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Journal of Chromatography A, 1088 (2005) 104–109
JOURNAL OF CHROMATOGRAPHY A
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Chiral separation of atropine by high-performance liquid chromatography

D. Breton [a,*], D. Buret [a], P. Clair [a], M. Lafosse [b]

[a] *Pharmacie Centrale des Armées, BP 04, 45998 Orleans Armées, France*
[b] *Institut de Chimie Organique et Analytique, UFR Sciences, BP 6759, 45067 Orleans Cedex 2, France*

Available online 13 March 2005

Abstract

The separation and quantitation of the enantiomers and also the determination of the enantiomeric purity are now current and indispensable tasks for the pharmaceutical analysis. Among the various techniques, liquid chromatography remains the best modality owing to several advantages. High speed, sensitivity, and reproducible results make LC the method of choice in almost all laboratories. Phases that contain α_1-acid glycoprotein as chiral selector are suitable for separation of charged and uncharged enantiomers with widely different structure. Atropine is widely used as parasympatolytic, anticholinergic and antiemetic drugs. It is one of the preferred antidote for immediate management of toxicity associated with nerve agents. Stereoselective separation was achieved with a prepacked α_1-acid glycoprotein column without any derivatization procedure. The liquid chromatography system is coupled to mass spectrometry with an atmospheric pressure chemical ionization interface in the positive-ion mode. The chromatographed analytes are detected in selective ion monitoring after optimisation using factorial experimental design. Small amount of enantiomeric composition can be evaluated either by MS or by UV spectrometry (less than 5%).

Keywords: Enantiomers; Atropine; Liquid chromatography; Mass spectrometry

1. Introduction

In the last decades liquid chromatography (LC) became one of the most powerful separation techniques in analytical chemistry. In recent years, the direct separation of enantiomers by chiral chromatography has been the target of intense research. Enantiomeric selectivity usually is achieved through the appropriate choice of a chiral stationary phase and mobile condition. Several interactions between the phases of the chromatographic system and the components of the sample can be utilized and there is usually a certain choice available to realize a concrete separation.

In order to separate a chiral compound such as atropine, several method have been used. LC is the first used with direct separation [1]. Enantiosepration of atropine was also achieved by capillary zone electrophoresis method [2] and thin layer chromatography [3].

An important method for separating enantiomers involves protein-based chiral stationary phases [4]. Proteins are natural polymers, made of amino acids, which are chiral molecules, except for glycine. Moreover, some proteins also contain sugar moieties. Protein stationary phases for LC were introduced in the early 1980s [5]. In many of them, α_1-acid glycoprotein (AGP) was found to recognize basic [6], acidic [7] and neutral compounds [8]. This protein is used very frequently for the chiral resolution of a wide variety of racemates.

Atropine, an alkaloid derived from belladonna root or produced synthetically, is an anticholinergic agent with various therapeutic uses. It has two main types of actions, one on the central nervous system to cause respiratory stimulation, and the other, to suppress smooth muscles and secretory glands innervated by parasympathetic (cholinergic) nerves [9]. This alkaloid is also the preferred antidote for immediate management of toxicity associated with nerve agents or other cholinergic syndrome [10]. Atropine is a mixture of optical isomers and is also referred to as *dl*-hyoscyamine.

* Corresponding author. Tel.: +33 2 38 60 73 21; fax: +33 2 38 60 73 24.
E-mail address: dbreton.pca@noos.fr (D. Breton).

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doi:10.1016/j.chroma.2005.02.073

The aim of this work is to describe a direct method to determine stereoselectivity of the enantiomers of atropine, by employing a commercially available AGP LC column. The effects of the principal operational parameters were characterized by using statistical experimental design.

Two detection systems were used: UV and mass spectrometry. The last one was used to confirm that there was no degradation product under one of the peaks of enantiomers. In fact, several related substances from atropine should be separated using hydrophilic column [11] and then have a negative effect on the UV detection [12].

2. Experimental

2.1. Chemicals and reagents

Racemic atropine and the L-enantiomer of atropine were purchased from Sigma (Saint Quentin Fallavier, France). Acetonitrile of LC grade was obtained from Merck (VWR, Fontenay sous bois, France). Water was purified with a Milli-Q system (Saint Quentin en Yvelines, France). Chemicals reagents such as ammonium acetate, acetic acid, sodium octanoate were analytical grade and purchased from Sigma.

2.2. Instrumentation

The LC system consisted of a 1100 Series liquid chromatograph equipped with a vacuum degasser, a binary pump, a thermostated column compartment, an autosampler and a diode array detector, all from Agilent Technologies (Massy, France). Mass spectrometric detection was carried out using a 1100 MSD simple quadruple instrument from Agilent Technologies equipped with an atmospheric pressure chemical ionization interface (APCI).

The chiral stationary phase used for the determination of atropine enantiomers was a Chiral-AGP column (150 mm × 2 mm I.D.) packed with α_1-acid glycoprotein coated on silica (5 μm) from Chromtech (Congleton, Cheshire, UK).

2.3. Factorial design

The factorial experiment included all parameters thought to be able to influence the response of a sample in the LC analysis. Each factor was applied at two different levels. The different factors and levels are described in Table 1.

Six factors were included in a 16 single experiment design in the form 2^{6-2} fractional design. All interactions are initially assumed to be small compared to main effects. One central point with six replicate was added to the factorial design.

Results were evaluated by means of Statistica (Statsoft, http://www.statsoft.com).

Table 1
The different factors and their levels included in the factorial design

	Factors	Level 1	Level 2
A	Flow rate (mL min^{-1})	0.15	0.25
B	Ionic strength of mobile phase (mM)	10	40
C	pH	5.7	6.7
D	Ion pair reagent (octanoic acid) (mM)	1	5
E	Acetonitrile content in the mobile phase (%)	1	5
F	Temperature of the column (°C)	10	20

2.4. Chromatographic conditions

All chromatographic experiments were carried out in the isocratic mode. The mobile phase for the enantioseparation of atropine was a mixture of ammonium acetate buffer and acetonitrile.

The stock standard solution of atropine and hyoscyamine (sulfate salts) was prepared by dissolving an appropriate amount of the compounds in purified water. Concentration for optimization was 0.1 mg mL^{-1}. The volume of sample solution injected was typically 20 μL. The UV detector operated at 230 nm.

Mass spectra were recorded using a full scan in the positive mode, with a scan range from *m/z* 100 to 300. The following parameters were investigated: fragmentor voltage (50–200 V), drying gas (N_2) flow rate (2–13 L min^{-1}), nebulizer pressure (20–60 psig), drying gas temperature (100–350 °C), vaporizer temperature (200–500 °C), capillary voltage (1000–5000 V) and corona discharge (2–12 μA).

3. Result and discussion

The mechanism of chiral recognition of proteins used as CSPs is still unclear but it seems reasonable to think that chiral specificity is related to conformational changes of the protein and effects of the sugar moiety of the selector [13]. First of all, the effects of the principal operational parameters have been statistically characterized.

3.1. Optimization of the chromatographic separation

The factorial design gives information about whether a probable variable really is affecting the response. An advantage with factorial design is that much information as possible is achieved from a limited number of experiments. It enables us to measure the individual effects of each factor in the analysis. This factorial experiment was analysed by means of analysis of variance techniques. The experiments were performed based on the design matrix as shown in Table 2. Randomization is a method of safeguarding the experiment from systematic bias that causes variation in response.

Two responses were considered: resolution and retention time of the L-enantiomer. Under our experimental conditions, the L-atropine always elutes before the D-enantiomer. The results of experiments are given in Table 2. Fig. 1 illustrates the

Table 2
Uncoded factorial design plan and results

Trial number (run)	Factors						Results	
	A	B	C	D	E	F	α[a]	Tr[b]
1	0.15	10	5.7	1	1	10	1.5	8.1
2	0.25	10	5.7	1	5	10	0.6	4.0
3	0.15	40	5.7	1	5	20	0.6	5.0
4	0.25	40	5.7	1	1	20	1.3	3.7
5	0.15	10	6.7	1	5	20	1.2	15.6
6	0.25	10	6.7	1	1	20	1.9	15.8
7	0.15	40	6.7	1	1	10	2.6	17.7
8	0.25	40	6.7	1	5	10	0.9	5.4
9	0.15	10	5.7	5	1	20	1.4	7.0
10	0.25	10	5.7	5	5	20	0.6	3.6
11	0.15	40	5.7	5	5	10	0.5	4.7
12	0.25	40	5.7	5	1	10	0.9	3.3
13	0.15	10	6.7	5	5	10	1.7	13.9
14	0.25	10	6.7	5	1	10	2.6	12.4
15	0.15	40	6.7	5	1	20	2.6	16.2
16	0.25	40	6.7	5	5	20	1.6	5.1
17[c] (1)	0.20	25	6.2	3	3	15	1.7	6.3
18[c] (8)	0.20	25	6.2	3	3	15	1.7	6.3
19[c] (19)	0.20	25	6.2	3	3	15	1.7	6.1

[a] α: separation factor.
[b] Tr: retention time of the L-enantiomer (min).
[c] Central points.

main effects of the control parameters on studied responses. A negative sign indicates a diminution effect of the resolution and/or the retention time. On this graph, the *p* value indicates the significance limit. Two main effects are judged to be statistically significant (pH and amount of acetonitrile).

pH appears to be relevant for resolution and retention time. The positive signs of this effect are in accordance with previous results [14]. On the other hand, a decrease of acetonitrile amount results in an increase of the two responses. Thus, an adequate enantioseparation of racemic atropine seems to be hard to achieve. Moreover, a long retention time is correlated with a decrease of the UV response.

Flow rate is also important for the retention time, but has a non-significant action on the enantioresolution. This could be relevant to minimize time of analysis. Nonetheless, an increase in the flow rate induces an increase in the column pressure. A too high pressure may damage the column. Reducing the flow rate results in a higher background.

Ionic strength, rate of octanoic acid and temperature have no influence on the two responses of interest. However, octanoate as a counter ion cannot be suppressed. As shown by Arvidsson and Janson [15], it has a very significant effect on the stereoselectivity of atropine and in its absence a very poor resolution is obtained.

After having examined the LC separation and retention behavior of the enantiomers of atropine, the following chosen optimized isocratic conditions for the efficient, economical, and time saving separation were: 3/97% (v/v) acetonitrile-buffer, with the pH buffer 6.2 containing 10 mM ammonium acetate and 1 mM sodium octanoate. The flow rate was fixed at 0.2 mL min^{-1} and temperature was 20 °C (Fig. 2). The separation for racemic compound is quite good. In fact, capacity of the column is linked to it internal diameter [4] and the proportion of the two enantiomers. In case of non-racemic composition (e.g. 80/20 L-/D-atropine), the resolution is greatly improved (see applications).

3.2. Optimization of the mass spectrometric detection

Mobile phase is composed of a large amount of aqueous buffer (97%). Moreover, atropine is a rather small compound (MM = 289.4 g mol^{-1}), so we decided to use APCI. This chemical ionisation is good for small molecules (polar to non-polar) and the heater system allows a better solvent evaporation than electrospray [16].

The chromatograms were firstly monitored using TIC (100–300 Th). For the optimization of MS conditions, atropine was directly introduced into the MS detector using flow injection analysis (FIA). Parameters such as corona discharge, capillary voltage, and fragmentor voltage were optimized in order to obtain the protonated molecule $[M+H]^+$. The predominant fragment corresponding to this protonated molecule was identified at *m/z* 290.1 (Fig. 3). The peak height

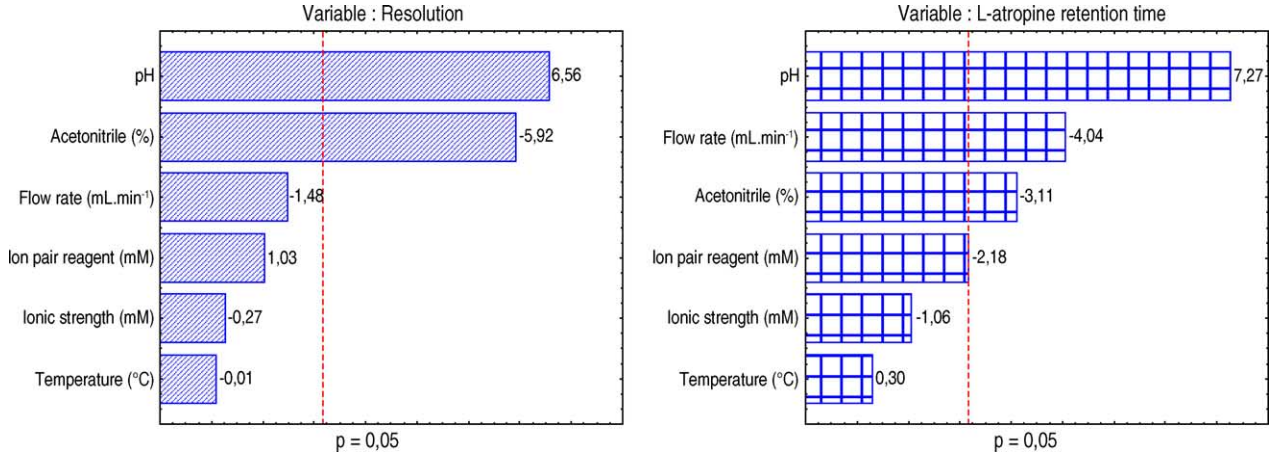

Fig. 1. Calculated effects of factors, *p* shows the significance level.

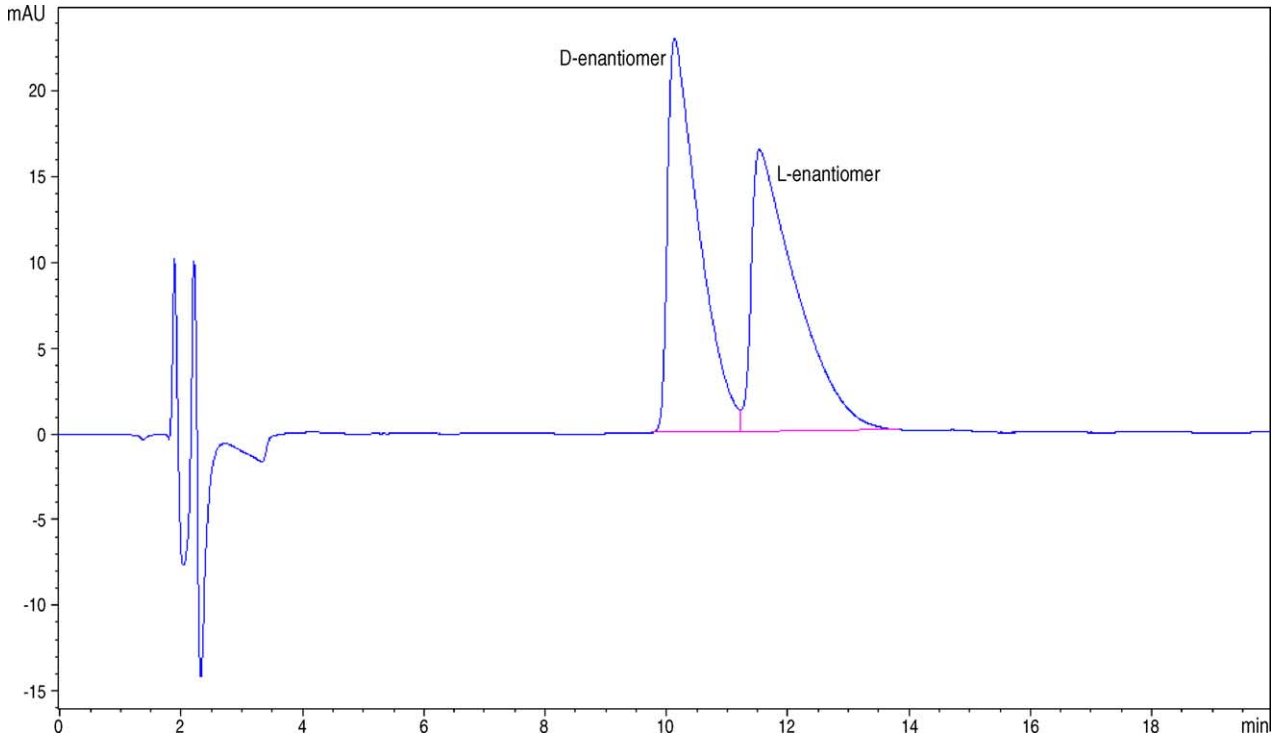

Fig. 2. Optimized chromatogram (UV response) of racemic atropine (0.1 mg mL^{-1}). The flow rate was 0.2 mL min^{-1} and the solvent composition was (3:97) acetonitrile: 10 mM aqueous ammonium acetate buffer (pH 6.2). Temperature was 20 °C and ion pair reagent amount was 1 mM.

of atropine varied with the fragmentor voltage and the optimum response was obtained at 100 V. Thus, the value for the fragmentor voltage was set to 100 V and the other parameters were evaluated in this way, the optimum conditions based on the maximum peak height were: fragmentor voltage (100 V), drying gas flow rate (12 L min^{-1}), nebulizer pressure (50 psig), drying gas temperature (300 °C), vaporizer temperature (400 °C), capillary voltage (2000 V) and corona discharge (10 μA).

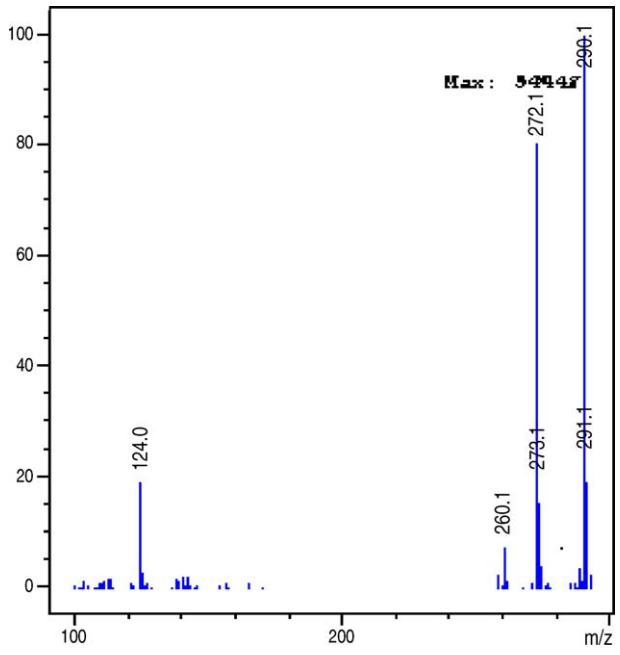

Fig. 3. Characteristic mass spectra of atropine obtained by APCI-MS. The base peak ion *m*/*z* 290.1 is the protonated molecular ion.

The results of this study also demonstrated that there were no overlaps in the mass spectra of the compound at the *m*/*z* value chosen after the optimization. The presence of sodium octanoate seems not to pose problems when coupling with mass detector. The counter ion only reduces very little the mass detector response and do not decreases it sensitivity. The base peak of *m*/*z* 124.1 indicated the cleavage between the tropine ring and the oxygen at the center of atropine. The same effect is shown on resolution as for UV detection: the less D-enatiomer in the mixture is, the best resolution is. It confirms that the capacity of this kind of column is tightly dependent of the mixture composition.

For analytical assays, the chromatograms were monitored using SIM (*m*/*z* = 290.1).

3.3. Applications

The method was used on different atropine. Some of them are active pharmaceutical ingredients. Fig. 4 shows a typical chromatogram of pure L-hyoscyamine (0.1 mg mL^{-1}). Under our analytical conditions, the L-enantiomer elutes before the D-one. However, we must notice that the sensibility of this method when using UV detection is quite good, so small amount of enantiomers could be detected. In the case of racemic atropine sulfate (Fig. 5), mass detec-

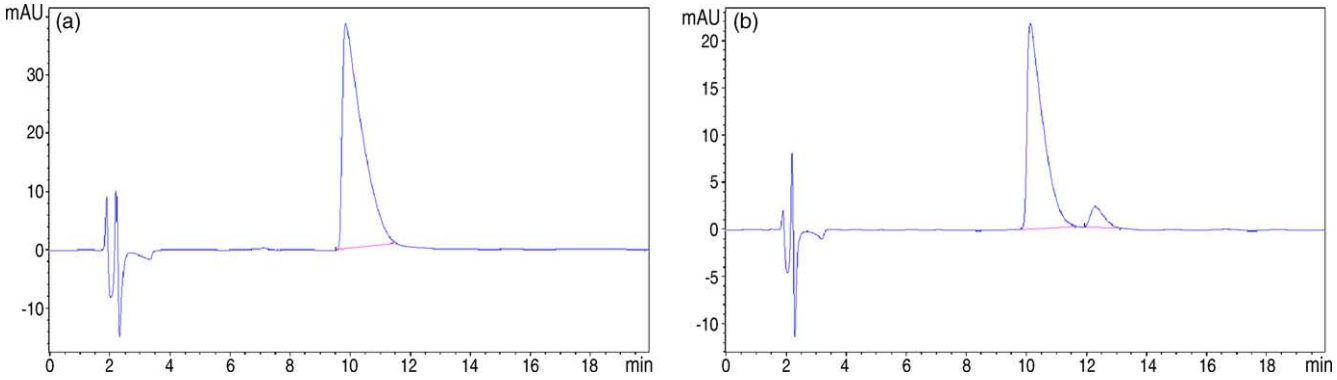

Fig. 4. Typical chromatograms (UV 230 nm) of hyoscyamine (a) and a composition of 90% of L-enantiomers and 10% of D-enantiomers (b).

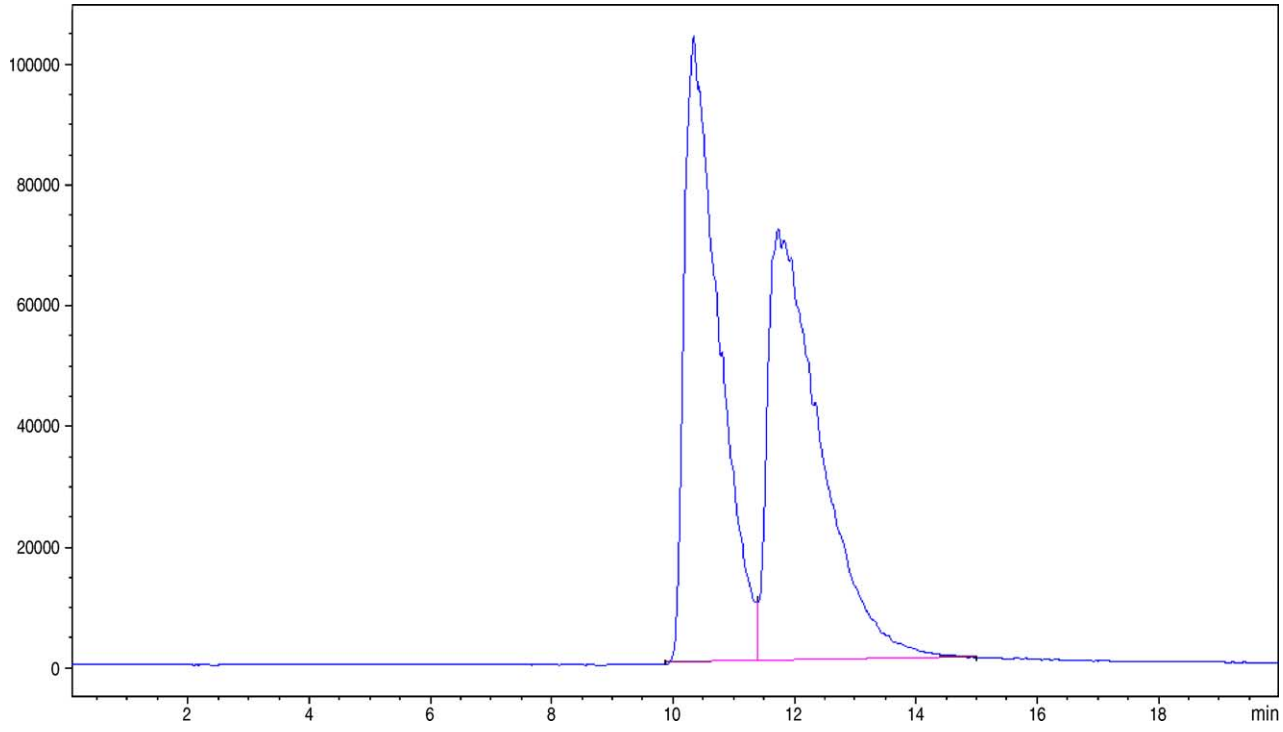

Fig. 5. Characteristic chromatogram of racemic atropine (MSD–SIM, $m/z = 290.1$).

tor cannot allow us to obtain a well-resoluted chromatogram. This detector could currently just be used as a qualitative detector.

The enantiomeric excess (e.e.%) is defined as follow [17]:

$$\text{e.e.\%} = \frac{C_+ - C_-}{C_+ + C_-}$$

where C_+ and C_- are the concentrations of the (+) and (−) or (D) and (L) isomers, respectively. Therefore, the term $C_+ - C_-$ represents the difference between the concentration of the two enantiomeric components in the mixture, and $C_+ + C_-$ is the total concentration. The method allows detecting minor compound in composition containing less than 10% D-enantiomer (Fig. 4), that is to say a e.e. of more than 80%. In this case, mass detector shows a sufficient resolution.

4. Conclusion

The chiral separation using a narrow bore AGP column (2 mm I.D.) depends to a high degree on the chromatographic conditions, particularly organic solvent amount and pH as shown in our study. On the other hand, the use of octanoic acid as a counter ion seems to be critical. Despite the low loading capacity of AGP, it allows a sufficient resolution of the enantiomers of atropine. Moreover, the less D-enantiomer is present, the best is the separation. Indeed, small amount

(less than 5%) of the distomer (inactive enantiomer) could be detected either in UV or mass-spectrometry. The last one is especially useful for the study of compounds during stress-condition study of pharmaceutical active ingredients. It must be shown that method is specific and that no degradation product could appear under one of the peaks.

Moreover, the use of narrow bore column with a low flow rate (0.2 mL min^{-1}) allows to reduce the use of mobile phase which decreases the cost of each analysis.

L-Hyoscyamine is the natural compound which has the activity of the molecule (eutomer) of the well-known atropine. The stability of L-hyoscyamine needs further investigation in order to replace racemic atropine in pharmaceutical formulation. The kinetic of L-hyoscyamine racemization could be evaluated without degradation product interference owing to our method.

Acknowledgement

We thank our colleague PharmD A. Pech for valuables comments and her constructive suggestions.

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