Journal of Chromatography A, 653 (1993) 253–259
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 483

Automated derivatization and high-performance liquid chromatographic analysis of ibuprofen enantiomers

D. Nicoll-Griffith*, M. Scartozzi and N. Chiem

Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe Claire-Dorval, Québec H9R 4P8 (Canada)

(First received February 5th, 1993; revised manuscript received July 22nd, 1993)

ABSTRACT

The automated pre-column derivatization and HPLC analysis of ibuprofen enantiomers was accomplished using a Gilson–Advanced Automated Sample Processor combination similar to the Bio-Fully Automated Sample Treatment system, and an (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine-derived Pirkle chiral column. In the derivatization reaction excess ethylchloroformate and anisidine were used to form the amide derivative of ibuprofen. After injection of the crude derivatized mixture through the AASP, strong cation-exchange cartridges efficiently trapped the excess anisidine and the enantiomeric ibuprofen amides passed directly into the analytical column where baseline separation was achieved. UV detection was accomplished at 254 nm. Linear standard curves were obtained for each enantiomer of ibuprofen in the range of 7.5–200 μg per derivatization with coefficients of correlation of 0.994 or greater. The quality control samples gave relative standard deviations of 17% for the low quality control (QC) samples (8.0 μg/enantiomer) and 7% for the middle and high QCs (60 μg and 160 μg/enantiomer respectively).

INTRODUCTION

Ibuprofen, [2-4-isobutylphenyl)propionic acid], is a non-steroidal anti-inflammatory drug which exists in two enantiomeric forms due to the presence of an asymmetric carbon atom α to the carbonyl function. Ibuprofen is frequently sold as a racemic mixture of (*R*)- and (*S*)-enantiomers, although it has been demonstrated that the anti-inflammatory activity resides predominantly in the (*S*)-enantiomer. Furthermore, it has been reported that this drug undergoes an *in vivo* uni-directional inversion from the inactive (*R*)-enantiomer to the active (*S*)-enantiomer [1–3]. This finding has sparked considerable research aiming to understand the mechanism of this inversion [4–7] and the organs responsible [8,9] for this conversion *in vivo*.

Three approaches have been used for the separation and quantification of ibuprofen enantiomers. The simplest method was direct resolution on a chiral column such as the α_1-glycoprotein column [10]. With this method it was necessary to develop a delicate compromise giving sufficient resolution, and short retention times using conditions which guaranteed an acceptable column lifetime [10]. A second approach was the derivatization of ibuprofen with a chiral reagent, resulting in two diastereomers, which were subsequently separated on a non-chiral column by gas chromatography [3,4] or HPLC [11–14]. The possible disadvantages of this approach were: (i) the rates of reaction for the formation of the two diastereomers could be different, resulting in an incorrect ratio of the two diastereomers, and (ii) impurity of the chiral reagent could result in four diasteromers instead of only two. A third approach was to prepare a non-chiral derivative of ibuprofen and separate the resulting enantiomers on a chiral column [15]. While analytical methods utilizing a derivatization are frequently quite efficient and

* Corresponding author.

0021-9673/93/$06.00

sensitive, the derivatization adds a time consuming step. The objective of this report is to describe the development of an approach for automating pre-column derivatizations using the analysis of ibuprofen enantiomers as a model system.

In a previous study ibuprofen was manually derivatized using ethyl chloroformate and *p*-anisidine to yield enantiomeric amides which were then resolved on a (3,5-dinitrobenzoyl)-phenylglycine (DNBPG) chiral column [15]. It was critical to do a liquid–liquid extraction with aqueous acid in order to remove excess anisidine prior to chromatography since it co-eluted with the ibuprofen amides. The liquid–liquid extraction of anisidine was very effective, however, this step was very time consuming and severely limited the number of samples which could be processed each day. An automated method involving on-line extraction of the anisidine would be more appropriate for the derivatization and analysis of large numbers of samples.

This paper describes the successful automation of this method using an autosampler to mix the reagents, on-line solid phase extraction to trap excess anisidine and the DNBPG column to separate the enantiomers. The instrumentation used is an adaption of the Bio-Fully Automated Sample Treatment (Bio-FAST) system [16,17]. The Bio-FAST is a sophisticated column switching system in which a fresh pre-column is used for each sample. In this application, the pre-columns were used to retain the excess anisidine from each derivatization while the ibuprofen amides passed directly onto the analytical column.

EXPERIMENTAL

Apparatus

A diagram of the Bio-FAST type HPLC system used is given in Fig. 1. The system is comprised of a Vista 5500 LC (Pump A) (Varian, Walnut Creek, CA, USA), a Gilson 323-401 sample processor and injector with a 25-μl loop (Gilson Medical Electronics, Middleton, WI, USA), an Advanced Automated Sample Processor (AASP) (Varian) and an AASP injector valve (Valve 2 in Fig. 1) (Varian), a Model 510

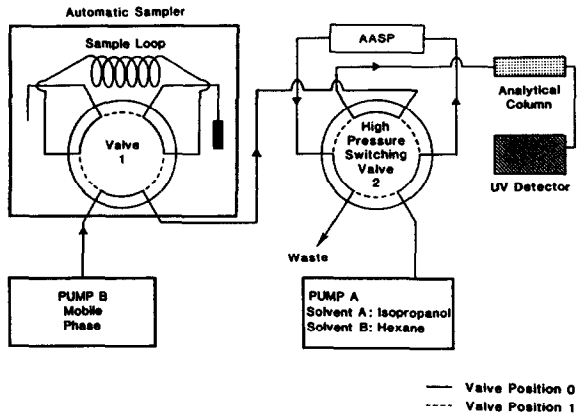

Fig. 1. Bio-FAST type HPLC system flow diagram.

pump (Pump B) (Waters Assoc., Milford, MA, USA), a UV 200 detector (Varian) and an HP 3396 Series II Integrator (Hewlett-Packard, Palo Alto, CA, USA). For manual injections, a Valco 6CW Valve (Valco, Houston, TX, USA), fitted with a 25-μl sample loop, was installed between the AASP injector valve and the HPLC column.

Reagents

Racemic ibuprofen, 2-phenylpropionic acid (internal standard), ethylchloroformate and *p*-anisidine were all purchased from Aldrich (St. Louis, MO, USA). The ibuprofen was recrystallized from heptane [18] and the *p*-anisidine was recrystallized from water–ethanol before use. Heptane, hexane, 2-propanol, methylene chloride and triethylamine (stored over KOH) were purchased from BDH (Montreal, Canada). The amide standard of racemic ibuprofen was prepared as previously described [19].

Derivatization procedures

The derivatization procedures were based on the method previously described [15], with minor modifications. All the reagents were prepared as solutions in methylene chloride.

For the manual derivatizations each sample consisted of a mixture of 100 μl of ibuprofen stock solution and 100 μl of internal standard stock solution (500 μg/ml of 2-phenylpropionic acid). After the addition of 200 μl of 100 m*M*

triethylamine and 100 μl of 60 m*M* ethylchloroformate the sample was vortexed for 15 s. After a 15-min interval (T1), 100 μl of 0.5 *M* *p*-anisidine were added to the sample mixture, which was then vortexed for 15 s. After a 5-min interval (T2) the sample was diluted with 600 μl of isopropanol–hexane (10:90, v/v) to give a total volume of 1200 μl. The sample was vortexed for 15 s, and a 25-μl aliquot was injected.

The automated derivatization was performed by the Gilson sample processor and injector. The procedure was identical to the manual procedure described above, except for sample mixing. After the addition of each reagent, half the sample was aspirated and dispensed back into the vial three times.

Bio-FAST HPLC procedure

The time programming of valves and pumps is shown in Table I. AASP cartridges were purchased from Analytichem (Harbor City, CA, USA) and the AASP washing and conditioning solvents were 2-propanol and hexane. The analytical column was a Rexchrom Regis Pirkle D-phenylglycine column (25 cm × 4.6 mm I.D., 5 μm particle size) (Regis, Morton Grove, IL, USA). The mobile phase consisted of 2-propanol–hexane (10:90, v/v) at a flow-rate of 2.0

TABLE I

SEQUENCE OF VALVE SWITCHING AND PUMP OPERATION

Time (min)	Operation	Effect
0.0	Valve 1: position 0 Valve 2: position 0	Load fresh AASP cartridge
0.05	Gilson automatic sample processor and injector is started	Derivatization procedure is started
15.05	Pump A: isopropanol (4.0 ml/min)	Isopropanol wash of AASP cartridges
16.56	Pump A: isopropanol–hexane (10:90) (4.0 ml/min)	Conditioning of AASP cartridges with mobile phase
18.56	Pump A is stopped	AASP cartridges are prepared
25.0	Valve 2: position 1	Mobile phase goes through AASP to effect final conditioning of cartridges
25.45	Sample is injected into loop filler port	Sample is loaded onto sample loop
25.57	Valve 1: position 1	Integrator is started and sample is carried through the AASP cartridges where excess anisidine is trapped and mobile phase elutes the derivatized ibuprofen on to column
25.60–29.30		Rinsing of injection needle and loop filler port
28.0	Valve 2: position 0	End of on line time for AASP cartridge with column
29.30	Valve 1: position 0	Sample loop ready for next sample
29.9–30.0	Automated advance to next AASP cartridge	Re-initiate cycle

ml/min and was pumped continuously by Pump B. UV detection was at 254 nm.

Anisidine trapping by AASP cartridges

Solutions of ibuprofen amide standard and anisidine were prepared in 2-propanol–hexane (25:75, v/v). These were injected using the Gilson autosampler through the AASP when it was connected directly on-line with the HPLC column. Silica (SI), phenylboronic acid (PBA), carboxylic acid (CBA), propylbenzenesulfonic acid (SCX) and propylsulfonic acid (PRS) cartridges were evaluated for their efficiency in trapping the anisidine.

Dilution of derivatization mixture with mobile phase

Solutions of ibuprofen amide standards were prepared with varying ratios of methylene chloride and mobile phase. These were injected directly onto the analytical column and the resolution determined according to the formula $R_s = 1.18\Delta t_R/(w_1 + w_2)$ where t_R indicates retention time and w_1 and w_2 are the peak widths at half height.

Derivatization time study

To optimize the derivatization times, T1 and T2 (see *Derivatization Procedures*) were evaluated. With T2 kept constant at 8 min, T1 was varied from 0.5 to 48 min. With T1 kept at 15 min, T2 was varied from 0.5 to 100 min. The total peak areas of the ibuprofen amides in triplicate analyses were used to evaluate the derivatization as a function of time.

Determination of percent derivatization

A standard curve from 40 to 170 μg/ml was prepared with synthetic ibuprofen amide standards. A quantity of ibuprofen was derivatized so that a 100% theoretical yield would have given a final derivative concentration of 100 μg/ml. These experiments were conducted using both the manual and automated procedures. From the calibration curve the true concentrations of derivatized product were determined. The percent yield was calculated as the ratio of experimental to theoretical concentrations of the amide derivatives.

Validation study; standard curves

Stock solutions of ibuprofen were prepared in methylene chloride at concentrations of 150, 300, 500, 1000, 2000 and 4000 μg/ml. Quality control (QC) solutions were prepared at concentrations of 160, 1200 and 3200 μg/ml. A 100-μl aliquot of each stock solution (see *Derivatization Procedures*) was processed through the fully automated procedure.

Standard curves were plotted using peak area ratios (each ibuprofen peak over the first internal standard peak) as a function of the amount of ibuprofen derivatized. Standard curves were generated on three different days, as well as on the same day, so as to give an indication of inter-day and intra-day reproducibility. Duplicate samples of three QC samples were run with each standard curve and were then back calculated using the best-fit linear regression of the standard curve for further statistical analysis.

RESULTS AND DISCUSSION

The research was conducted to determine whether an HPLC system such as the Bio-FAST [16] could be used to effect a fully automated pre-column derivatization requiring solid phase work-up prior to analysis. The analysis of ibuprofen amides on the DNBPG column was chosen as a model system to develop this approach. The previously described manual derivatization procedure required a liquid–liquid extraction of excess anisidine since this reagent masked the ibuprofen peaks during chromatography [15]. Two approaches for the Bio-FAST method were envisaged. The first was to operate the column-switching so that the ibuprofen amides were retained on the pre-column and the anisidine transferred to waste. Unfortunately, none of the AASP cartridges selectively retained the ibuprofen amides. The second approach was to trap the excess anisidine on the cartridge while the ibuprofen amides passed directly onto the analytical column. Solutions of the ibuprofen amide standards injected directly onto the column compared with injections which passed through the AASP first, demonstrated that the retention times were retarded by about 0.5 min but the resolution did not deteriorate (data not

shown). This suggested that this approach would be viable, providing an appropriate cartridge could be found to retain the anisidine.

It was anticipated that anisidine could be trapped by an acidic solid phase. This was tested by injecting a mixture of the ibuprofen amide standards and anisidine through the AASP prior to chromatography. SI and PBA cartridges caused increased retention time of the anisidine but did not trap it. The addition of acetic acid to the injection solution did not increase the retention of anisidine. CBA cartridges had no apparent effect on the anisidine. SCX and PRS cartridges both effectively retained the anisidine. The extraction mechanisms for the SCX and PRS cartridges, proposed in Fig. 2, suggest that the interaction is ionic. The effect of a $\pi-\pi$ interaction between the aromatic ring of the anisidine and the sorbent group of the SCX cartridge is probably minimal since the PRS and SCX cartridges were equally efficient. Since the Bio-FAST HPLC uses a new cartridge for each sample the extraction efficiency for anisidine will be constant for a batch of samples. Fig. 3A and B compare a blank manual derivatization injected directly onto the analytical column with an automated blank derivatization processed through the PRS AASP cartridge. This demonstrates the effectiveness of the extraction.

Methylene chloride is used as the solvent for derivatization. A standard solution of ibuprofen amides in methylene chloride injected directly onto the analytical column gave broad peaks. The addition of mobile phase to the injection solution improved the peak shape and resolution (Fig. 4). Although the graph suggests that even 10% added mobile phase gave adequate resolution, fronting was observed on the peaks and baseline separation of the enantiomers was not achieved. It was concluded that a 1:1 ratio of methylene chloride and mobile phase gave good

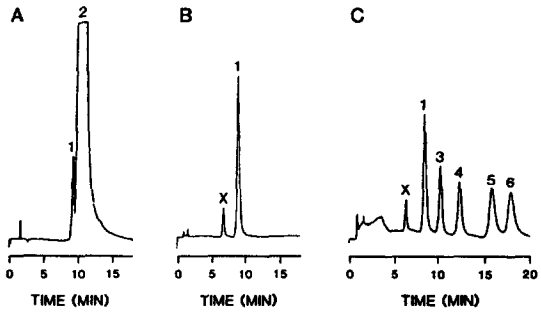

Fig. 3. Typical chromatograms. (A) Manual blank derivatization mixture (diluted 1:1 with mobile phase) injected directly onto the analytical column. (B) Automated blank derivatization (diluted as above) processed through PRS AASP cartridge. (C) Automated derivatization, dilution, extraction and analysis of racemic ibuprofen and internal standard (50 μg of each). Peaks: X = unknown impurity seen in automated derivatization procedure; 1 = urethane by-product [19]; 2 = anisidine; 3 = (*S*)-ibuprofen amide derivative; 4 = (*R*)-ibuprofen amide derivative; 5 = (*S*)-internal standard amide derivative; 6 = (*R*)-internal standard amide derivative. See Experimental section for HPLC conditions.

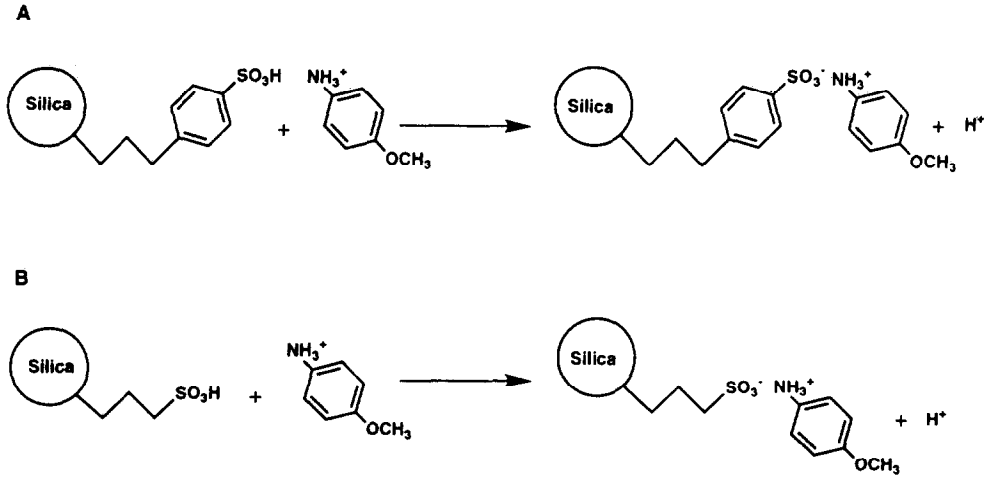

Fig. 2. Proposed extraction mechanisms for (A) SCX and (B) PRS cartridges.

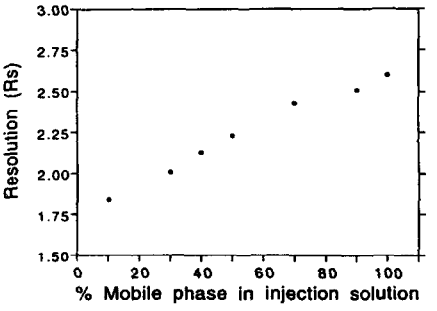

Fig. 4. Graph showing determination of optimal dilution of the methylene chloride solution with mobile phase. Chromatographic resolution of the racemic ibuprofen amide standard peaks is plotted against the percent mobile phase present in the injection solution.

compromise between maximum peak area and adequate peak shape. Therefore, before injection of the automated derivatizations, each crude mixture was diluted with an equal volume of mobile phase.

The Gilson sample processor and injector has been used in combination with the AASP for several bioanalytical applications [20,21]. In this application for ibuprofen derivatization the Gilson injector was preferred since flexible programming allowed for the optimization of some important parameters, including: (i) the rate of aspirating and dispensing the reagents, (ii) proper mixing of the samples and (iii) rinsing of the needle and injection port to avoid cross contamination. A study of the timing for T1 and T2 (see methods) indicated that each step of the derivatization could be as short as 5 min and longer periods of T1 or T2 did not increase the amount of derivatized product (data not shown). The percentage yields for the derivatization were $61.4 \pm 0.7\%$, $n = 2$ and 61.2 ± 3.4, $n = 3$ for the manual and automated derivatizations, respectively. Fig. 3C shows a typical chromatogram of ibuprofen and the internal standard processed through the entire automated derivatization, dilution, extraction and analysis procedure.

A validation study of the automated method was done in order to evaluate the accuracy and reproducibility of the assay. Intra-day reproducibility was assessed by using back-calculated QC samples as seen in Table II. These results indicate acceptable reproducibility and accuracy.

TABLE II

INTRA-DAY PRECISION (USING BACK-CALCULATED QC SAMPLES)

	Nominal amount (μg/derivatization)		
	8.0	60.0	160.0
(S)-Ibuprofen			
Mean observed	7.8	55.9	168.3
±S.D.	1.32	3.89	11.15
R.S.D. (%)	16.9	6.96	6.62
% Nominal amount	97.5	93.2	105.2
n	6	6	6
(R)-Ibuprofen			
Mean observed	7.2	54.4	167.8
±S.D.	1.22	3.55	11.40
R.S.D. (%)	17.1	6.53	6.79
% Nominal amount	90.0	90.7	104.9
n	6	6	6

Linearity and inter-day reproducibility were assessed by comparing the six point standard curves obtained using racemic ibuprofen (15 μg–400 μg/derivatization) on three different days. The coefficients of correlation exceeded 0.994 for each enantiomer (*i.e.* 7.5 μg–200 μg range for each enantiomer). Average equations of the lines were: (*S*)-enantiomer $y = 0.029763x - 0.043725$ (slope relative standard deviation, R.S.D. = 4.72%, $n = 3$). (*R*)-Enantiomer $y = 0.031372x - 0.057435$ (slope R.S.D. = 6.54%, $n = 3$). The linearity range, reproducibility and accuracy were comparable to results obtained with the manual derivatization [15]. The limit of detection was 0.5 μg/ml (S/N ratio = 2) for a racemic ibuprofen amide derivative standard solution injected directly onto the analytical column. For biological applications, a sample would need to be extracted and dried down before derivatization [15]. The limit of an assay would be governed by the volume of the biological fluid extracted and the presence of interfering peaks at the retention times of interest.

As seen in Table I, the derivatization process, the washing and conditioning of the AASP cartridges, and the HPLC analysis were all synchronized. Thus, as one sample was being

chromatographed, the next was undergoing the derivatization. During a 24-h run, up to 48 samples could be processed completely unattended. It would be difficult to do this many samples per day by the original manual method [15].

CONCLUSIONS

The automated derivatization and HPLC analysis of ibuprofen enantiomers was achieved on a HPLC system similar to the Bio-FAST. The process was optimized by trapping the excess anisidine using either SCX or PRS solid-phase extraction cartridges prior to chromatography. Linear standard curves were obtained, with a detection range of 7.5–200 μg per derivatization for each enantiomer. Statistical analysis proved that the accuracy, intra-day and inter-day reproducibility of the assay were within acceptable limits.

This method demonstrates an application of a Gilson-AASP combination similar to the Bio-FAST wherein AASP cartridges are used for the efficient trapping of excess derivatizing reagent. It could be applied to the analysis of ibuprofen enantiomers in drug samples or in biological samples such as urine [15]. It could also be adapted for the normal-phase analysis of other drugs requiring pre-column derivatization and extraction of excess basic reagents, such as amines, prior to chromatography.

REFERENCES

1 W.J. Wechter, D.G. Loughhead, R.J. Reischer, G.J. VanGiessen and D.G. Kaiser, *Biochem. Biophys. Res. Commun.*, 61 (1974) 833.
2 S.S. Adams, P. Bresloff and C.G. Mason, *J. Pharm. Pharmacol.*, 28 (1976) 256.
3 D.G. Kaiser, G.J. VanGiessen, R.J. Reischer and W.J. Wechter, *J. Pharm. Sci.*, 65 (1976) 269.
4 S.M. Sanins, W.J. Adams, D.G. Kaiser, G.W. Haltead, J. Hosley, H. Barnes and T.A. Baillie, *Drug Met. Dispos.*, 19 (1991) 405.
5 M.P. Knadler and S.D. Hall, *Chirality*, 2 (1990) 67.
6 R.D. Knihinicki, K.M. Williams and R.O. Day, *Biochem. Pharmacol.*, 38 (1989) 4389–4395.
7 Y. Nakamura, T. Yamaguchi, S. Takahashi, S. Hashimoto, K. Iwatari and Y. Nakagawa, *J. Pharm. Dyn.*, 4 (1981) S-1.
8 T. Yamaguchi and Y. Nakamura, *Drug Met. Dispos.*, 15 (1987) 535.
9 F. Jamali, N.N. Singh, F.M. Pasutto, A.S. Russell and R.T. Coutts, *Pharmaceutical Res.*, 5 (1988) 40.
10 S. Menzel-Soglowek, G. Gleisslinger and K. Brune, *J. Chromatogr.*, 532 (1990) 295.
11 E.J.D. Lee, K.M. Williams, G.G. Graham, R.O. Day and G.D. Champion, *J. Pharm. Sci.*, 73 (1984) 1984.
12 S. Fournel and J. Caldwell, *Biochem. Pharm.*, 35 (1986) 4153.
13 J.M. Maître, G. Boss and B. Testa, *J. Chromatogr.*, 299 (1984) 397.
14 A.C. Rudy, K.S. Anliker and S.D. Hall, *J. Chromatogr.*, 528 (1990) 395.
15 D.A. Nicoll-Griffith, T. Inaba, B.K. Tang and W. Kalow, *J. Chromatogr.*, 428 (1988) 103–112.
16 D. Lessard, D. Nicoll-Griffith and H.M. Hill, *Am Lab.*, October (1990) 42.
17 D. Nicoll-Griffith, R. Zamboni, J.B. Rasmussen, D. Ethier, S. Charleson and P. Tagari, *J. Chromatogr.*, 526 (1990) 341.
18 V.J. Capponi, G.W. Halstead and D.L. Theis, *J. Labelled Compd. Radiopharm.*, 23 (1986) 192–193.
19 D. Nicoll-Griffith, *J. Chromatogr.*, 402 (1987) 179.
20 E. Doyle, R.D. McDowall, G.S. Murkitt, V.S. Picot and S.J. Rogers, *J. Chromatogr.*, 527 (1990) 67–77.
21 D. Nicoll-Griffith and R. Zamboni, *Prostaglandins*, 43 (1992) 523–532.