Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1055 (2004) 55–62
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Highly sensitive and rapid normal-phase chiral screen using high-performance liquid chromatography–atmospheric pressure ionization tandem mass spectrometry (HPLC/MS)

María Luz de la Puente*

European Analytical Technologies, Centro de Investigación Básica, Lilly S.A., Avenida de la Industria 30, 28108 Alcobendas, Spain

Received 11 June 2004; received in revised form 30 July 2004; accepted 1 September 2004

Abstract

In the last years, there has been an increasing demand on the development of quantitative assays for determination of enantiopurity. Herein, we present a methodology based on a direct linking of an atmospheric pressure ionization mass spectrometer (MS-APCI) with a high-performance liquid chromatography HPLC (DAD) system, operated under normal-phase mode and without post-column addition of MS-compatible solvents, which provides the high specificity/selectivity (identification of isomers in complex mixtures) and accuracy (1–2% area level) required for daily chiral studies. As result of the success of our screen, the preparation of individual enantiomers or the racemic mixture in our Drug Discovery Research Laboratories at Lilly, Spain is usually not required. Therefore, additional non-valuable synthetic work is eliminated.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Gradient elution; Normal phase; Chiral; HPLC/MS; High-throughput; Screening

1. Introduction

Since chiral molecules are at the forefront of strategies for the development of safer and more effective drugs, the number of chiral pharmaceutical candidates has been increasing steadily for the last few years. These factors contribute to the need for new analytical methods to characterize chiral molecules more efficiently, providing quick answers to chemists and eliminating any additional synthetic work.

Recently, we have described an automated HPLC screening approach based on gradient elution, which affords accurate enantiomeric excess determinations in a very short timeframe. Baseline resolution of enantiomers is obtained in most of the cases (>80%). For the remaining cases, complete enantioseparation by isocratic elution is generally achieved in a single shot [1] without the need of additional studies, as this methodology allows the direct transferring of conditions to isocratic mode by just checking retention time and selectivity yielded by standard gradient. The work described herein, deals with the benefit of using mass spectrometry coupled to HPLC for identification and quantitation of isomeric species, and presents a further step in our continuing effort to increase efficiency of chiral HPLC analytical work.

On the one hand, it is well known that polysaccharide derivatives such as Chiralpak® AD, Chiralpak® AS, Chiralcel® OD and Chiralcel® OJ, belong to the most widely used chiral stationary phases (CSP) and have confirmed their effectiveness in the separation of most racemic compounds for HPLC analytical and preparative purposes [2–14]. In addition, these materials may be used not only under standard reversed (buffered and unbuffered) and normal-phase (alcohol–hydrocarbon mixtures) elution conditions, but also in polar mode using pure polar organic solvents [15–18].

On the other hand, high-performance liquid chromatography (HPLC) coupled to atmospheric pressure mass spectrometry (API-MS) has become a powerful tool for the analysis of pharmaceutical compounds due to its sensitivity, speed, and

* Fax: +34 91 623 3561.
E-mail address: de_la_puente_maria_luz@lilly.com.

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.09.007

specificity. For chiral purity determination, MS easily identifies isomeric impurities separated by HPLC because they have the same mass as the major component, which reduces significantly the time and effort to identify and/or quantify those species. Particularly when exploring a new synthetic route, chemists only want a preliminary monitoring of the chirality of the compound. In cases where multiple components with the same molecular weight are observed, separation of enantiomers may be assumed and in these cases, HPLC/MS can play an indispensable role in providing a quick answer to their synthesis without requiring pure racemates.

However, HPLC/MS has been traditionally performed using reversed-phase separations. In contrast, there are just very few references concerning the use of normal-phase with atmospheric pressure ionization, as it has been reported to be, in many applications, incompatible when linked with atmospheric pressure ionization mass detection (API) due to several incompatibilities including mobile phase, additive, and flow rate selection. Thus, solvents such as hexane have been described to not supporting the formation of ions, critical step in ESI [19]. Further, the use of hexane as the main component of the mobile phase seems to introduce a possible explosion hazard in the presence of the high voltage of the ESI needle [20].

Lately, the need to quickly develop chiral separation methods suitable not only for analysis but also for purification applications has increased significantly. While the use of reversed-phase HPLC/MS is commonplace within the industry, solvent consumption rates and work up times, result in the exclusive use of such methods for purification purposes becoming prohibitive. Therefore, normal-phase HPLC chromatography is the best method of choice for the analysts, as it offers many advantages such as a much greater sample loading or a minimal dry down. Further on, when doing enantiomeric analysis, simply changing the mobile phase and/or the additive type to one that is MS-compatible can decrease or even eliminate enantiomeric resolution and/or selectivity.

At this point, analysts have focussed their efforts in overcoming the difficulties of coupling MS detection with normal-phase HPLC by employing post-column addition of MS-compatible solvent systems [21–24]. However, the compatibility of the normal-phase polar mode with LC–MS interfaces and detection without the need for post-column treatment remains unexplored, even though the atmospheric pressure chemical ionization (APCI) interface shows good compatibility with the organic solvents used in normal-phase. Further on, the application range of APCI includes non-polar and medium-polarity molecules with mass range between 100 and 1000 u (MM), which represents traditional targets in Drug Discovery.

Herein, we present an optimization of the normal-phase chiral screen (polysaccharide CSPs) developed and applied in our labs for identification, quantification and purification of enantio/diastereoisomers [1] by direct linking of the HPLC (DAD) system with APCI-MS detection.

2. Experimental procedure

2.1. Chemicals

Model compounds were selected on the basis of their differing structural properties. All of them showed suitable polarity for resolution under normal-phase. Those products included a commercial drug compound (comparative studies) but mainly investigational drug compounds internal of Ely Lilly Pharmaceutical Research and Development. Warfarin was the commercially available drug, supplied by Sigma–Aldrich (Steinheim, Germany). This standard was included in our previous paper [1] and has been selected again in this work in order to show reproducibility of the new methodology concerning chromatographic resolution. The names and structures of Lilly compounds used to illustrate chromatographic separations cannot be disclosed due to proprietary reasons.

HPLC grade hexane and ethanol are purchased from Merck (Darmstadt, Germany), while isopropanol was supplied by LabScan (Dublin, Ireland). Spectrophotometric grade trifluoroacetic acid (TFA) and dimethylethylamine (DMEA) are also purchased from Sigma–Aldrich.

2.2. Instrumentation

All the analytical studies are performed on a Series 1100 liquid chromatography/mass selective detector LC/MSD (Agilent, Waldbronn, Germany) driven by ChemStation software Rev. A.09.03 (1417)®, 1997–2000, Agilent Technologies). This system is composed of a solvent degasser (G1379A), a quaternary pump (G1311A), an auto-sampler (G1313A), a column compartment (G1316A), a diode array detector (G1315B) and a mass spectrometer (G1946D). Connections from the autosampler to column inlet and from column outlet to detector are made with 0.005 in. i.d. Stainless steel connections are made as short as possible in order to minimise dead volume. For automation of the system, two Agilent 6-position selection valves (G1159A-6), controlled by the ChemStation, are installed.

2.3. Chromatographic conditions

The wavelength of UV detection is monitored from 210 to 380 nm although chromatograms are recorded at 215 and 254 nm signals. Experiments are carried out at room temperature.

2.3.1. Stationary phases

The enantioselective separations are accomplished on the following polysaccharide CSPs: Chiralpak® AD [amylose tris (3,5-dimethyl-phenyl carbamate)], Chiralpak® AS [amylose tris ((*S*)-1-phenylethyl carbamate)], Chiralcel® OD [cellulose tris (3,5-dimethylphenyl carbamate)] and Chiralcel® OJ [cellulose tris (4-methyl benzoate)] columns from Daicel® (Chiral Technologies Europe). The column dimension is

250 mm × 4.6 mm with the enantioselective phase coated onto a 10 μm silica-gel substrate.

2.3.2. Mobile phases

Mixtures of hexane and isopropanol or ethanol, have proved to be the most suitable eluent for efficient separation of a wide variety of racemates on polysaccharide-based CSPs under low polar mode, therefore they are the selected mobile phases for our screen. In addition, successful enantioseparations on those CSPs with pure polar organic mobile phases have been also reported [15–18], as it may offer different chiral recognition mechanisms and higher solubility of some analytes, being a useful choice in preparative scale applications. Among the selected CSPs, Chiralpak® AD using neat EtOH has been recognized as the most universal column for this elution mode, and thus it is included in our protocol. We need to notice that, in the last months, we have obtained successful results on Chiralcel® OJ using MeOH. However, these chromatographic conditions are only applied in our laboratories when facing lack of resolution under any of our standard conditions.

2.3.3. Mobile phase additives

In our standard analysis under low polar elution mode, TFA (CF_3COOH) and DMEA (Me_2EtN) are added to hexane in standard concentrations of 0.05 and 0.2% (v/v), respectively. Neutral elution conditions can be also applied if required.

For high polar mode, addition of any of those two modifiers directly depends on mobile phase composition (see following section for details).

2.3.4. Elution program

2.3.4.1. Low polar mode (alkane–alcohol mixtures). For standard screening under gradient conditions, a range gradient from 20 to 60% alcohol over 15 min is defined. This latter solvent composition is held for 2 min and the return to the initial conditions is performed within 3 min. After 5 min of isocratic equilibration, the next sample is injected. The flow rate is held constant at 0.75 ml min^{-1} on all column types and gradients. After loading a new method, the selected new column is equilibrated for 15 min before injection. For Chiralpak® AD there is a restriction on the use of hexane/ethanol mixtures from 85/15 to 40/60 due to UV absorption and stability issues, as it is described by manufactures, and thus it is not included in the hexane/ethanol gradient studies.

Extremely complex mixtures sometimes required isocratic elution. In those cases, mobile phase composition is defined on the basis of the results afforded by gradient screen (direct transferring) [1].

2.3.4.2. High polar mode. Elution on Chiralpak® AD with acidic modifier is established as a hexane-TFA 0.05%/ethanol gradient going from 70 to 100% alcohol in 15 min. For basic elution conditions EtOH-DMEA 0.2% is used. When using methanol, acidic conditions are discarded, and either neutral or basic elution is preferred.

2.4. Mass detector parameters

Mass spectra are recorded using a full scan in positive/negative modes simultaneously. The optimized parameters are set as follows.

- MSD spray chamber: drying gas flow (ml/min) and temperature (°C): 10/350; nebulizer pressure (psgi): 35; vaporizer temperature (°C): 415; capillary voltage (V): 2500 (for either positive or negative mode); corona current (uA): 4 (positive mode)/15 (negative mode).
- MSD signal settings: mass range (*m*/*z*): 100–800; fragmentor: 85; gain: 1; threshold: 80; step size: 0.1; peak width (min): 0.30; cycle time (s/cycle): 3.04; cycle time (%): 50/50 positive/negative.

2.5. System automation

Two valves (6-position/unit) are installed in order to provide support for the two sets of experiments, which are defined on the basis of the additive, TFA or DMEA, selected. In each valve, five positions are occupied by the four CSPs selected for low polar mode elution and the Chiralpak® AD units focused in high polar mode using ethanol. The remaining position is used to flush the system when changing modifier in order to avoid any additive cross-contamination. Unfortunately, and due to the lack of any available position in the quaternary pump, those experiments requiring the use of MeOH cannot be performed sequentially overnight.

2.6. System suitability

System suitability (HPLC/DAD) and column performance tests are performed, monthly, by injecting the standard mixture (*trans*-stilbene oxide) on each column unit. The main parameters under control are retention time, tailing factor and resolution.

Mass spectrometry instrument Check Tune is performed weekly. Check tune provides a way to quickly determine whether the LC/MSD is correctly tuned without performing a complete Auto Tune. Check Tune performs a single profile scan of the tune masses and compares the peak widths and mass axes with target values. If the values so obtained are outside of acceptable ranges, Check Tune suggests adjusting peak widths or calibrating the mass axis.

In addition, on-flow injection of a solution of Warfarin is also performed (injection volume: 80 uL; standard solution: 1 mg/1 mL EtOH; mobile phase hexane-TFA 0.05%/IPA 9/1; flow rate: 0.3 mL/min).

2.7. Sample preparation

Sample is completely dissolved in the minimum amount of ethanol (if required) and diluted with hexane to a

concentration of 1 mg/mL. However, it has to be noticed that the presence of impurities in amine additives tends to reduce the usable UV range, and thus lower sensitivity at low wavelengths is observed. For this reason, and depending on the UV absorbance of the analyte, screening under basic conditions sometimes may require a 2 mg/ml concentration.

Standard injection volume is 5 μL (of a 1 mg/mL sample solution) for studies under acidic conditions, and 15 μL (of a 2 mg/mL sample solution) for screening with basic modifiers.

3. Results and discussion

3.1. Selection of elution program in low polar mode

We have already reported the success of our screen [1]: baseline resolution of peaks is accomplished under gradient conditions over 80% cases. In the remaining cases, signs of chiral recognition are detected and baseline resolution is, most of the times, achieved in a second experiment by direct transferring conditions from gradient to isocratic elution. Those results prompted us to keep our gradient step.

It is well known that CSPs are very sensible to backpressure. As expected, the coupling of the mass detector to the HPLC system resulted in a significant increase in this parameter. The values so registered did not exceed the limit recommended by manufactures (50 bar for a 4.6 mm × 250 mm/10 μm column). However, we preferred to slightly refine the elution program to decrease pressure and avoid the risk of damaging the stationary phase. Further on, we took advantage of this opportunity to improve the performance of our methodology by decreasing cycle time, an idea that had been under consideration for a few months.

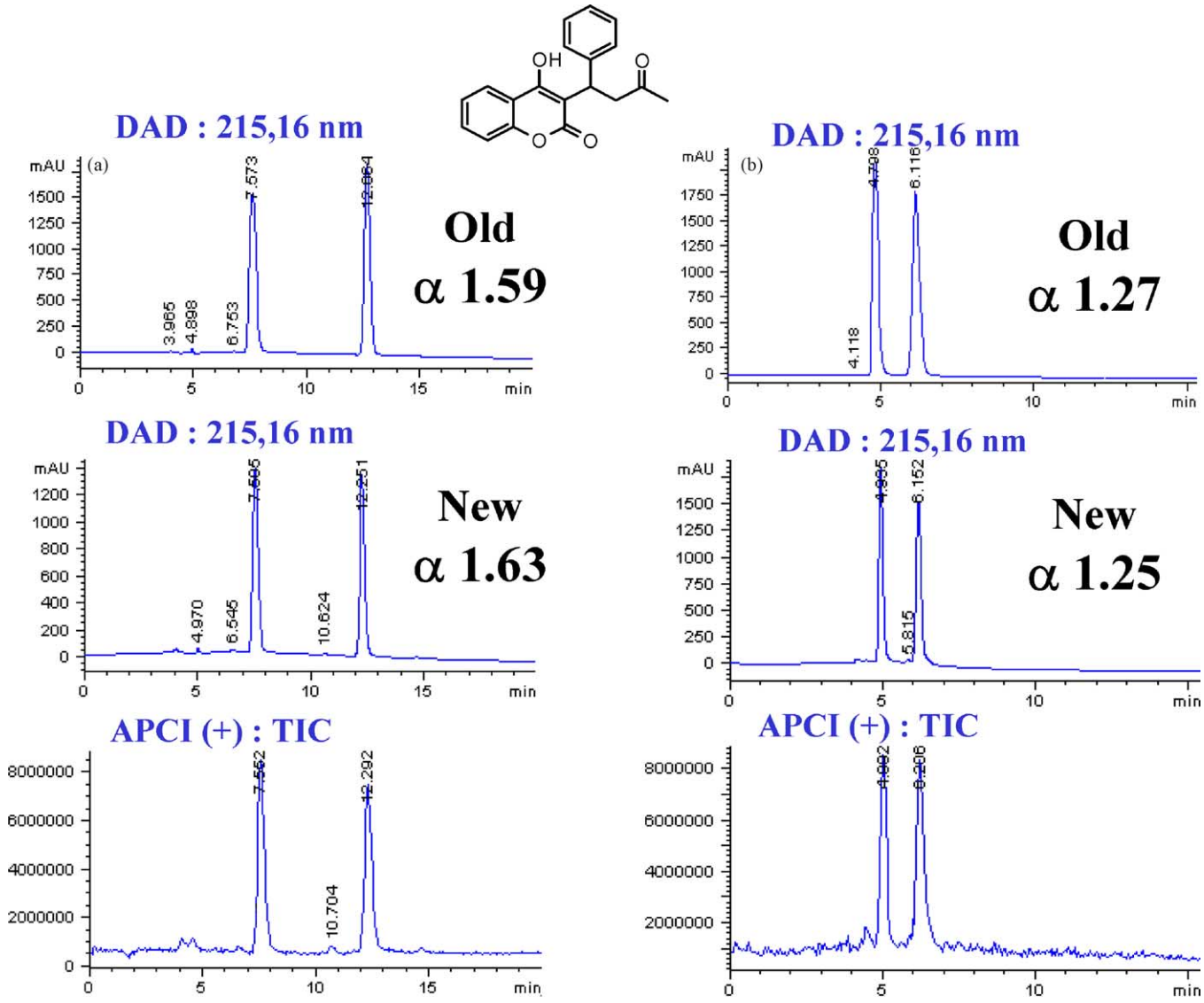

Fig. 1. Comparison of chiral resolution for analysis of commercially available Warfarin (racemic mixture) under old (HPLC) and new (HPLC/MS) gradient elution programs. Chromatographic conditions: column, Chiralpak AD; mobile phase and gradient program; (**1a**) Hexane-TFA 0.05% (A)/IPA (B), gradient from 20 to 70% (B) in 20 min (old) and from 20 to 60% (B) in 15 min (new); (**1b**) Hexane-TFA 0.05% (A)/EtOH (B), gradient from 70 to 100% (B) in 20 min (old) and from 70 to 100% (B) in 15 min (new); flow rate, 0.75 ml/min; detection, DAD 215 nm and APCI (TIC + mode).

Thus, the most straightforward way to match our goal was decreasing final alcohol concentration and run time while keeping gradient step and flow rate. New elution program for low polar mode was defined as a gradient from 20 to 60% alcohol in 15 min at 0.75 mL min^{-1} flow rate. Under those elution conditions, we could observe reproducibility of chiral resolution compared to the previous standard gradient mode. See Fig. 1a for comparison of results achieved by comparative analysis of Warfarin under the two different gradient programs. Selectivity (α) achieved in the two comparative studies is also given, confirming success of the new approach under low polar mode.

3.2. Selection of elution program in high polar mode

As we described in our previous communication [1], high polar elution mode under acidic conditions was defined using a binary mobile phase, hexane–EtOH, in which TFA was added to the alkane. Elution program was defined as a gradient from 70 to 100% alcohol in 20 min at 0.75 ml min^{-1}. Due to stability issues on Chiralpak® AD when using ethanol, we could not modify the aforementioned initial and final mobile phase composition. However, we reduced gradient time from 20 to 15 min. No impact on chiral resolution was observed after running comparative studies. We want to remark that, although there is an increase in backpressure due to the coupling of the MS detector, we have been working with the same column unit (in a daily basis) for 2 years without detecting any impact on column performance. See Fig. 1b for comparison of results achieved by analysis of Warfarin under the two different gradient programs. As it can be observed, reduction of run time did not impact selectivity.

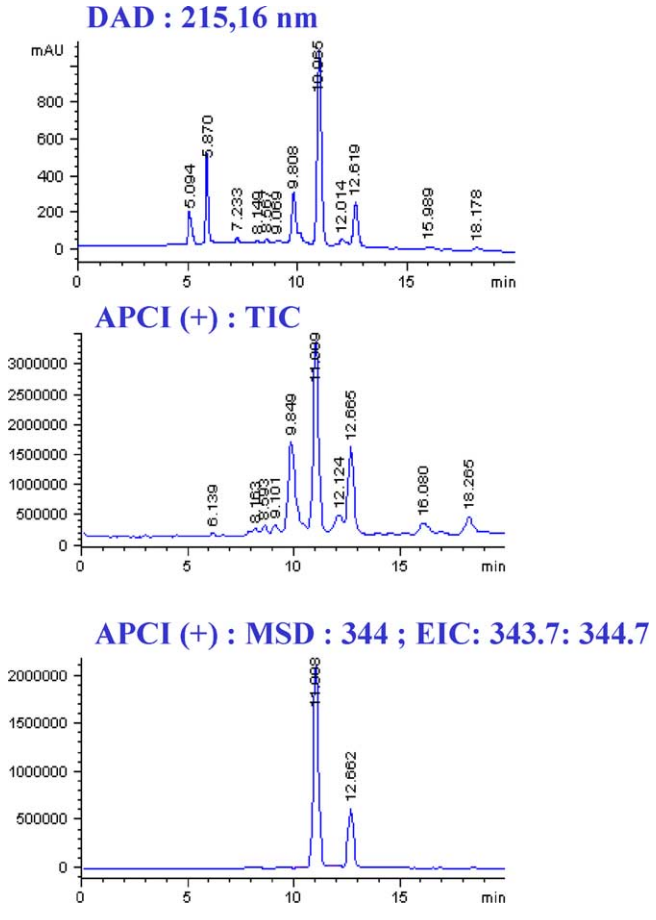

Fig. 2. Analysis of a pharmaceutical intermediate prepared in our Drug Discovery Research Laboratories by standard screen under acidic elution conditions. Ion extraction in APCI (+mode) confirmed identification of isomeric species even on complex mixtures. Chromatographic conditions: column, Chiralpak AD; mobile phase, Hexane-TFA 0.05% (A)/IPA (B); gradient from 20 to 60% (B) in 15 min; flow rate, 0.75 ml/min; detection, DAD 215 nm and APCI (TIC + mode and extracted ion).

3.3. Selection of mobile phase additives

As we mentioned in our previous work [1], the addition of mobile phase modifiers has a dramatic impact on retention and enantioselectivity, and plays an important role in any enantioseparation process. While trifluoroacetic acid (TFA) shows excellent UV transparency, basic modifiers in normal-phase gradient HPLC lead to a severe drifting of the baseline as well as to a lower sensitivity at low wavelengths. This was one of the main reasons why TFA was selected as standard modifier for routine analysis of any structural type of compounds (neutral, basic or acidic). Unlike traditional HPLC (UV/DAD) chiral analysis, in which pure isomeric mixtures are required for unambiguous identification, the HPLC/MS approach has the potential of reducing significantly the effort

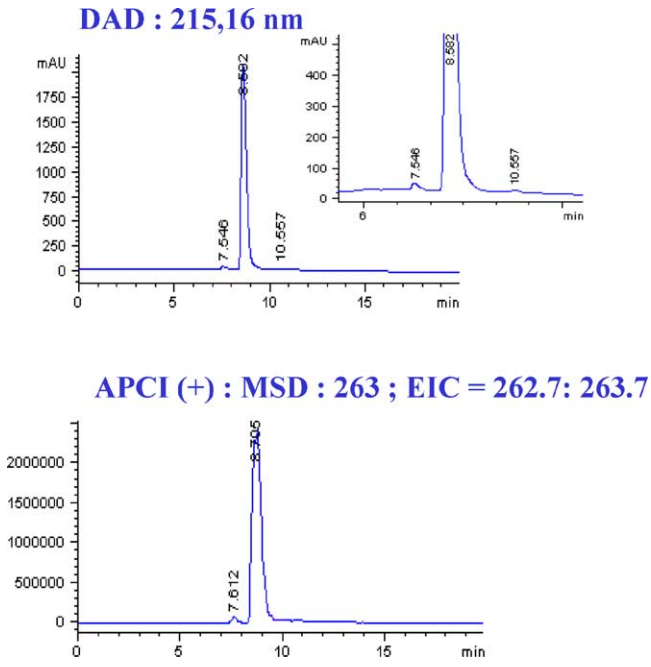

Fig. 3. Chiral purity determination of a pharmaceutical intermediate prepared in our Drug Discovery Research Laboratories by standard screen under acidic elution conditions. Chromatographic conditions: column, Chiralcel OJ; mobile phase, Hexane-TFA 0.05% (A)/IPA (B); elution mode, gradient from 20 to 60% (B) in 15 min; flow rate, 0.75 ml/min; detection, DAD 215 nm and APCI (extracted ion); *ee* (UV peak area integration) >98%.

needed for purification purposes as identification of isomers can be efficiently achieved by ion extraction. Fig. 2 illustrates results achieved by analysis of one internal compound. The name and structure are not disclosed for proprietary reasons. Undesired epimerization was easily detected without purification of the crude material but a simple silica-gel filtration.

In addition, the stability of the baseline achieved using TFA allows accurate integration of UV peaks (area) for enantiomeric excess determination under our standard gradient program. A representative example of chiral purity determination of a pharmaceutical intermediate prepared in our research laboratories is shown in Fig. 3. As above cited, the name and structure cannot be included due to proprietary reasons.

Nevertheless, for purification purposes we need to take into account not only stability issues but also the form of the final isolated species. Sometimes, degradation of the compound is observed under acidic conditions and/or the TFA

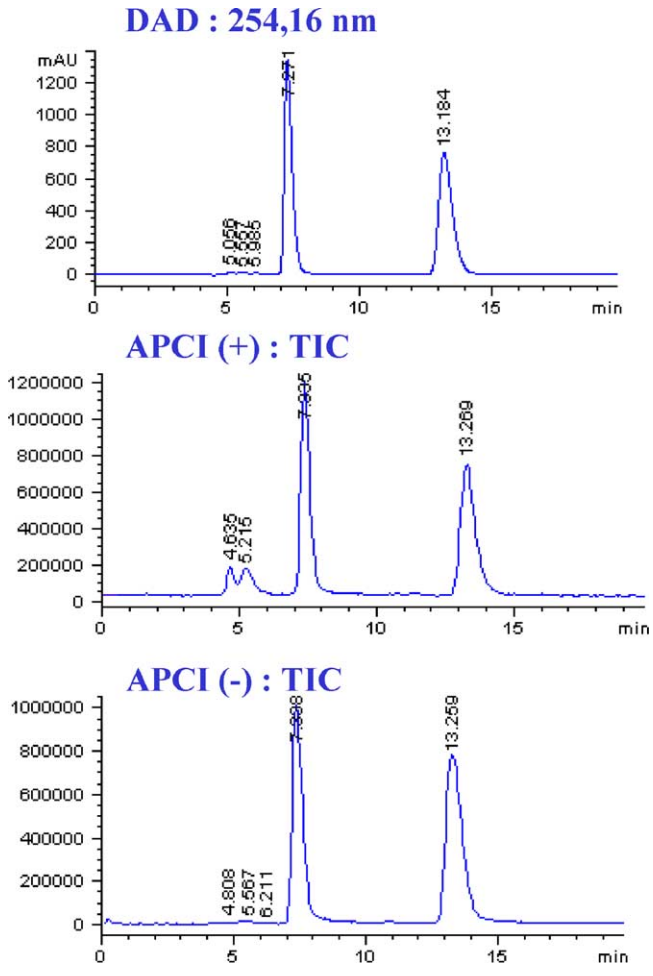

Fig. 4. Analysis of a racemic pharmaceutical intermediate prepared in our Drug Discovery Research Laboratories by standard screen under basic elution conditions. Depending on the nature of the compound, ionization can be achieved in positive and/or negative modes. Chromatographic conditions: column, Chiralpak AD; mobile phase, EtOH-DMEA 0.2%; elution mode, isocratic; flow rate, 0.75 ml/min; detection, DAD 254 nm, and APCI (TIC +/− modes).

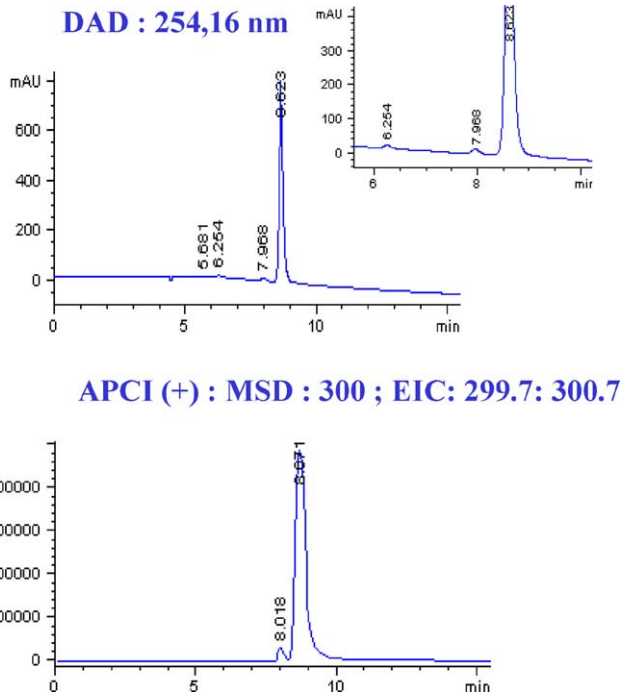

Fig. 5. Chiral purity determination of a pharmaceutical intermediate prepared in our Drug Discovery Research Laboratories by standard screen under basic elution conditions. Chromatographic conditions: column, Chiralpak AD; mobile phase, Hexane-DMEA 0.2% (A)/IPA (B); gradient from 20 to 60% (B) in 15 min; flow rate, 0.75 ml/min; detection, DAD 254 nm, and APCI (Extracted ion positive mode); *ee* (UV peak area integration) 98%.

salts are not viable for biological testing. In those cases, the use of a basic additive is required and DMEA is added to hexane in standard concentrations for routine screening. High proton affinity of basic modifiers has been reported to induce suppression of ion production [25]. We have also observed that, in some cases, ionization under basic elution conditions is poorer compared to the ionization yielded when TFA is the selected additive. However, this phenomenon is directly dependent on the chemical structure. Working under the chromatographic conditions defined in our screen, ionization has been successfully achieved on all the different pharmaceutical intermediates prepared in our laboratories that have required analysis using basic modifiers (DMEA). Figs. 4 and 5 include two representative examples. Appropriate ionization can be observed not only on racemic mixtures (Fig. 4) but also on highly enantiomerically pure species (Fig. 5).

In addition, neutral conditions can be also applied if required (i.e. unstable compounds) on those mixtures lacking ionisable groups. Removal of additives does not have impact on resolution. See Fig. 6 for chiral analysis of an internal compound under standard neutral gradient elution.

3.4. Coupling of MS detector

As we have aforementioned, some authors claim that hexane-containing mobile phases are potentially explosive

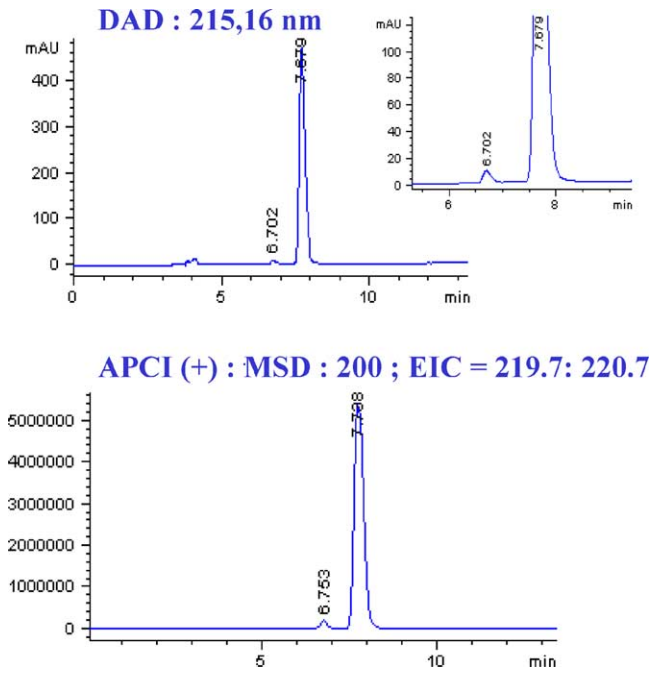

Fig. 6. Chiral purity determination of a pharmaceutical intermediate prepared in our Drug Discovery Research Laboratories by standard screen under neutral conditions. Chromatographic conditions: column, Chiralcel OJ; mobile phase, Hexane (A)/EtOH (B); elution mode, gradient from 20 to 60% (B) in 15 min; flow rate, 0.75 ml/min; detection, DAD 215 nm, and APCI (TIC + mode and Extracted ion); *ee* (UV peak area integration) 97%.

in LC–MS (APCI). Therefore, post-column solvent addition is usually implemented.

In contrast, in the last years, some scientists have reported that eluent containing up to 95% hexane–5% alcohol can be safely coupled to the API source in both positive/negative ion modes, without addition of any water-containing solvent [26]. In agreement with these results, we have to remark that there has been no evidence at all of any explosion or any related phenomenon over the 24 months that we have been working with our Agilent system, even though full flow goes into APCI interface (as it can manage flow rates in the range of 1 ml/min without splitting the effluent) without using any make-up solvent. We want to notice that nitrogen is used as auxiliary and nebulizing gas type in our MS detector.

Further on, it has been also reported that systems run under normal-phase conditions do not incorporate the ion concentrations required to achieve ionization in the APCI source, and post-column introduction of make-up mobile phase is needed to overcome the problem [24]. Working under the conditions described herein, stable low noise is achieved all through the experiment as it has been reflected in the different examples included in this work.

4. Conclusions

Hyphenation of liquid chromatography with mass spectrometry combines powerful separation methods with sensitive and selective detection techniques. Herein, we have reported that normal-phase Chiral HPLC can be successfully coupled to APCI-MS detector without post-column reagent addition. Further on, neither explosions nor lack of ionization have been detected, although traditionally described in literature. For those compounds showing poor ionization due to their nature, ion extraction provides a smooth solution to this challenge.

Following this approach, chiral purity can be accurately determined in an easy, fast and accurate manner, even on complex mixtures. When separation of two species with the same molecular weight is observed, the analysis of the corresponding pure racemic mixture can be avoided. Of course, success of the method is a function of the amount of enantiomer contained in the mixture, as well as its ionization behavior. Experience accumulated for 2 years applying this refined screen has shown that a compound with the isomeric impurity present at the 1–2% area level can be detected without any adjustment of the experimental conditions. In addition, analytical chromatographic conditions can be easily transferred for its application to semi-preparative or preparative HPLC purifications (up to 20 g), as it was reported in our previous work.

Acknowledgments

I would like to thank to my collaborators Amelia Gonzalez, Pilar Lopez and Leticia Cano, for their daily contribution to the validation of this screen. The author thanks Alfonso Espada and Joseph Kennedy (Lilly), for careful evaluation of the manuscript.

References

[1] M.L. de la Puente, C. White, A. Rivera-Sagredo, J. Reilly, K. Burton, G. Harvey, J. Chromatogr. A 983 (2003) 101.
[2] M.E. Andersson, D. Aslan, A. Clarke, J. Roeraade, G. Hagman, J. Chromatogr. A 1005 (2003) 83.
[3] I. Kartozia, M. Kanyonyo, T. Happaerts, D.M. Lambert, G.K.E. Scriba, B. Chankvetadze, J. Pharm. Biom. Anal. 27 (2002) 457.
[4] C. Perrin, V.A. Vu, N. Matthijs, M. Maftouh, D.L. Massart, Y. Vander Heyden, J. Chromatogr. A 947 (2002) 69.
[5] H.Y. Aboul-Enein, I. Ali, Il Farmaco. 57 (2002) 513.
[6] E. Yashima, J. Chromatogr. A 906 (2001) 105.
[7] H.Y. Aboul-Enein, J. Chromatogr. A 906 (2001) 185.
[8] E. Francotte, J. Chromatogr. A 906 (2001) 379.
[9] Y. Okamoto, E. Yashima, Angew. Chem. Int. Ed. 37 (1998) 1020.
[10] E. Yashima, C. Yamamoto, Y. Okamoto, Synlett (1998) 344.
[11] Y. Okamoto, Y. Kaida, J. Chromatogr. A 666 (1994) 403.
[12] J. Dingenen, J.N. Kinkel, J. Chromatogr. A 666 (1994) 627.
[13] E. Francotte, A. Junker-Buchheit, J. Chromatogr. 576 (1992) 1.
[14] T. Shibata, K. Mori, Y. Okamoto, Polysaccharide phases, in: A.M. Krstulovic (Ed.), Chiral Separations by HPLC: Applications to Pharmaceutical Compounds, Ellis Horwood Ltd., Chichester, 1989, p. 336.
[15] B. Chankvetadze, I. Kartozia, C. Yamamoto, Y. Okamoto, J. Pharm. Biom. Anal. 27 (2002) 467.
[16] B. Chankvetadze, C. Yamamoto, Y. Okamoto, J. Chromatogr. A 922 (2001) 127.

[17] M. Meyring, B. Chankvetadze, G. Blaschke, J. Chromatogr. A 876 (2000) 157.
[18] L. Miller, C. Orihuela, R. Fronek, J. Murphy, J. Chromatogr. A 865 (1999) 211.
[19] R.D. Voyksner, Electrospray Ionization Mass Spectrometry, Fundamentals, Instrumentation and Applications, Wiley, New York, 1997, p. 323.
[20] K.V. Penmetsa, C.D. Reddick, S.W. Fink, B.L. Kleintop, G.C. DiDonato, K.J. Volk, S.E. Klohr, J. Liq. Chromatogr. Relat. Technol. 23 (2000) 831.
[21] C. Miller-Stein, C. Fernandez-Metzler, J. Chromatogr. A 964 (2002) 161.
[22] C.J. Welch, B. Grau, J. Moore, D.J. Mathre, J. Org. Chem. 66 (2001) 6836.
[23] A.P. Zavitsanos, T. Alebic-Kolbah, J. Chromatogr. A 794 (1998) 45.
[24] T. Alebic-Kolbah, A.P. Zavitsanos, J. Chromatogr. A 759 (1997) 65.
[25] B. Toussaint, B. Streel, A. Ceccato, Ph. Hubert, J. Crommen, J. Chromatogr. A 896 (2000) 201.
[26] C. Li, C. Dufour, Proceedings of the 222nd ACS National Meeting, Chicago, IL, USA, August 2001.