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Liquid chromatographic chiral separations of the N-6-(*endo*-2-norbornyl)-9-methyladenine enantiomers

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ABSTRACT

Attempts to separate the enantiomers of the novel adenosine antagonist N-0861, the racemic mixture of N-6-(*endo*-2-norbornyl)-9-methyladenine, by high-performance liquid chromatography are described. Owing to the very low efficiency and the lack of selectivity of the α-AGP column, the direct separation method did not give satisfactory results. The indirect separation method involved derivatization of N-0861 with (+)-1-(9-fluorenyl)ethylchloroformate. The method is easy to perform and aqueous solutions can be used. The calibration graphs showed that the reaction is linear. A resolution between the formed diastereoisomers of 1.13 within 30 min was obtained on a C_8 column with an acetonitrile–water eluent. The identities of the reaction products were checked by LC–MS.

INTRODUCTION

The racemic mixture of N-6-(*endo*-2-norbornyl)-9-methyladenine (N-0861), (Whitby Research, Richmond, VA, USA), the structure of which is depicted in Fig. 1, is under study as a novel adenosine antagonist. The adenosine receptor can be divided in to several subtypes [1]. Although it is known that biologically active molecules, such as receptors and enzymes, tend to be stereoselective in their binding to, and activation by, *e.g.* drugs [2], N-0861 is under study as a racemate.

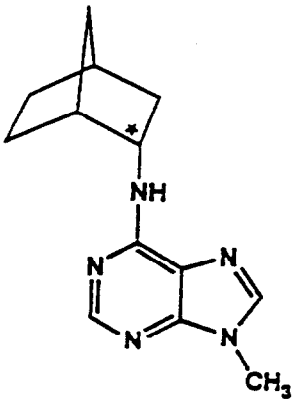

Fig. 1. Structure of N-0861. The asterisk denotes the chiral centre.

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This paper describes a direct and an indirect high-performance liquid chromatographic (HPLC) method for the separation of the enantiomers of N-0861. As N-0861 seemed to have no suitable functional groups close to the chiral centre that may facilitate an interaction with a particular chiral stationary phase, the choice of a suitable stationary phase was difficult. For protein-based chiral columns the enantioselective properties are based on principles of bioaffinity towards the protein [3]. A protein-based column was chosen because this bioaffinity involves a combination of hydrogen bonding, electrostatic interaction and hydrophobic interaction [4].

The indirect separation method [5] involved a derivatization reaction with (+)-1-(9-fluorenyl)ethylchloroformate [(+)-Flec]. Chloroformates are known for their reactivity towards amino acids [6], primary and secondary amines [6–8] and tertiary amines [9,10]. The supposed reaction scheme is depicted in Fig. 2 as reaction A.

The influences of pH and buffer on the derivatization reaction were studied together with the reaction time. The formation of the hydroxy product of (+)-Flec, which is shown as reaction B in Fig. 2, needs to be controlled by appropriate pH adjustment of the reaction mixture.

To check the linearity of the derivatization reaction, calibration graphs were constructed for both diastereoisomers in the range 80–0.625 μg/ml of racemic N-0861. The influence of concentration on the resolution of the diastereoisomers was studied using the same calibration graphs.

EXPERIMENTAL

HPLC equipment

Unless indicated otherwise, the solvent-delivery system was a Model 9010 HPLC pump and 20-μl injections were made with a Model 9095 autosampler, both purchased from Varian (Walnut Creek, CA, USA). Detection at 270 nm was carried out with a Model 1000 S diode-array detector from Applied Biosystems (Foster City, CA, USA). Integration of the chromatograms was effected with an SP-4270 integrator (Spectra-Physics, San Jose, CA, USA). The experiments to improve the separation and to prepare the calibration graphs were carried out with a Model 2150 LKB HPLC solvent-delivery system (Pharmacia–LKB Biotechnology, Uppsala, Sweden).

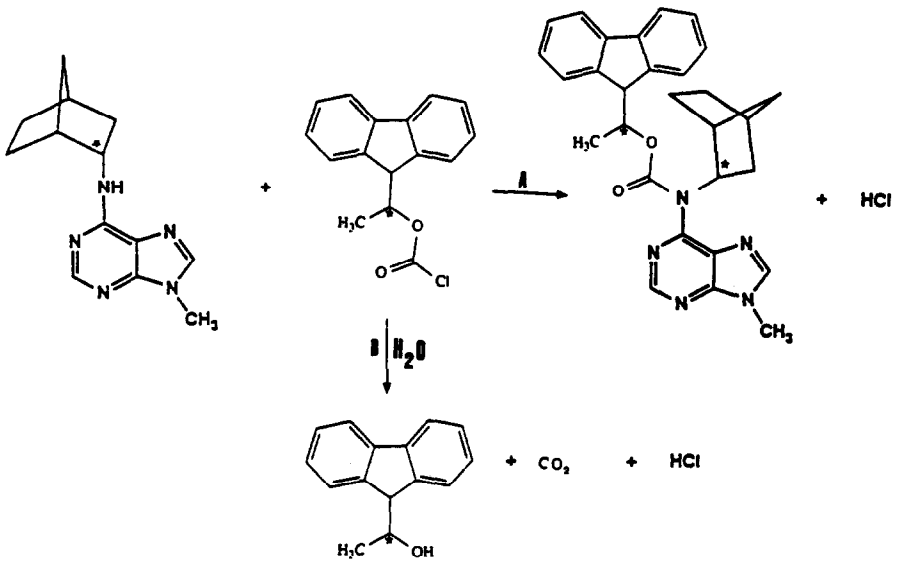

Fig. 2. (A) The expected reaction between N-0861 and (+)-Flec; (B) the hydrolysis of (+)-Flec resulting in (+)-Flec-OH. The asterisks denote the chiral centres.

This pump was used in combination with a Model 710 A WISP (Waters, Milford, MA, USA) and detection was carried out with a Model 770 spectrophotometric detector at 270 nm (Spectra-Physics, Santa Clara, CA, USA). For integrating the chromatograms a C-R3A Chromatopac (Shimadzu, Kyoto, Japan) and for recording a BD 40 recorder (Kipp, Delft, Netherlands) was used. If the column temperature was controlled the column was immersed in a water-bath. A combination of a Thermomix 1442 D thermostat (Braun, Rijswijk, Netherlands) and a cooling device (HETO, Birkerod, Denmark) was used to regulate the temperature the bath.

Mass spectrometers

Two types of liquid chromatographic–mass spectrometric (LC–MS) apparatus were used. In the following they will be referred to as LC–MS-I and LC–MS-II, respectively.

LC–MS-I. An API III triple quadrupole from Perkin-Elmer Sciex (Thornhill, Ontario, Canada) with an ion-spray interface was used. The repeller potential was +50 V for positive ions. The liquid chromatographic system used in combination with the Perkin-Elmer Sciex spectrometer consisted of a Hewlett-Packard (Palo Alto, CA, USA) Series 1050 pump and an autosampler, in combination with a Hewlett-Packard Series 1050 variable-wavelength UV detector. A 33 × 4 mm I.D. C_{18} column with 5-μm particles (Supelco, Bellefonte, PA, USA) was used for LC–MS-I.

LC–MS-II. A Finnigan MAT (San Jose, CA, USA) TSQ-700 mass spectrometer with an electrospray LC–MS interface was used. The mass detector was in the positive-ion mode. The eluent-delivery system was a Phoenix from Carlo Erba (Milan, Italy). A 900 × 0.25 mm column filled with 5-μm particles of Kromasil C_8 (Eka Nobel, Bohus, Sweden) was used for LC–MS-II.

Chemicals

Racemic N-6-(*endo*-2-norbornyl)-9-methyladenine was a gift from Whitby Research, according to whom the drug was an optically neutral mixture of the (+)- and (−)-enantiomers. (+)-1-(9-Fluorenyl)ethyl chloroformate, which was a 18 m*M* solution in acetone, was obtained from Aldrich Chemie (Steinheim, Germany). Deuterium oxide was purchased from Dr. Glaser (Basle, Switzerland), acetonitrile of HPLC grade from Rathburn (Walkerburn, UK) and acetic acid, sodium acetate, sodium borate and sodium phosphate, all of analytical-reagent grade, from Merck (Darmstadt, Germany). Four different buffer solutions were prepared in distilled water, namely 0.25 *M* borate (pH 8.1), 0.2 *M* phosphate (pH 7.0 and 6.3) and 0.2 *M* acetate (pH 5.2). These four buffers will be referred to as buffers a, b, c and d, respectively.

Direct separation method

For the direct separation a Chiral-AGP (α-AGP) column (100 × 4 mm I.D.) (ChromTech, Stockholm, Sweden) with 5-μm particles was used. The eluents studied consisted of different ratios of an organic modifier with 0.01 *M* phosphate buffer. Two phosphate buffers with corresponding pH values of 6.95 and 5.30 were used. The organic modifiers tested were methanol, ethanol, acetonitrile and 2-propanol. Both phosphate buffers were also tested without the addition of an organic modifier. Chromatographic runs were carried out with all eluent combinations at various temperatures in the range 0–50°C. The eluent flow-rate for all experiments was 0.5 ml/min. Injections of 20 μl from a solution of 5 μg/ml of N-0861 in the corresponding eluent were made.

Indirect separation method

Indirect separations, unless indicated otherwise, were carried out with a 150 × 4 mm I.D. Nucleosil C_{18} column with 5-μm particles (Macherey–Nagel, Düren, Germany) in combination with a gradient. The gradient started at 50% acetonitrile–50% 0.1 *M* acetic acid (pH 2.9) for the first 22 min, and from 22 to 40 min it was increased linearly to 100% acetonitrile. After each run the starting conditions were equilibrated for at least 15 min.

General derivatization procedure

The derivatization reactions were performed in 250-μl autosampler microvial inserts. These inserts were placed in larger vials, which were

then sealed with Teflon seals. To 80 μl of a solution of 200 μg/ml of N-0861 in acetonitrile were added 80 μl of one of the four buffers and 40 μl of an 18 m*M* (+)-Flec solution in acetone. The vial was closed and the reaction was allowed to take place at room temperature for different time periods. The percentage of N-0861 still present in the sample was related to a reference solution of N-0861. This reference solution consisted of 80 μl of 200 μg/ml of N-0861 in acetonitrile, 80 μl of the corresponding buffer and 40 μl acetone. Because of the absence of (+)-Flec, this sample was considered to contain 100% N-0861. The mean value of the chromatographic peak area from these reference samples was used for further calculations.

Procedure for studying the influence of type of buffer and pH on the derivatization reaction

To study the influence of the type of buffer and the pH on the reaction rate, four different buffers (a, b, c and d) were studied. Injections were made 2 h after the reaction mixture was prepared. These experiments were carried out in duplicate on different days. The HPLC conditions are given under *Indirect separation method* and the detailed procedure for the derivatization reaction is outlined under *General derivatization procedure*.

Procedure for studying the derivatization reaction

To study the reaction time, samples were prepared as described under General derivatization procedure. The 0.2 *M* phosphate buffer of pH 7.0 was used. Injections were made at different times after the reaction had started. The corresponding derivatization times were in the range 1–1440 min. The HPLC conditions and the column were the same as those given under Indirect separation method, except that the acetic acid was omitted from the eluent, resulting in an eluent containing acetonitrile and water.

Procedure for preparing the calibration graphs and studying the influence of different concentrations of N-0861 on the resolution between the diastereoisomers

The solutions used to prepare the calibration graphs were prepared by adding 40 μl of 18 m*M* (+)-Flec in acetone to 80 μl of phosphate buffer b and to 80 μl of an N-0861 solution in acetonitrile. The concentration of racemic N-0861 in acetonitrile varied from 200 to 1.56 μg/ml, which corresponds to a concentration range of racemic N-0861 in the reaction vial of 80–0.625 μg/ml. The concentration range for each enantiomer therefore ranged from 40 to 0.313 μg/ml. The HPLC system used is described under *Improvement of the separation*. Injections of 20 μl were made more than 2 h after the preparation of the derivatization mixture. Calibrations graphs were constructed twice on different days. The mean peak area on the chromatogram of each diastereoisomer was used to prepare the calibration graph. The influence of different concentrations N-0861 on the resolution between the diastereoisomers was also studied.

Liquid chromatographic–mass spectrometric procedure

For the LC–MS experiments, derivatization samples were prepared using 0.2 *M* phosphate buffer (pH 7.0). Injections were made more than 2 h after the start of the derivatization reaction.

LC–MS-I. The eluent used was a gradient of a mixture of A (50% acetonitrile–50% 2 m*M* ammonium acetate–0.1% formic acid) and B (100% acetonitrile–0.1% formic acid). The gradient started at 100% A for 5 min, from 5 to 10 min it was changed to 10% A–90% B, this ratio was kept constant from 10 to 15 min, then from 15 to 20 min the gradient was returned to its starting point. The eluent flow-rate was 1.0 ml/min and 20-μl injections from the derivatization mixture were made. The above conditions are further referred to as LC–MS-I.

LC–MS-II. The eluent used for these experiments was 70% acetonitrile–30% 5 m*M* ammonium acetate in D_2O. The flow-rate with this narrow-bore column was *ca.* 1 μl/min. The amount injected was 100 nl.

RESULTS AND DISCUSSION

Direct separation

The direct method using the α-AGP column for the separation of the N-0861 enantiomers was not satisfactory. The best results obtained with the tested eluents and temperatures are depicted

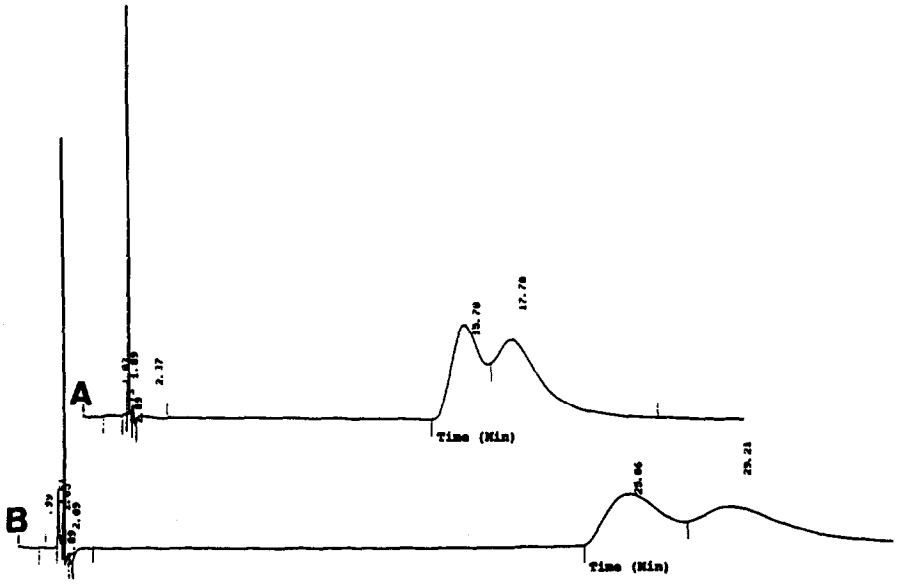

Fig. 3. Separation of the N-0861 enantiomers on an α-AGP column. (A) Eluent acetonitrile–0.01 *M* phosphate buffer (pH 6.95) (3:97). Temperature, 20°C. (B) Eluent acetonitrile–0.01 *M* phosphate buffer (pH 6.95) (1:99). Temperature, 20°C.

in Fig. 3. With an eluent of 1% acetonitrile–99% 0.01 *M* phosphate buffer (pH 6.95) and a column temperature of 20°C, a resolution of 0.78 could be obtained.

Indirect separation

Fig. 4 shows a chromatogram obtained from a derivatized sample during the initial phase of the studies. Peak 1 is the N-0861 still present in the sample, peak 2 the hydrolysis product of (+)-Flec, denoted as Flec-OH, peaks 3 the diastereoisomeric derivatization products and peak 4 the remaining (+)-Flec. These peaks were tentatively identified by comparing this chromatogram with reference chromatograms of the different solutions used for the derivatization. It should be

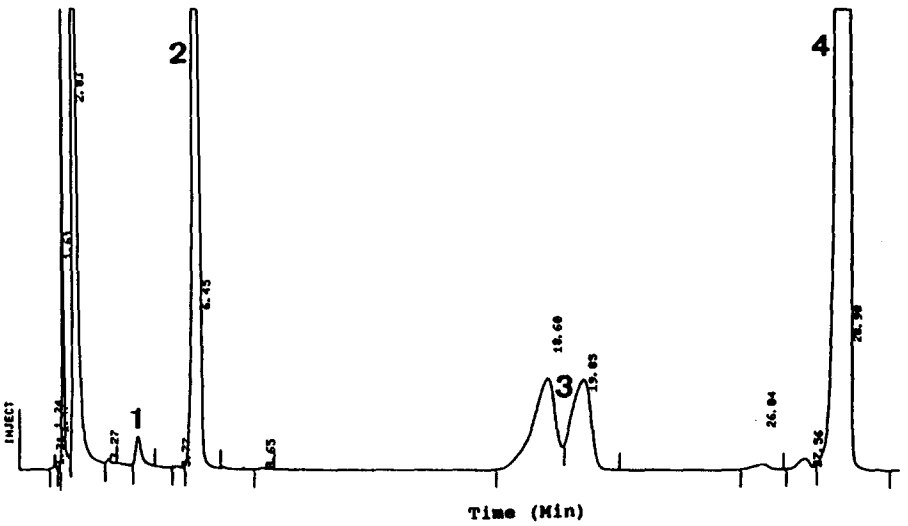

Fig. 4. Chromatogram obtained on a C_{18} column after derivatization of N-0861 with (+)-Flec. Conditions as given under Indirect separations. 1 = N-0861; 2 = (+)-Flec-OH; 3 = the two diastereoisomers resulting from the derivatization reaction; 4 = (+)-Flec.

noted that peaks number 3 and 4 exhibit pronounced fronting which does not occur in the other peaks; we have no explanation for this phenomenon. To be certain about the structure of the eluted compounds, LC–MS experiments were performed. The improvement of the initial separation is described later.

Liquid chromatography–mass spectrometry

The structures of three of the main peaks in the chromatogram in Fig. 4 could be elucidated with LC–MS-I. The resulting mass/charge (m/z) ratios from LC–MS-I together with the molecular masses (M_r) of the expected products are given in Table I.

Peak 1, which is underivatized N-0861, could not be detected with LC–MS-I because the derivatization reaction was almost complete. The m/z ratios of the ions of peaks 2 and 4 correspond to the ammonium adducts of the hydrolysis product of (+)-Flec and unchanged (+)-Flec, respectively. The ions found at peak 3 were different from the $M_r + 1$ of the expected products, and they did not correspond to the ammonium adducts of the expected products. The ion of m/z 498 suggest the addition of H_2O during the experiment. Under the influence of the chloroformate, the methyladenine ring structure present in N-0861 may have opened on addition of H_2O. The addition of H_2O will increase the M_r by 18. If the resulting molecule is protonated, the m/z will be 498. This supposed mechanism results in two possible products, as can be seen in Fig. 5, routes A and B. To verify this mechanism and to be able to distinguish between the products of routes A and B, LC–MS-II studies with an eluent containing ^2H_2O instead of H_2O were performed [11]. With this eluent the number of protons linked to heteroatoms in the molecule can be determined. These protons will be exchanged with the

TABLE I

MOLECULAR MASSES (M_r) OF THE EXPECTED PRODUCTS FROM THE DERIVATIZATION REACTION AND THE ACTUALLY FOUND m/z RATIOS OF THE IONS DETECTED BY THE MASS SPECTROMETER CORRESPONDING TO THREE OF THE FOUR PEAKS IN FIG. 4

Parameter	Peak 1	Peak 2	Peak 3	Peak 4
M_r	243	210	479	272
m/z	n.d.[a]	228	498	290

[a] Not detected.

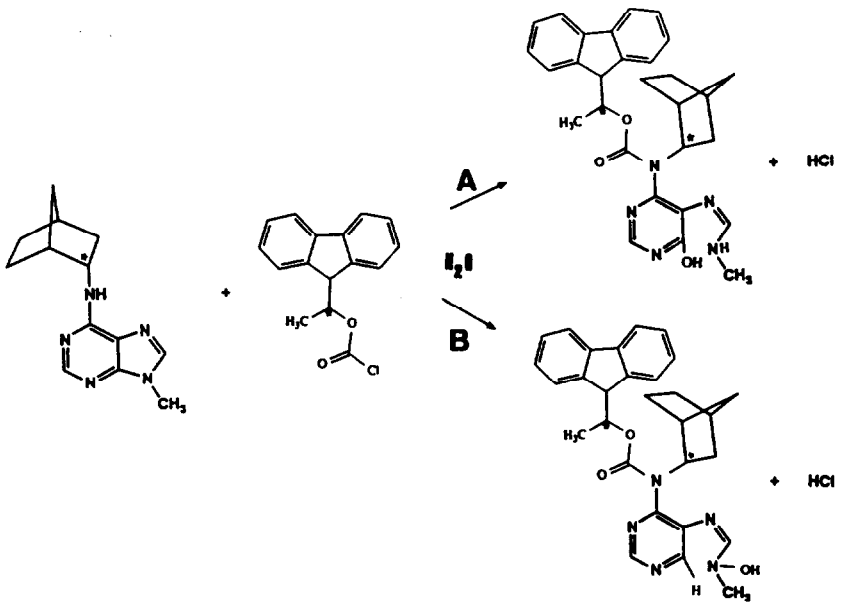

Fig. 5. The two possible derivatization reactions resulting in products that will yield m/z 501 (route A) and 500 (route B) in LC–MS-II. The asterisks denote the chiral centres.

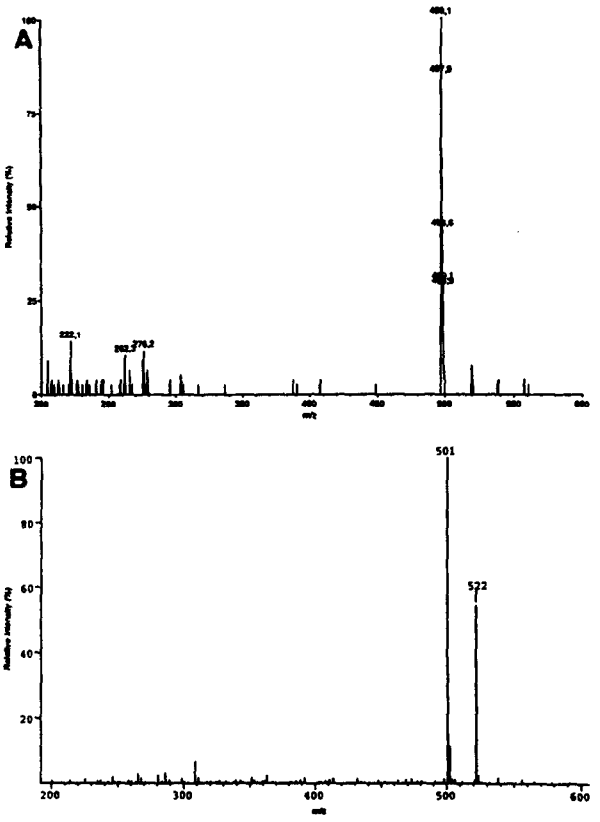

Fig. 6. Mass spectra of peak 3 in Fig. 4. (A) LC–MS-I with H_2O in the eluent; (B) LC–MS-II with ^2H_2O in the eluent instead of H_2O.

deuterium ions present in the eluent, resulting in an increase in the m/z of 1 for each exchangeable proton present in the molecule. The proton which gives the molecule its charge will also be exchanged by a deuterium ion. The LC–MS-I results showed an m/z of 498 for peak 3, as can be seen in Table I and Fig. 6A. Deuterium for the charge instead of a proton gives m/z 499. If there is one exchangeable proton in the molecule as in the product of route B in Fig. 5, the m/z will be 500, and if there are two exchangeable protons the m/z must be 501. Fig. 6B shows the ions corresponding to peak 3 found in the LC–MS-II experiment. The ion of m/z 501 suggests that there are two protons linked to heteroatoms (OH and NH) present in the derivative. Hence it can be concluded that the structure of the derivative is that depicted in Fig. 5, route A.

The ions of m/z 520 and 522 in Fig. 6A and B, respectively, may result from complexation with sodium instead of protonation. Traces of sodium may have been present in the mobile phase even without it having been added.

Influence of type of buffer and pH on the derivatization reaction

Table 2 shows the results of the derivatization reaction in different buffers at various pH values. The use of a phosphate buffer of pH 6.3 or 7.0 gives the highest yield after 2 h. There is no significant difference between the reaction yields with these two buffers.

When the acetate buffer was used, an extra peak was observed in the chromatogram, as can be seen in Fig. 7. LC–MS-I showed that this peak contained an ion of m/z 314. This m/z suggests the formation of an ammonium adduct with the product from the reaction between acetate and (+)-Flec. The reaction between

TABLE II

EFFECT OF THE BUFFER AND pH ON THE DERIVATIZATION REACTION AND ON THE CONVERSION OF (+)-FLEC TO (+)-FLEC-OH ($n = 2$)

Buffer	pH	N-0861 left (%)	Total area of the two diastereoisomers formed ($n = 2$)	(+)-Flec-OH formed (%) ($n = 2$)[a]
(a) borate	8.1	5.7	3 690 240, 3 442 788	93, 82
(b) phosphate	7.0	1.0	4 387 925, 3 952 745	28, 30
(c) phosphate	6.3	1.1	4 329 186, 400 895	31, 23
(d) acetate	5.2	46.1	n.d.[b]	77, 80

[a] % (+)-Flec-OH = [area (+)-Flec-OH/area (+)-Flec-OH + area (+)-Flec] · 100.
[b] Not detected; see text.

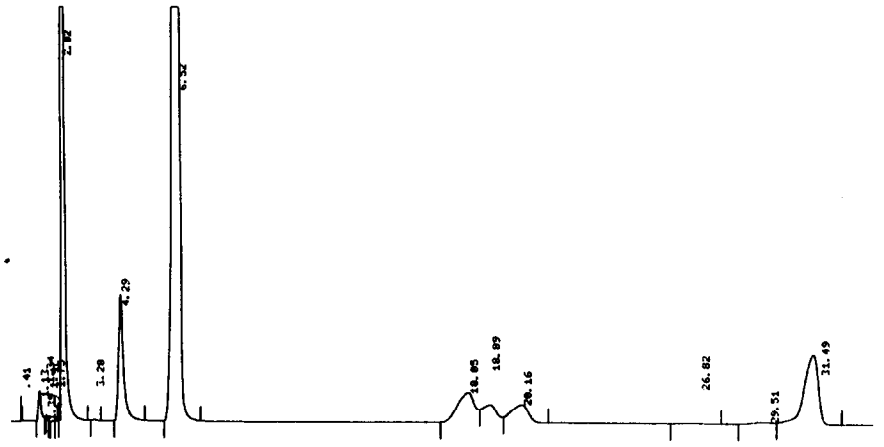

Fig. 7. Chromatogram obtained from the product of the derivatization reaction in the acetic acid buffer (pH 5). The disturbing peak elutes at 18 min.

acetate and (+)-Flec may result in the formation of an anhydride [12]. This anhydride, which is unstable in an aqueous environment, co-elutes with the diastereoisomers with a retention time of 18 min. The degradation product of the anhydride in aqueous solution is probably Flec-OH. This explains the high percentage of Flec-OH in Table II for the experiment with the acetate buffer. Acetate present in the reaction medium reacts with the Flec before the desired reaction between Flec and N-0861 is complete, and the resulting anhydride gives a disturbing peak in the chromatogram. With the knowledge of the reaction between the acetate and the (+)-Flec in mind, it was checked whether the acetate in the eluent reacts with (+)-Flec during the chromatographic run. A reference (+)-Flec, namely a solution of 80 μl of acetonitrile with 80 μl of phosphate buffer (pH 7) and 40 μl of a 18 m*M* (+)-Flec solution in acetone, was injected into the chromatographic system. A small peak eluted at the same time as the product between acetate and (+)-Flec. Therefore, to avoid this reaction, the acetic acid was removed from the eluent. This had no effect on the retention and/or peak shape of the diastereoisomers, (+)-Flec and (+)-Flec-OH, but the retention of N-0861 was delayed by 1.5 min to 6 min and the disturbing peak had disappeared.

Output of the derivatization reaction

Fig. 8 shows the results after different reaction times. It can be seen that the reaction is more than 90% complete after 1 h. The variation in the percentage of the diastereoisomers formed after a reaction time of more than 1 h may be an indication for the precision of the method. The average percentage of the formed diastereoisomers after a reaction time of more than 59 min was 94% with a relative standard deviation of 3%

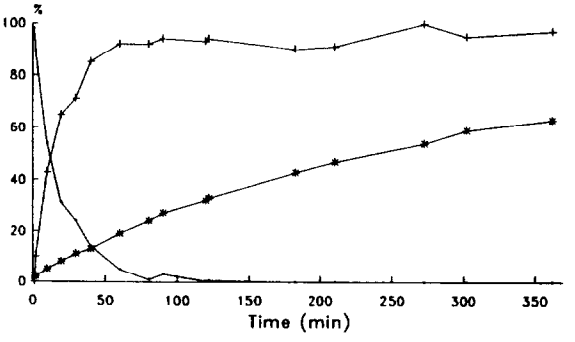

Fig. 8. Influence of reaction time on the derivatization yield. (●) % N-0861 = (area N-0861 after derivatization/area of the reference N-0861) · 100. (+) % derivatives = (total area of the two diastereoisomers at a certain time/total area of the two diastereoisomers after a time of 272 min) · 100. (*) % Flec-OH = [area (+)-Flec-OH/area (+)-Flec-OH + area (+)-Flec] · 100.

($n = 11$). After 1 h N-0861 was no longer detectable and differences in the amount of the reaction product will reflect the random errors in the procedure. The reaction product is stable for at least 1 day.

Improvement of the separation

The highest resolution within acceptable retention times, t_{R_1} and t_{R_2} of 27 and 30 min respectively, was obtained with a 125 × 4 mm I.D. LiChrospher RP Select B C_8 column with 5-μm particles (Merck). The combination of this C_8 column, which was thermostated at 30°C, with an eluent of water–acetonitrile (60:40, v/v) and a flow-rate of 1 ml/min resulted in a resolution (R_s) of 1.13. A chromatogram obtained under these conditions is shown in Fig. 9. Underivatized N-0861 could not be detected because the derivatization reaction was almost complete at the time the sample was injected into the HPLC system. The long run time is necessary to allow the unreacted (+)-Flec to elute from the column. If the remaining (+)-Flec could be removed before the injection, the run time could be decreased substantially. The disadvantage of removing anything from the reaction sample is the risk that some of the diastereoisomers will also be removed. If the latter happens it will decrease the sensitivity of the method. Another method to decrease the run time may be to use gradient elution after the diastereoisomers have eluted from the column. However, the easiest way to remove Flec is to inject samples one day after the derivatization. The derivatization products are sufficiently stable but Flec will be converted completely into Flec-OH, which elutes at about 7 min.

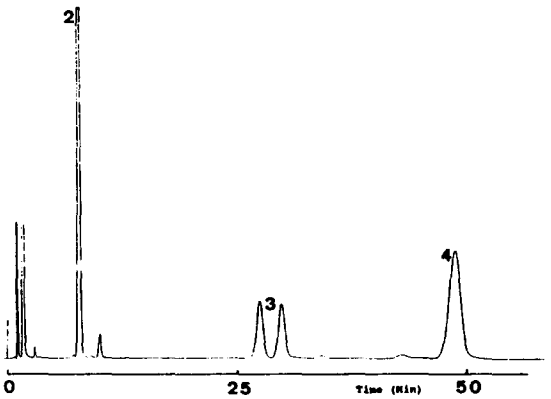

Fig. 9. Chromatogram obtained on a C_8 column, after derivatization of N-0861 with (+)-Flec. Conditions as given under Improvement of the separation. 2 = (+)-Flec-OH; 3 = the two diastereoisomers resulting from the derivatization reaction; 4 = (+)-Flec.

Calibration graphs and influence of different concentrations of N-0861 on the resolution between the diastereoisomers

The calibration graphs are described by the equation

$$y = Ax + B \quad (1)$$

where for the first-eluting isomer $A = 7.64 \cdot 10^{10}$, $B = 432$ and the correlation coefficient $r = 0.9999$ and for the second-eluting isomer $A = 8.54 \cdot 10^{10}$, $B = 5659$ and $r = 0.9999$. As the individual enantiomers of N-0861 were not available, the calibration graphs were prepared using racemic N-0861. The elution order therefore could not be established. The calibration graphs for the single diastereoisomers show good linearity with $r =$ 0.9999 in each instance. This shows that the derivatization reaction resulting in the formation of the diastereoisomers is linear, at least in the concentration range studied. The lowest concentration studied corresponds to a total amount injected of 6 ng for each N-0861 enantiomer.

It should be noted that there is no significant influence of concentration on the resolution. Nevertheless, for enantiomeric purity studies, calibration graphs with different ratios of the enantiomers should be prepared because a large amount of the first-eluting diastereoisomer can easily disturb the measurement of a small peak of the second-eluting diastereoisomer.

CONCLUSIONS

Owing to the very low efficiency and the lack of selectivity of the α-AGP column for the enantiomers of N-0861, the direct separation

method did not give satisfactory results. On the other hand, even if a better separation had been possible, applications to biological samples might have been limited because α-AGP stationary phases may easily be ruined by a biological matrix if not properly removed.

For the enantiomeric separation of N-0861, derivatization with (+)-Flec showed good potential. The method is easy to perform and aqueous solutions can be used. This, in combination with the use of a reversed-phase HPLC system, may make the method suitable for biological samples to study enantioselectivity in the kinetics of N-0861. However, for bioanalytical work an exhaustive clean-up step of the sample will remain necessary before the derivatization reaction can take place. The calibration graphs showed that the reaction is linear. A resolution between the formed diastereoisomers of 1.13 within 30 min was obtained on a C_8 column with an acetonitrile–water eluent.

The identities of the reaction products were checked by LC–MS. The derivatization product appeared to be different from the expected product. Under the influence of the chloroformate the methyladenine ring structure present in N-0861 opened on addition of H_2O. This was established by LC–MS experiments in which the H_2O in the eluent was replaced with ^2H_2O. With this eluent the number of protons linked to heteroatoms, such as nitrogen and/or oxygen, could be calculated. This allowed the exact structure of the derivatization product to be elucidated.

As the individual enantiomers of N-0861 were not available, the elution order of the enantiomers could not be established. However, it should be noted that if enantiomeric purity studies require a reversal of the elution order, this can easily be achieved because the individual enantiomers of Flec are commercially available.

A drawback of the indirect method is the relatively high price of the optically pure derivatization reagent.

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