of methanol. A 10-μl volume was injected into the LC–CL system; the remainder was evaporated under nitrogen.

A portion of the evaporated sample was introduced into a glycerol–trifluoracetic acid (0.1%) matrix to measure the fast atom bombardment (FAB) mass spectrum with a MAT 90 mass spectrometer (Finnigan MAT, Bremen, Germany), equipped with an Ion Tech saddle-field FAB gun (operated at 0.2 mA and 7 kV, and employing xenon). Another portion was introduced into the same instrument on a platinum wire to measure the desorption chemical ionization (DCI) mass spectrum (emission current, 0.2 mA; electron energy, 150 eV; indicated source pressure, 0.053 Pa) with ammonia as the reagent gas.

Factorial design analysis

To obtain a better insight into the influence of the individual parameters on the derivatization reaction and to discover interactions between these parameters, full factorial designs [9] were set up. The height of the ABEI-labelled ibuprofen peak served as the response factor. The experiments were performed in duplicate and randomized.

TABLE I

SET-UP OF THE FIRST FACTORIAL DESIGN

Level	HOBT[a] (mg/ml)	EDC[a] (mg/ml)	ABEI[a] (mg/ml)	Time (min)	Temperature (°C)
−1	0.1	0.50	1.0	10	20
0	0.3	1.25	3.0	20	–
+1	0.5	2.00	5.0	30	50

[a] Concentration of stock solution.

In the first factorial design five parameters were selected. Four of these, the concentrations of HOBT, EDC and ABEI, and the derivatization reaction time, were varied at three levels, and one, the reaction temperature, at two levels (Table I). Three levels were chosen for four of the parameters because the dependence of the response on them was expected to be non-linear.

In the second factorial design four parameters were varied at three levels (Table II); the concentration of the stock solution of ABEI was kept constant at 4 mg/ml.

RESULTS AND DISCUSSION

Optimization of the derivatization reaction

Because derivatization of carboxylic acids with ABEI using 2-bromo-1-methylpyridium iodide (BMP) and 3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidin-2-one (DPP), as proposed by Yuki *et al.* [5],

TABLE II

SET-UP OF THE SECOND FACTORIAL DESIGN

Level	HOBT[a] (mg/ml)	EDC[a] (mg/ml)	Time (min)	Temperature (°C)
−1	0.005	0.50	15	50
0	0.100	1.25	30	60
+1	0.195	2.00	45	70

[a] Concentration of stock solution.

did not give satisfactory results in our hands, a carbodiimide method was selected. EDC was chosen as the carbodiimide and HOBT as the pre-activator for the carboxylic acid function. Fig. 2 shows the reaction of carboxylic acids with ABEI in the presence of EDC and HOBT.

First of all, the nature of the solvent used as reaction medium was investigated. Because, in principle, reactions of carboxylic acids with amines are incompatible with water [10], an organic solvent had to be used. Because ABEI does not dissolve well in organic solvents, a methanolic solution of potassium hydroxide was used instead. The potassium hydroxide additionally served as the base catalyst for the derivatization reaction. From the results in Table III, it is clear that acetonitrile gave the best results; chloroform and dichloromethane can also be used. Acetonitrile was used in all further experiments. Concentrations of potassium hydroxide higher than 0.04 *M* were found to decrease the derivatization yield, and at lower concentrations ABEI did not dissolve. For derivatization the addition of 10 μl of the ABEI solution was found to be optimal. The use of either a smaller (5 μl) or a larger (30 μl) volume of the ABEI solution, and use of 30 μl of a three-fold lower ABEI concentration, all gave 15–25% lower derivatization yields.

TABLE III

EFFECT OF SOLVENT ON THE DERIVATIZATION YIELD OF IBUPROFEN WITH ABEI

For conditions, see Experimental.

Solvent	Peak height[a] (mm)
Acetonitrile	60
Chloroform	54
Dichloromethane	53
Dichloroethane	44
Ethyl acetate	44
Diethyl ether	44
Tetrahydrofuran	42
Ethanol	36
Methanol	30
Acetone	23

[a] Mean of two experiments.

To identify the ABEI derivative of ibuprofen (ABEI-IBU), the product was isolated by TLC on silica gel and analysed by FAB and DCI mass spectrometry (MS). In FAB-MS the isolated

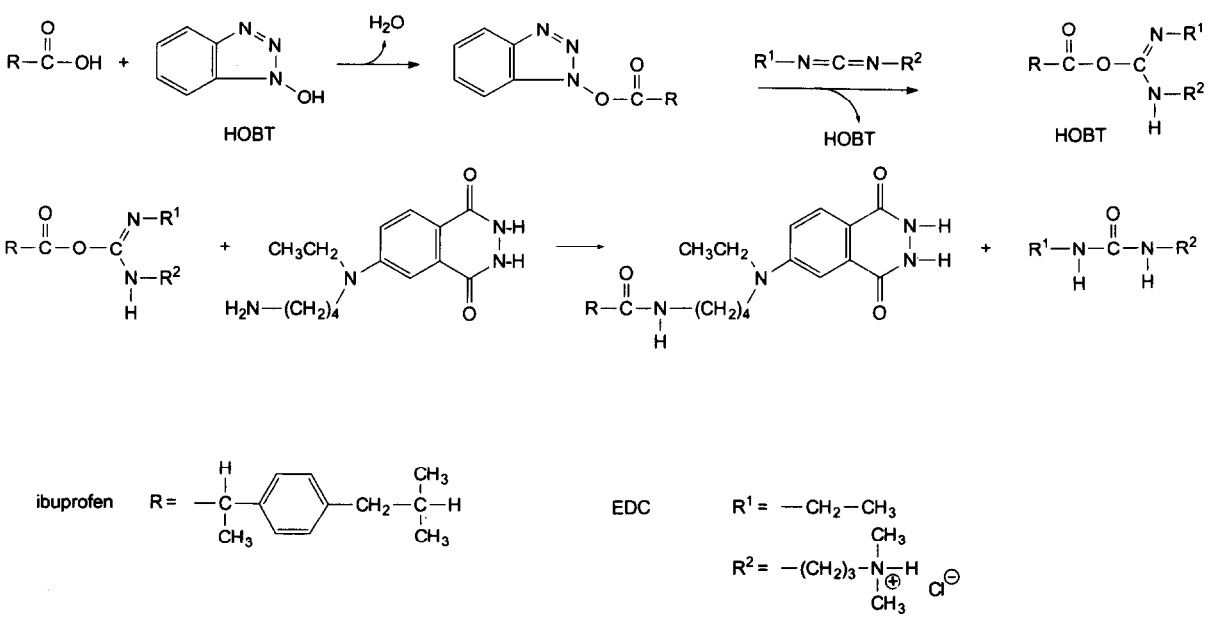

Fig. 2. Derivatization of carboxylic acids (ibuprofen) with ABEI in the presence of the pre-activator HOBT and the carbodiimide EDC.

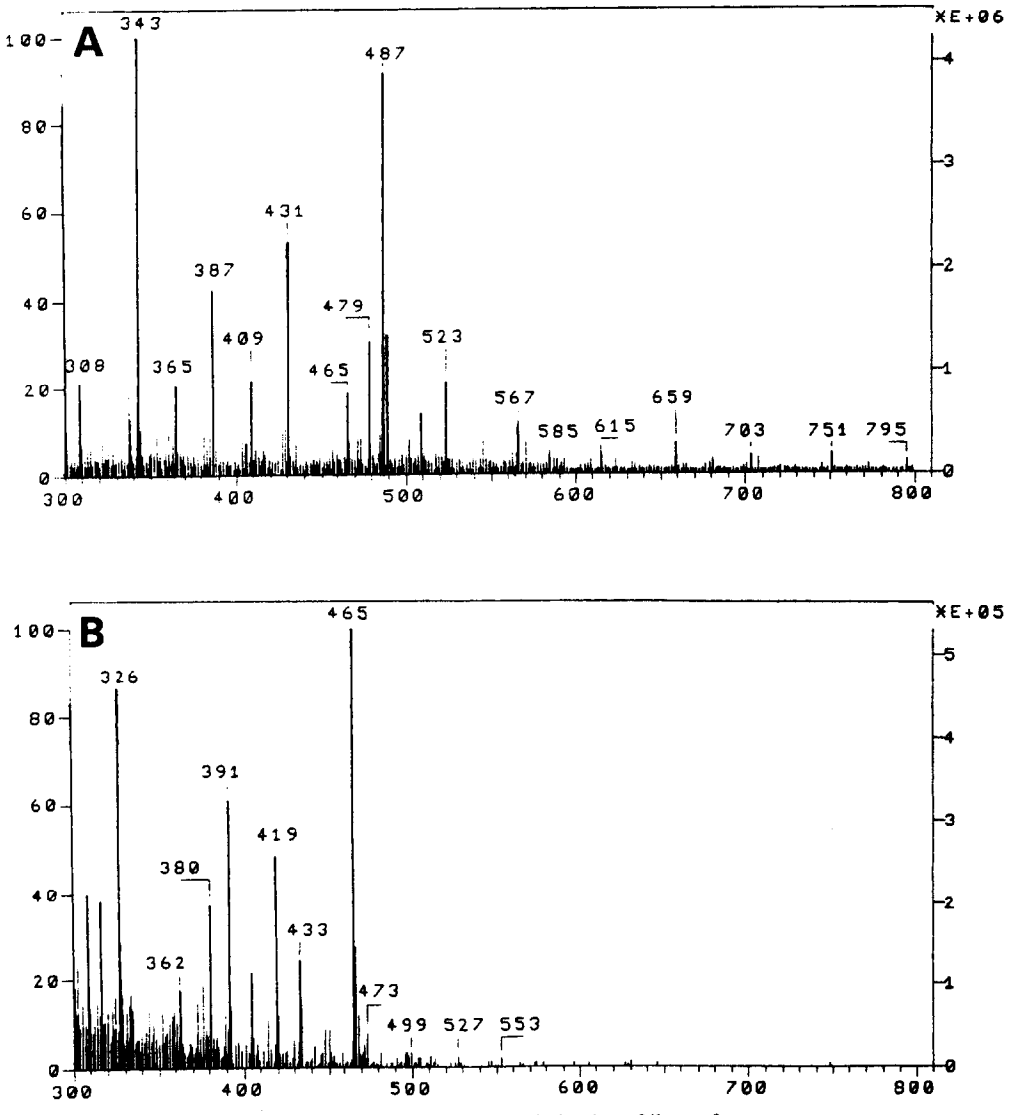

Fig. 3. FAB (A) and DCI (B) mass spectra of the ABEI derivative of ibuprofen.

product gave an $[M+Na]^+$ signal at m/z 487, while in both the FAB and DCI mass spectra the $[M+H]^+$ ion at m/z 465 showed up (Fig. 3). Therefore, the derivative formed after reaction of ibuprofen with ABEI is ABEI-IBU as shown in Fig. 2.

To optimize the derivatization yield and to discover relationships between the parameters involved in it, factorial designs were set up. For the first factorial design, which consisted of four parameters measured at three levels and one parameter measured at two levels, 162 ($3 \times 3 \times 3 \times 3 \times 2$) experiments were carried out in duplicate. From the results of these experiments, a model was calculated with the natural logarithmic values of the peak height of ABEI-IBU as the response factor. Natural logarithms were used because regression analysis can be carried out only when the absolute error is constant. The peak heights measured in this experiment have, however, a constant rela-

TABLE IV

FACTORIAL ANALYSIS OF THE FIRST EXPERIMENT

Parameter	Parameter coefficient	Standard error	t for H_0[a]	P[b]
Intercept	3.8111	0.0124	306.38	0.0001
HOBT	0.0144	0.0100	1.45	0.1495
EDC	0.4011	0.0058	69.65	0.0001
ABEI	0.0184	0.0058	3.19	0.0017
Time	0.3316	0.0058	57.57	0.0001
Temperature	0.5146	0.0081	63.18	0.0001
EDC^2	−0.2113	0.0100	−21.19	0.0001
ABEI^2	−0.0194	0.0100	−1.95	0.0535
Time^2	−0.1100	0.0100	−11.03	0.0001
HOBT × time	0.0173	0.0071	2.46	0.0152
EDC × time	−0.0728	0.0071	−10.32	0.0001
EDC × temperature	−0.1436	0.0058	−24.94	0.0001
Time × temperature	−0.0607	0.0058	−10.54	0.0001
EDC^2 × temperature	0.0275	0.0100	2.76	0.0066
Time^2 × HOBT	−0.0385	0.0122	−3.16	0.0019
EDC × time × temperature	−0.0338	0.0071	−4.80	0.0001

[a] Value of the t-test for the null hypothesis (H_0, parameter is zero).

[b] Probability that the null hypothesis is falsely rejected.

tive error. Calculation of the model was performed by regression analysis using the SAS software, in which the response factor is expressed as a function of the parameters and the relationships. For every parameter and relationship this computer program successively calculates the parameter coefficient, the standard error, the t-value for the null hypothesis and the probability (P).

The best model of the dataset with fifteen model terms was obtained by calculating a model with all parameters and interactions, the terms of values with $P > 0.06$ then being removed (Table IV). The model was kept hierarchical, *i.e.* HOBT was not removed, because a relationship of HOBT (HOBT × time) is included in the model. The model gives a root mean square error of 0.060, which corresponds to a relative error of 6.0% in the measured peak heights, and an adjusted r^2 of 0.9929, explaining 99.29% of the variance in the response values. There is no lack of fit, because the root mean square pure error of 0.066, calculated from the duplicate experiments, is greater than the root mean square error of the model. At a level of significance of 95%, the model shows three significant third-order relationships (with small coefficients), four significant second-order relationships and three quadratic effects. A relationship between parameters means that changing one of them at a constant level of the other parameter, or *vice versa*, will cause the response factor to change.

The data in Table IV also show that the concentration of HOBT is not significant in the area measured, and that there are no relationships between ABEI and the other parameters.

From the model, the optimum parameter settings were calculated by a specially written FORTRAN computer program. The model was represented by a polynomial, of which the maximum can be iteratively calculated within a hyperbox. The optimum of HOBT was found to be at the level −1.0 (0.10 mg/ml), but this parameter was not significant (see above). The optimum of EDC was found to be at the level 0.4 (1.55 mg/ml), and

that of ABEI at 0.5 (4.0 mg/ml) and of the time at the level 1.0 (30 min). The optimum of the temperature was found to be at the level 1.0 (50°C). However, because this parameter was measured at two levels, this implies only that the overall effect of the temperature was positive.

A second factorial design was then set up, because the concentration of HOBT seemed not to be significant, while the optima of time and temperature were at the border of the measured area. In the second experiment, the concentration of ABEI was kept constant at 4 mg/ml (ABEI gave no interactions), and the concentrations of EDC were the same as in the previous experiment. The levels of HOBT were chosen at lower concentrations than in the first experiment, because it is expected that HOBT will then be of significance. The levels of time were chosen at higher values and the temperature was measured at three levels to discover the presence of squared effects of the temperature (Table II). For the second factorial design experiment, 81 ($3 \times 3 \times 3 \times 3$) experiments were performed in duplicate.

The model was calculated in the same way as described for the first model (Table V); with nineteen model terms it has a root mean square error of 0.063 and an adjusted r^2 of 0.9617. There is no lack of fit, because the root mean square pure error is 0.060, which results in a ratio close to 1 (0.063/0.060). This model shows that, in the range 0.005–0.195 mg/ml, the concentration of HOBT is indeed significant. The reaction time, however, appears not to be of significance between 15 and 45 min. All parameters show a squared term, which means that serious curvature was present, and six third-order relationships appeared to be significant: as an example, one of these is presented in Fig. 4. Three-dimen-

TABLE V

FACTORIAL ANALYSIS OF THE SECOND EXPERIMENT

Parameter	Parameter coefficient	Standard error	t for H_0[a]	P[b]
Intercept	5.0055	0.0209	239.18	0.0001
HOBT	0.1749	0.0148	11.82	0.0001
EDC	0.0571	0.0085	6.69	0.0001
Time	−0.0166	0.0148	−1.12	0.2655
Temperature	−0.1179	0.0226	−5.21	0.0001
HOBT^2	−0.2218	0.0148	−14.99	0.0001
EDC^2	−0.1627	0.0148	−11.00	0.0001
Time^2	−0.0805	0.0148	−5.44	0.0001
Temperature^2	−0.1290	0.0148	−8.72	0.0001
HOBT × EDC	−0.0338	0.0104	−3.23	0.0020
HOBT × temperature	0.0722	0.0104	6.90	0.0001
EDC × time	−0.1400	0.0104	−13.38	0.0001
EDC × temperature	−0.1389	0.0104	−13.27	0.0001
Time × temperature	−0.1317	0.0104	−12.59	0.0001
HOBT^2 × temperature	−0.0743	0.0181	−4.10	0.0001
EDC^2 × HOBT	0.0938	0.0181	5.18	0.0001
EDC^2 × temperature	0.0761	0.0181	4.20	0.0001
Time^2 × temperature	0.0449	0.0181	2.48	0.0160
Temperature^2 × time	0.0460	0.0181	2.54	0.0137
HOBT × EDC × temperature	0.0368	0.0128	2.87	0.0056

[a] Value of the t-test for the null hypothesis (H_0, parameter is zero).

[b] Probability that the null hypothesis is falsely rejected.

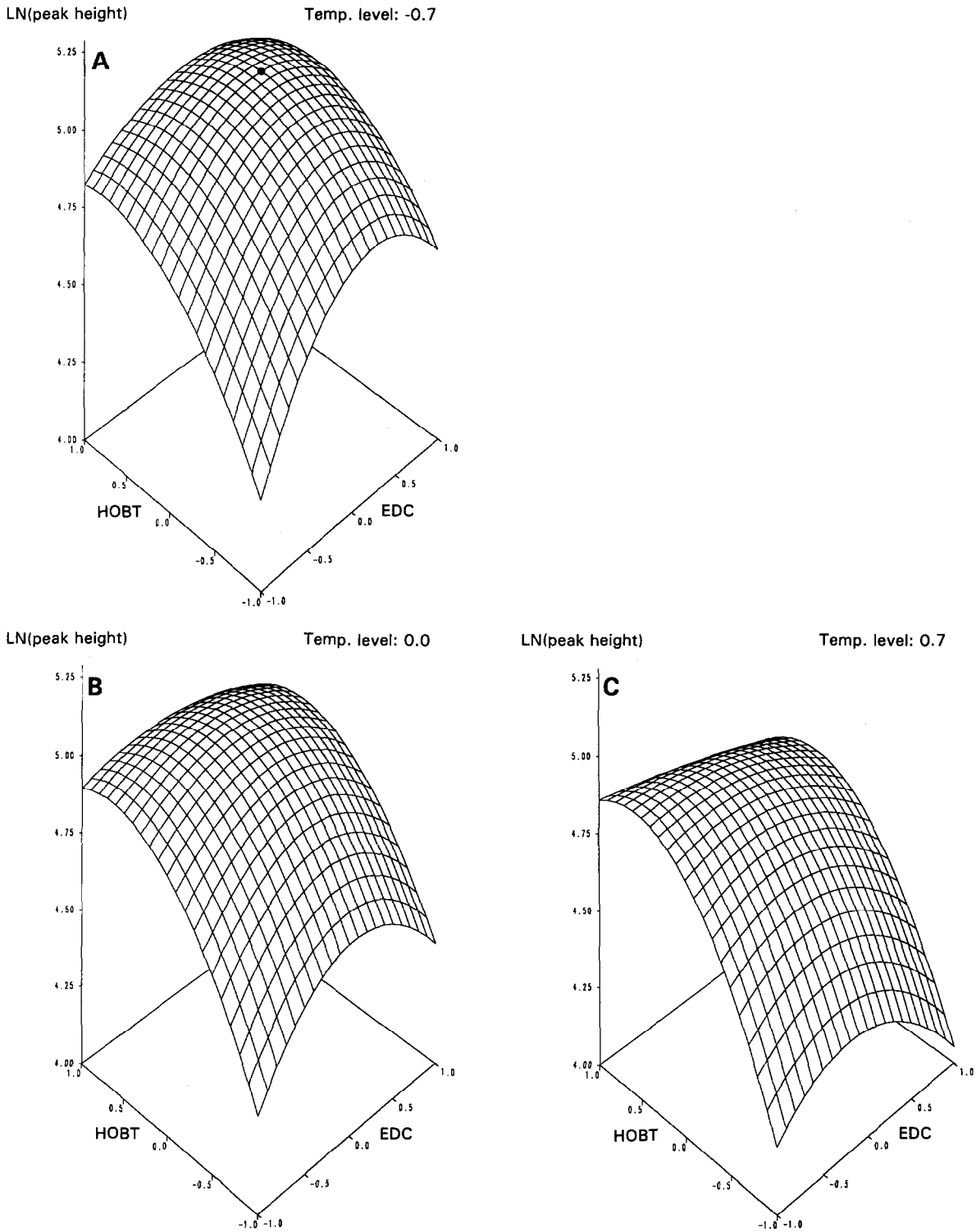

Fig. 4. Plots of the natural logarithm of the peak height of ABEI-IBU *versus* the levels of HOBT and EDC at the levels 0.3 (35 min) of the reaction time and the levels (A) −0.7 (53°C), (B) 0.0 (60°C) and (C) 0.7 (67°C) of the temperature. The absolute optimum is indicated by a black dot in A.

sional plots of the natural logarithms of the calculated response factor *versus* the calculated levels of HOBT and EDC are shown for three temperatures at a constant level of 0.3 for the reaction time. The relationship between the three parameters, HOBT, EDC and temperature, is apparent because the response surface is curved. The absolute optimum was calculated to be at a level of 0.3 (0.13 mg/ml) for HOBT, 0.3 (1.5 mg/ml) for EDC, 0.3 (35 min) for the reaction time and −0.7 (53°C) for the temperature. This optimum is indicated by the black dot in Fig. 4A [(ln(peak height) = 5.078 ± 0.063].

The optimum conditions, which are summarized in Experimental, were experimentally tested by measuring the peak height of the ABEI-IBU derivative at the optimum conditions and at both a higher and lower level for all the parameters involved. As shown in Table VI, higher concentrations of HOBT and EDC gave comparable peak heights, all other conditions (especially a lower EDC concentration and higher or lower temperatures) resulted in lower heights of the ABEI-IBU peak. Although the experimentally determined optimum peak heights are 30% lower than the calculated optimum of 160 mm, which may be partly due to small changes in the LC system, the calculated optimum appears to be close to the real optimum. The derivatization yield of ibuprofen (25 ng) with ABEI under the optimized conditions was 76 ± 3% (n = 2).

The repeatability of the ibuprofen derivatization procedure (2.5 μg) with ABEI was determined by injecting six samples with and without the internal standard flurbiprofen (2.5 μg), and analysis by LC with UV detection. The relative standard deviations (R.S.D.) of the measured peak heights were the same in both instances (1.6–2.1%). These values are of the same order as the R.S.D. of the precision of the LC–UV system (1.9%, n = 6). The relative errors of the factorial design analyses (6.0 and 6.3%) are quite satisfactory. They are, however, higher than the R.S.D. values of the repeatability and the precision. This is probably caused by a low inter-day variance of the LC and derivatization conditions.

In this study, ibuprofen was used as the test component. However, other drugs with a carboxylic acid function can also be labelled with ABEI by the present method. Both aliphatic and aromatic acids can be derivatized, although no product was observed with nalidixic acid and cromolyn sodium as the analytes (Table VII). This is probably due to the presence of an electron-withdrawing carbonyl group close to the carboxylic acid group.

TABLE VI

EXPERIMENTAL TEST OF THE CALCULATED OPTIMUM OF THE DERIVATIZATION REACTION OF IBUPROFEN WITH ABEI

HOBT[a] (mg/ml)	EDC[a] (mg/ml)	ABEI[a] (mg/ml)	Time (min)	Temperature (°C)	Peak height (mm)
0.13	1.5	4.0	35	53	112
0.23	1.5	4.0	35	53	112
0.03	1.5	4.0	35	53	109
0.13	2.5	4.0	35	53	113
0.13	0.5	4.0	35	53	86
0.13	1.5	6.0	35	53	109
0.13	1.5	2.0	35	53	110
0.13	1.5	4.0	55	53	109
0.13	1.5	4.0	15	53	98
0.13	1.5	4.0	35	68	87
0.13	1.5	4.0	35	38	77

[a] Concentration of stock solution.

TABLE VII

DERIVATIZATION OF DRUGS WITH A CARBOXYLIC ACID GROUP

+, derivative peak observed; −, no derivative peak observed.

Aliphatic acid		Aromatic acids	
Ibuprofen	+	Benzoic acid	+
Flurbiprofen	+	Nicotinic acid	+
Fenoprofen	+	Cinchophen	+
Ketoprofen	+	Nalidixic acid	−
Naproxen	+	Cromolyn sodium	+
Valproic acid	+		
Indomethacin	+		
Sulindac	+		

LC–CL system

Chromatography of standard solutions of ibuprofen was performed with a mobile phase consisting of 26 vol.% of acetonitrile in a 10 m*M* carbonate buffer (pH 10.5) at a flow-rate of 0.8 ml/min. In a previous study [8] this pH was found to be optimal for CL detection. In the same study, an optimum potential of −600 mV was found for the electrochemical generation of hydrogen peroxide. The optimum concentration of the catalyst, microperoxidase, was found to be 5 μM at a flow-rate of 0.4 ml/min. At higher concentrations of microperoxidase a decrease of the CL intensity was observed, possibly caused by quenching of the CL signal.

The precision of the LC–CL system was determined by derivatizing 25 ng of ibuprofen; the R.S.D. was 2.2% (n = 6).

The calibration graph of standard solutions in the range from 1.25 ng (6 pmol) to 20 ng (100 pmol) of ibuprofen in the presence of 5 ng of flurbiprofen as internal standard (five data points measured in duplicate) had a correlation coefficient (r) of 0.997 with an intercept of −0.061 ± 0.221 and a slope of 0.520 ± 0.021 with t = 24.3 (P <0.001). Fig. 5 shows an LC–CL chromatogram of a standard solution of ibuprofen derivatized with ABEI and a corresponding blank.

Determination of ibuprofen in saliva

Anti-inflammatory drugs are frequently

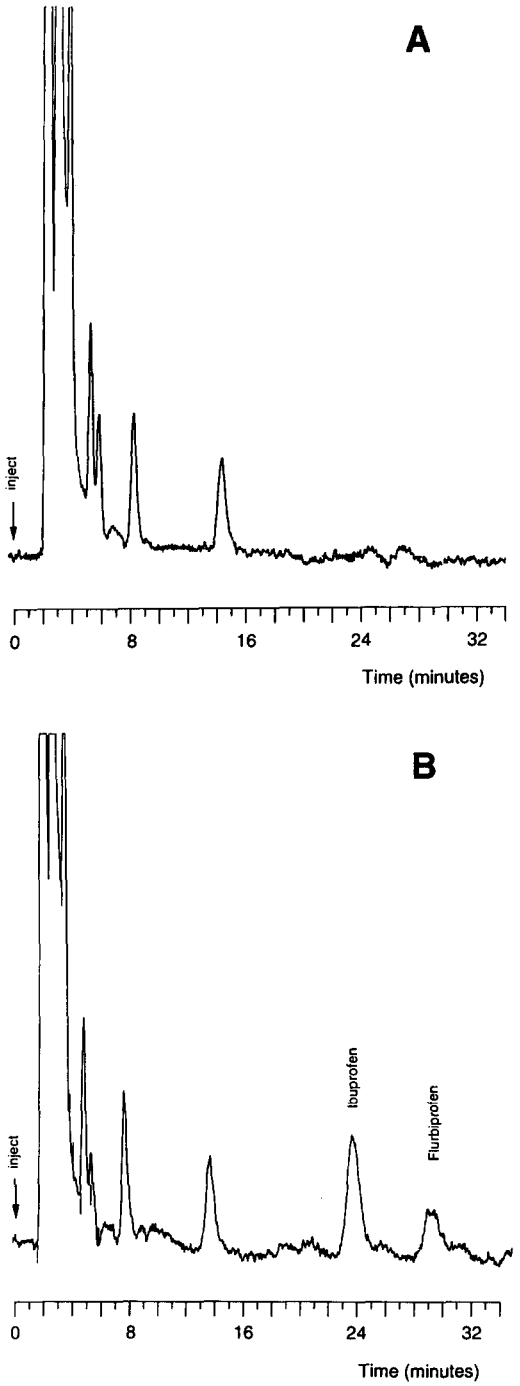

Fig. 5. LC–CL chromatograms of standard solutions of ibuprofen after derivatization with ABEI: (A) a blank and (B) ibuprofen (5 ng) with 5 ng of flurbiprofen as internal standard. LC eluent, acetonitrile–10 m*M* carbonate buffer pH 10.5 (26:74, v/v). Other conditions as Experimental.

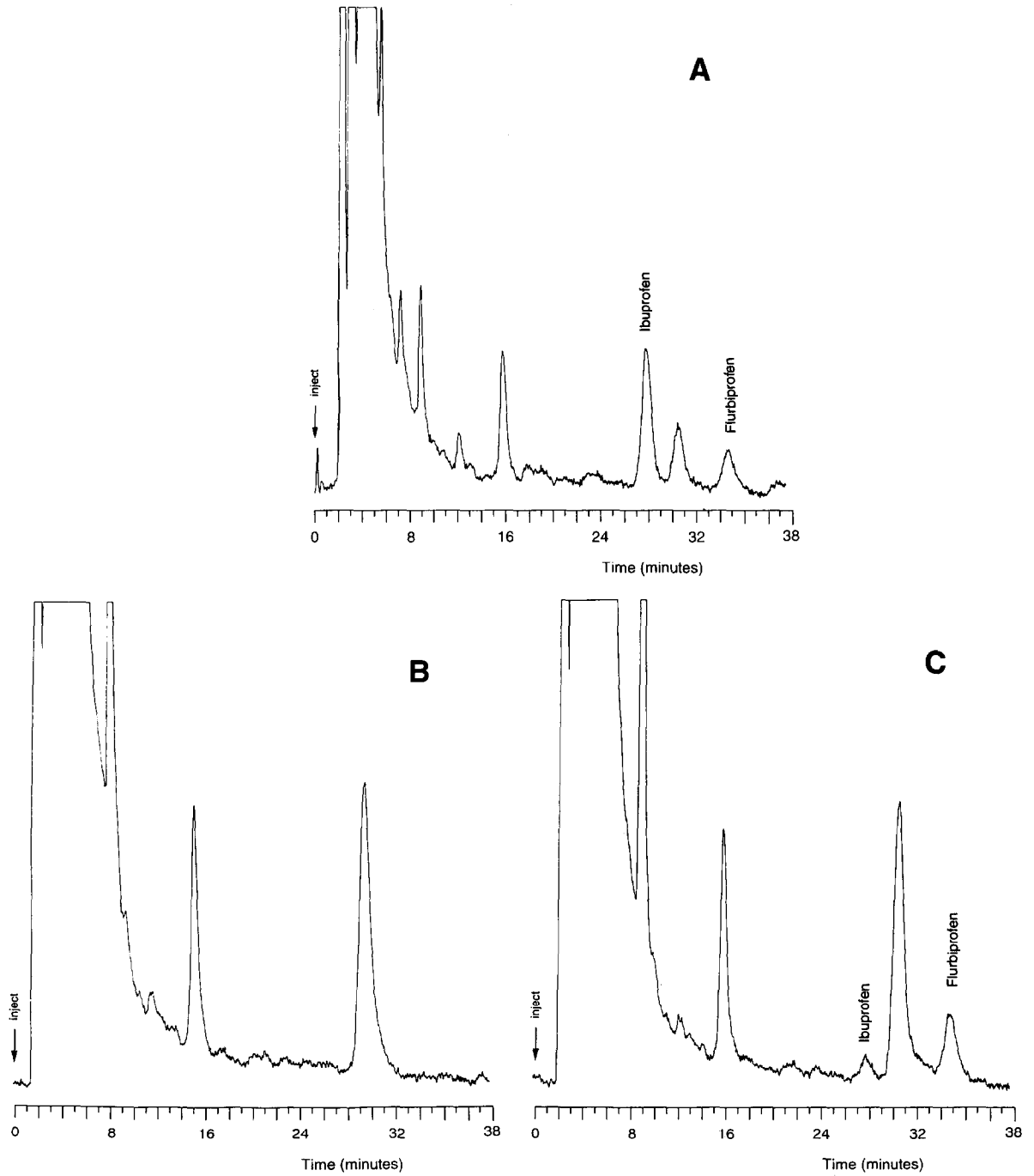

Fig. 6. LC–CL chromatograms obtained after derivatization of ibuprofen in saliva (0.5 ml) containing 10 ng of flurbiprofen as internal standard, with ABEI (A) 1 h after a 400-mg oral administration (calculated concentration, 14.5 ng per 0.5 ml saliva), (B) blank saliva, (C) saliva spiked with 1.25 ng of ibuprofen per 0.5 ml. LC eluent, acetonitrile–10 m*M* carbonate buffer pH 10.5 (25:75, v/v). Other conditions as in Experimental.

strongly bound to plasma proteins and, as a result, only a small fraction of the administered drug will be present in saliva. The analysis of saliva samples offers advantages in comparison with plasma samples, because saliva sampling is easy, non-invasive and stress-free. Ibuprofen is 99% bound to plasma proteins [11], and the maximum concentration of ibuprofen in plasma after an oral administration of 400 mg is *ca.* 30 μg/ml, which is reached after *ca.* 3 h, and the half-life is *ca.* 2 h for a normal healthy human [12]. In a pH range of 6–8 for saliva, the saliva concentration of ibuprofen (pK_a = 4.4) will be in the range 0.04–4% of the plasma concentration [13], and therefore a sensitive analytical method is required.

In order to extract ibuprofen from saliva a liquid–liquid extraction was performed. Extraction with two 1-ml volumes of hexane–2-propanol (90:10, v/v) was found to give recoveries of 96.1 ± 1.3% (n = 6). Extraction with an additional millilitre of this mixture gave a recovery of 0.5–1.0% only.

After the extraction of ibuprofen from saliva, derivatization with ABEI was performed as described above. To determine ibuprofen at low concentrations with the LC–CL system, the excess of the label had to be removed before injection. Different SPE cartridges, containing C_2, C_8 and C_{18} bonded silica, were tested for the removal of excess ABEI. It was found that C_2 cartridges gave the best result. Although the recovery of ibuprofen after SPE (78.5%) was not extremely high, the repeatability (R.S.D. 4.2%, n = 4) for 2.5 μg of ibuprofen derivatized with ABEI was acceptable; the amount of ABEI left in the remaining sample solution was only *ca.* 15%. To investigate the analyte recovery at lower levels, 25 ng of ibuprofen were derivatized and detected by LC–CL, while a external standard of phenol (50 μl of 0.1 mg/ml acetonitrile) was detected by a UV detector, placed at the outlet of the LC column. The recovery was of the same order as before, 79.2% with an R.S.D. of 5.2% (n = 4).

The calibration graph of ibuprofen [2.5 ng to 2.5 μg (12 pmol to 12 nmol); four data points measured in duplicate] in 0.5 ml of saliva using the internal standard flurbiprofen (10 ng) showed that the LC–CL method was linear [r = 0.999996; intercept, 0.018 ± 0.018; slope, (0.75 ± 0.15) · 10^{-2} with t = 518 (P < 0.001)] over at least three decades. This means that the results of the experiments performed by LC–UV at the level of 2.5 μg of ibuprofen can be extrapolated to lower levels measured by LC–CL. The limit of quantification was 1.25 ng (6 pmol) of ibuprofen per 0.5 ml of saliva with an R.S.D. of 11.8% (n = 4), and the detection limit was 0.7 ng (3.3 pmol) of ibuprofen per 0.5 ml of saliva, which corresponds to 17.5 pg (85 fmol) injected (signal-to-noise ratio, 3:1).

Fig. 6 shows typical LC–CL chromatograms obtained from saliva 1 h after oral administration of 400 mg of ibuprofen, and of a spiked and a blank saliva sample. With saliva samples the mobile phase had to be changed to contain 25 instead of 26 vol.% of acetonitrile, because an unknown peak appeared between those of ibuprofen and flurbiprofen, its height being different for different samples. The concentration of this unknown compound probably depends on the food intake before saliva sampling.

CONCLUSION

This LC–CL method allows the sensitive and selective determination of drugs with a carboxylic acid function, such as ibuprofen. The derivatization with ABEI in the presence of the preactivator HOBT and the carbodiimide EDC in dry acetonitrile appears to be suitable for trace analysis after removal of excess ABEI by SPE.

Optimization of the derivatization reaction by factorial design analysis provides information on relationships between the reaction parameters, and can also be used to calculate the optima of the various parameters. The method is especially useful for systems with a relatively high number of parameters or relationships. The present LC–CL system is easy to operate because of the on-line post-column generation of the oxidative reagent instead of the addition of hydrogen peroxide by means of a separate pump. Current re-

search is investigating the development of a total on-line post-column system.

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