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Fast-liquid chromatography using columns of different internal diameters packed with sub-2 μm silica particles

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ABSTRACT

Capillary columns (50, 75 and 100 μm I.D.) were packed with silica C_{18} sub-2 μm particles for 50 mm and were employed in nano-liquid chromatography (nano-LC) for fast chromatographic separations. Mixtures of nonsteroidal anti-inflammatory drugs (NSAIDs) and steroids were used as models. Separations no longer than 3 min were obtained with the three capillary columns employing a mobile phase of 35/65 (v/v) and 45/55 (v/v) ACN/H_2O in 0.1% formic acid (HFo) for steroids and NSAIDs, respectively. Among the capillary columns used, best results, in terms of retention factor and selectivity, were achieved with the 50 μm I.D. column. The same sample mixtures were analyzed by micro-liquid chromatography (μ-LC), employing a commercial microbore column Hypersil GOLD™ (50 mm × 1.0 mm I.D.) packed with C_{18} 1.9 μm particles, and working at the same linear velocity. The results were compared with those obtained with the 50 μm I.D. column in terms of analysis time, efficiency and selectivity. An evaluation of Van Deemter curves was also done for both columns. Finally both nano-LC and μ-LC methods, developed for the separation of the steroid mixture, were validated and applied to the determination of dexamethasone in commercial tablets. Relative standard deviation (RSD%), intra and inter days, of retention time were in the 0.1–1.0% and 2.3–3.5% ranges respectively, for nano-LC, while for μ-LC they were in the 0.6–1.4% and 0.9–1.6% ranges. Peak areas RSD% were also satisfactory (not higher than 6.3% for inter days values). LOD were between 0.010 and 0.040 μg/mL for nano-LC and between 0.1 and 0.5 μg/mL for μ-LC. To assess the linearity of both methods, six concentration levels were injected for three times in two different concentration ranges. Correlation coefficients (r^2) of 0.998 and 0.997 were obtained for μ-LC and nano-LC respectively. Good recovery data were also found from analysis of real samples, utilizing 11α-hydroxyprogesterone as internal standard.

1. Introduction

The need of fast separations of compounds in chromatography has seen unbelievable growth in the last few years. Several analytical laboratories, as pharmaceutical ones, put significant efforts in developing faster and more efficient analytical methods, for both qualitative and quantitative investigations. When a large number of samples have to be analyzed, even a minimal reduction in the analysis time brings benefits in all other steps of drugs production. This leads, in turn, to a faster samples screening, higher productivity rates, lower delivery times, and therefore a much cheaper procedure as a whole [1].

In High Performance Liquid Chromatography (HPLC), the combination of short columns and high flow rates is the simplest approach for reducing the analysis time [2].

Columns packed with 3–5 μm particles are generally employed in HPLC, because they can be easily conditioned, exhibiting good efficiency and a relatively low back-pressure.

The model proposed by Van Deemter [3] and Knox [4,5] shows that the use of silica particles of small diameter offers the best results for improving the chromatographic performance.

The reduction of particles diameter causes a remarkable decrease of mass transfer resistance modifying the *C* term in the Van Deemter equation. Consequently a flatter flow profile can be observed. This effect offers additional advantages for achieving optimum chromatographic performances, e.g., working at higher flow velocities without sacrificing the efficiency and thus reducing analysis time. Therefore particles with smaller internal diameter, i.e. sub-2 μm, have been introduced because capable for obtaining good analytes resolution, high efficiency and short analysis time [1,6–9]. Besides the above mentioned advantages in decreasing the particles diameter, it is worth mentioning that some drawbacks

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have to be expected, e.g. back-pressures higher than 400 bar are usually recorded [1,10]. In addition undesirable effects such as frictional heating on the mobile phase, [11] causing radial temperature gradient into the column, are generated [12].

In the last decade miniaturized versions of conventional Liquid Chromatography (LC) have been developed using columns with reduced I.D. [13]. Although so far there is no uniform classification of the different miniaturized LC, when capillary columns with I.D. 0.5–1.0 mm, 100–500 μm, ≤100 μm are employed, they are called micro-liquid chromatography (μ-LC), Capillary Liquid Chromatography (CLC), and nano-Liquid Chromatography (nano-LC), respectively.

These miniaturized techniques, as well as offering fast separations with a significant reduction in conditioning time, make use of low flow rates through the column. Consequently, one of the main advantages of setting a μL or nL/min flow in the chromatographic arrangement is the low consumption of mobile phase. Therefore, a system equipped with columns of reduced diameter is a good attempt to achieve a cheaper and also greener chromatography due to the lower environmental impact. Although some drawbacks related to low mass loadability, reduced sensitivity and higher skill in column preparation, capillary columns are widely employed. In addition, recently, they were also packed with sub-2 μm particles stationary phases to exploit advantages of both reduced column internal diameter and small particles size towards speed of analysis, efficiency and, consequently, selectivity and sensitivity.

Examples of applications using sub-2 μm stationary phases packed in 1.0 mm I.D. columns [14,15] or in smaller capillary columns have been reported [16,18].

The term fast-liquid chromatography refers to chromatographic separation obtained in a very short time, i.e. few minutes. In some reports, quite fast analyses have been obtained with 2.1 mm I.D. columns, containing sub-2 μm particles stationary phase for the baseline separation of steroids in 2 min [19–21] and non-steroidal anti inflammatory drugs (NSAIDs) in less than 4 min [21,22]. Such pharmaceutical compounds are largely utilized and therefore widely studied, i.e. for both pharmacodinamic and analytical purposes. For this reason, in addition to traditional methods of analysis, fast separative techniques should be developed.

These drugs have also been separated employing micro/nano-liter flow by either capillary electrochromatography (CEC) or nano-liquid chromatography (nano-LC), but with analysis time not too short [23–27]. To the best of our knowledge μL–nL/min flow systems combined with sub-2 μm packing material has not yet been applied to fast chromatographic studies of both steroids and NSAIDs. Therefore aim of this work is to study the usefulness of capillary columns laboratory packed with sub-2 μm C18 silica particles for fast chromatographic separations. The effect of some experimental parameters, e.g., capillary I.D. and linear velocity, on the separation of two selected analytes model mixtures (steroids and NSAIDs) was investigated. The results were compared with those achieved for the separation of the same model mixtures using a commercially available microbore C 18 sub-2 μm column of 1 mm I.D.

Finally, both μ- and nano-LC developed methods were validated and applied to the analysis of a pharmaceutical formulation containing a steroidal drug.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received without further purification. Acetonitrile (ACN), methanol (MeOH), formic acid (HFo), glacial acetic acid (HAc, >99%) were purchased from Carlo Erba (Rodano, Milan, Italy). Ammonia solution (30%, v/v) was from Riedel-de Heän (Seelze, Germany). Water was freshly prepared using a Milli-Q® system (Millipore, Bedford, MA, USA).

Some compounds of pharmaceutical interest, belonging to the family of steroids and nonsteroidal anti-inflammatory drugs (NSAIDs), were selected for this study. The following neutral compounds were purchased from Sigma–Aldrich (St. Louis, MO, USA): triamcinolone, (9-Fluoro-11,16,17,21-tetrahydroxypregna-1,4-diene-3,20-dione); prednisolone, min. 99%, ((11β)-11,17, 21-trihydroxypregna-1,4-diene-3, 20-dione); cortisone, min. 98%, (17α-21-dihydroxy-4-pregnene-3,11,20-trione); dexamethasone, min. 98%, ((11β, 16α)-9-fluoro-11, 17, 21,-trihydroxy-16-methylpregna-1, 4-diene-3, 20-dione); corticosterone, min. 98%, ((11β)-11, 21-dyhydroxypregn-4-ene-3, 20-dione); triamcinolone acetonide, (9-fluoro-11, 21-dihydroxy-16,17–[1-methylethylidenebis(oxy)]pregna-1,4-diene-3,20-dione); 11α-hydroxyprogesterone, min. 95%, (11α-hydroxy-4-pregnene-3,20-dione); cortisone 21-acetate, min. 99%, (17α, 21-dihydroxy-4-pregnene-3,11,20-trione 21-acetate).

Indoprofen, ketoprofen, fenoprofen, flurbiprofen, ibuprofen were obtained from Sigma–Aldrich (St. Luis, MO, USA), while cicloprofen was kindly supplied by Dr Cecilia Bartolucci (Istituto di Cristallografia, C.N.R. Montelibretti, Italy). DF2008Y (2-(3′-carbossiphenyl) propionic acid), DF2107Y (2-(3′-carbossiphenyl) propionitrile), DF1770Y (2-[(4′-benzoyloxy-2′-hydroxy)phenyl]propionic acid) were kindly provided by Dompè (L'Aquila, Italy).

Fig. 1a and b shows the chemical structures of the studied compounds.

Decadron® tablets (Visufarma S.p.A., Rome, Italy), containing 0.5 mg of dexamethasone, were analyzed by nano-LC and μ-LC. The mobile phases were daily prepared by mixing appropriate volumes of ACN and water containing 0.1% (v/v) HFo.

2.2. Instrumentation

An Accela® HPLC pump (Thermo Electron Corporation, St. Josè, CA, USA) was used to deliver the mobile phase at both micro or nano-flow. Nano-flows, in the 40–500 nL/min range, were obtained using a passive mechanical split. The split system was connected with an injection valve (Sepaserve, Munster, Germany); a 50 μL loop was used for both sample injection and mobile phase reservoir.

The split system was realized using a stainless steel T piece (Vici, Valco, Houston, TX, USA) connected to the injector by using a stainless steel tube (5 cm × 500 μm I.D.). The other two ports of the T were tied through a fused silica capillary (70 cm × 50 μm I.D.) and a peek capillary tube (50 cm × 130 μm I.D.) with the pump and waste, respectively. The pump continuously delivered acetonitrile into the injection valve containing the selected mobile phase mixture.

The mobile phase was loaded time to time considering flow rate and analysis time. In order to minimize band broadening caused by dead volumes, the capillary column was directly connected to the injector.

The flow rate was estimated by measuring the volume of the mobile phase coming out from the column in a fixed time. In this respect, a 10 μL syringe was connected to the outlet of the column by a Teflon tube (TF-350; LC-Packings, CA, USA).

For experiments performed with 1 mm I.D. column, the pump was directly connected with the injection valve; the mobile phase, delivered in isocratic mode, was pre-mixed using Xcalibur™ 1.3 (Thermo-Finnigan) software. The column was connected to the injector valve by capillary PEEK tube (5 cm × 130 μm I.D.). Optimum separation flow rate was 100 μL/min.

Detection was done by using a Spectra 100 UV detector from Thermo Separation Products (St. Josè, CA, USA).

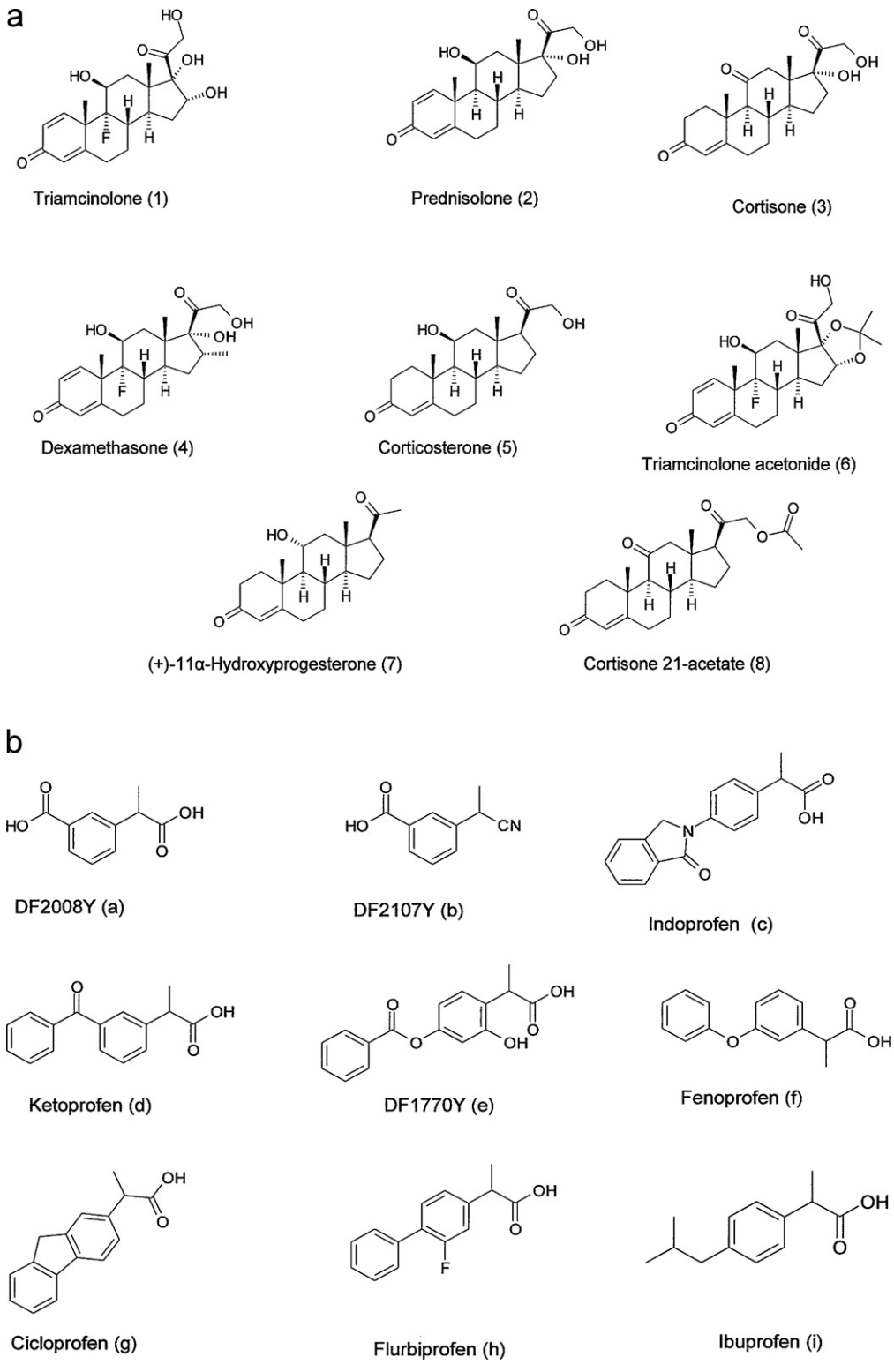

Fig. 1. (a) and (b) Chemical structures of studied compounds.

In nano-LC, the detection window was prepared directly on the capillary column, removing the polyimide layer, at about 2 cm from the outlet frit; in μ-LC the outlet end of the column was joined to an empty silica capillary (100 μm I.D.) and at 10 cm was prepared the detection window, as previously described.

Detector operated at 254 and 200 nm for the analysis of steroids and NSAIDs, respectively; rise time and data rate were set at 0.5 s and 20 Hz respectively.

The data from detector were acquired with ChromQuest version 3.0 software (Thermo Finnigan, St. Josè, CA, USA).

The Van Deemteer's plot and A, B, C values were calculated by using Curve expert 1.38 from Microsoft Corporation [28].

2.3. Chromatographic columns

Experiments were carried out utilizing columns differing for the I.D. and for the brand of sub-2 μm particle stationary phases.

Hypersil GOLD ™ (50 mm × 1.0 mm I.D.) packed with C_{18} (1.9 μm, pore size: 175 Å) purchased from Thermo Fisher Scientific and three laboratory packed fused silica capillary columns (50 mm × 0.100, 0.075, 0.050 mm I.D.) containing hydride-based RP-C18 (1.8 μm, pore size 100 Å) were used.

The latter stationary phase was a silica bidentate C_{18} material manufactured starting from a high purity silica and converted to silica hydride surface (Type-C™) (Microsolv Technology, Eatontown, NJ, USA). It has (i) high stability at extremes pH, (ii) high temperature resistance and (iii) extremely low silanol activity, with a 16% of carbon load [24].

2.3.1. Capillary columns preparation

In nano-flow experiments, fused silica capillaries (100, 75, 50 μm I.D. × 375 μm O.D.), purchased from Metal Composite Metal Services (Hallow, Worcestershine, UK), were packed in our laboratory following a packing procedure previously described [27,29] As reported in literature [17], the main problem raising from the packing of small diameter silica particles, that is a very high back-pressure, is generally avoided utilizing special packing pumps and low viscosity solvents for slurry preparation (acetone or hexane).

However these problems have not been met when preparing short columns (50 mm), and it is precisely for this reason that it was possible to use a conventional LC pump for the packing procedure.

10–15 mg of stationary phase were suspended in 1 mL of acetone and used as the slurry; the packing solvent was methanol. A Perkin-Elmer series 10 LC-pump (Palo Alto, CA, USA) was used for packing or flushing the capillary columns.

The two frits (inlet and outlet) were prepared by heating at 700 °C for 5–10 s with a laboratory made heated wire, while flushing continuously the capillary with a 5 mM NaCl aqueous solution. A detector window was prepared by removing about 0.5 cm of polyimide layer on the capillary wall with a razor.

The packed columns were cut at desired length and equilibrated with mobile phase.

2.4. Chromatographic conditions

All experiments were carried out in isocratic mode. Based on our experience and on the data reported in literature [20,22,24,29] mixtures containing ACN and water with 0.1% HFo, at different ratio (30–60% ACN in water), were tested in order to find optimum conditions and to achieve the highest resolution of analytes in the shortest time. Working with the nano-LC system, best results in terms of fast separation were obtained with a mobile phase composition of 35/65 (v/v) and 45/55 (v/v) ACN/H_2O in 0.1% HFo for steroids and NSAIDs respectively. The same mobile phases were then used in the μ-LC system in order to compare both techniques.

2.5. Standards and sample preparation

The stock standard solutions (1 mg/mL) were prepared by dissolving appropriate weighted amount of the studied compounds in MeOH; the injected mixtures were daily prepared after suitable dilution of stock standard solutions with deionized water to the desired concentrations.

Ammonium acetate buffer solution was prepared by titrating the appropriate volume of acetic acid (500 mM) with 1 M ammonia at pH 4.5 and diluting with water. Four tablets of Decadron®, each one containing 0.5 mg of dexamethasone, were weighed, crushed to fine powder, accurately mixed and divided in four portions.

Samples were suspended to a final volume of 4 mL with ACN. The suspension was mixed with vortex for 1 min and ultrasonicated for 10 min and then centrifuged at 2500 rpm (10 min). Finally, 11α-hydroxyprogesterone (selected as internal standard, I.S.) was added to the supernatant, and the mixture was diluted 100 or 1000 times with water (0.5 and 5 μg/mL final concentration of I.S. respectively), and injected into the chromatographic system.

The extraction/dissolving mixture was also injected to verify that no interferences occurred during analyses.

Samples were also spiked with dexamethasone at three different concentration levels for recovery studies.

All standards and buffer solutions were stored at −20 and +4° C respectively.

3. Results and discussion

A discussion, focused on columns packed with particles of reduced diameter, is often associated with the terms such as high-speed, ultrahigh performance, ultra-high pressure liquid chromatography. These are in fact the main aspects that characterize the new trend of utilizing sub-2 μm particles in liquid chromatography.

Nano-LC and μ-LC offer fast analyses without incoming in high back-pressure and for this reason both techniques were selected for the current investigation.

Initially, a considerable attention was devoted to verify if the lab-made system for micro/nano-LC (6000 psi maximum pressure tolerated), developed for columns packed with traditional particles size (3–5 μm), was suitable for studies on sub-2 μm stationary phases, such as Hypersil GOLD™ and hydride-based RP-C18, 1.9 and 1.8 μm particle size, respectively. Due to the fact that Hypersil GOLD™ was not available in bulk, a stationary phase with similar characteristics (hydride-based RP-C18) was selected for packing capillary columns.

Using mobile phases exceeding 50% in water content and delivered at about 100 μL/min flow rate in the 1 mm I.D. column, and at some hundreds nL/min in 50, 75, 100 μm I.D. columns, a maximum of 250 bar (3600 psi) pressure was recorded during the overall analyses. Such low backpressure values (comparing with other commercial columns for UPLC analyses) are reported also in the Hypersil GOLD™ (50 mm × 1.0 mm I.D.) data sheet, working with similar mobile phase.

Preliminary results of the separation of a mixture of two pharmaceutical model compounds (one mixture of steroids and one of NSAIDs) were characterized by high chromatographic performance and reduced analysis time, not exceeding 2.5 min.

The chromatographic system was capable of taking the advantages of reduced particle size and short analysis time without occurrence of high back pressure.

The reasons of this behavior, which appears as an exception in sub-2 μm particles issue, are likely to be found in two intrinsic aspects of the columns: its length and the structure of the stationary phases.

Very short columns are the perfect candidate for getting fast analysis with low backpressures as demonstrated by Darcy's law (see Eq. (1)) reporting a linear correlation between column length and pressure:

$$\Delta P = \frac{u \cdot L \cdot \eta \cdot \phi}{d_P^2} \quad (1)$$

where ΔP is the pressure drop, L is the column length, η the mobile phase viscosity, and Φ the flow resistance [1].

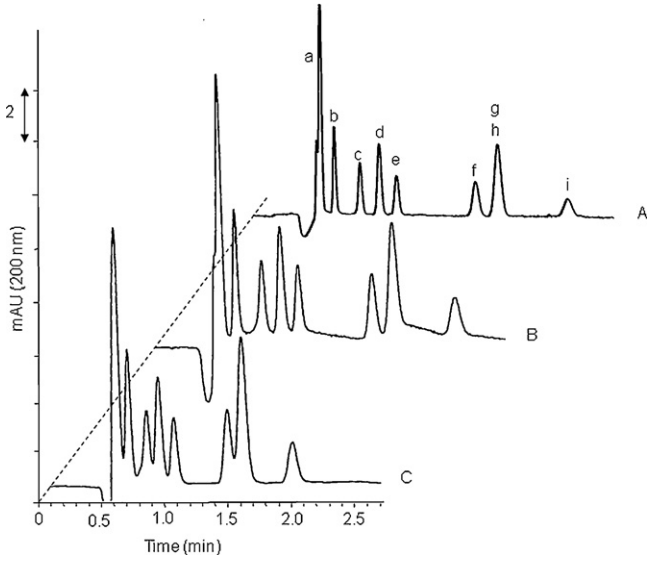

Fig. 2. Comparison of nano-LC separation of NSAIDs obtained by using capillary column with different I.D. packed with C_{18} Hydride Based silica (1.8 μm) (Lpack = 5.0 cm, Leff = 7.0 cm). *Experimental conditions*: mobile phase, 45:55 (v/v) ACN/water 0.1% HFo, injection volume, 100 nL, detection wavelength, 200 nm, room temperature (25 °C); sample, 10 μg/mL diluted in water, (a) DF2008Y, (b) DF2107, (c) indoprofen, (d) ketoprofen, (e) DF1770Y, (f) fenoprofen, (g) cicloprofen, (h) flurbiprofen, and (i) ibuprofen. Capillary column: (A) 50 μm I.D.; flow rate, 0.3 μL/min; (B) 75 μm I.D.; flow rate, 0.8 μL/min; (C) 100 μm I.D.; flow rate, 1.3 μL/min.

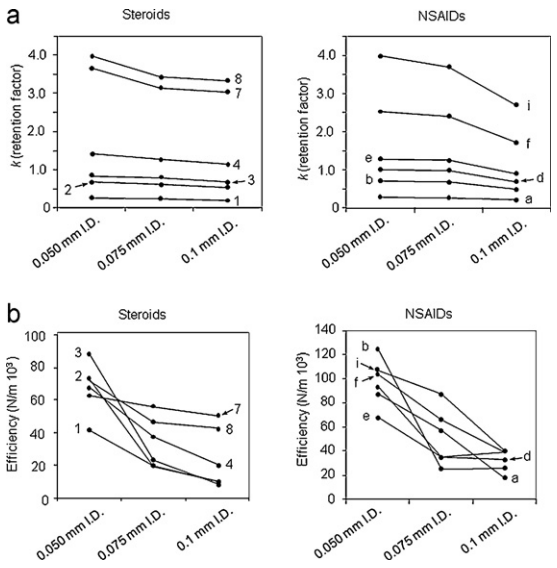

Fig. 3. (a) and (b) Effect of capillary internal diameter on the (a) retention factor, (b) chromatographic efficiency of studied compounds (steroids and NSAIDs). For experimental condition see Fig. 2.

Regarding the structure of the stationary phases, the Hypersil GOLD™ and hydride-based RP-C18 make possible to obtain columns with a resistance coefficient in lower than that of other commercial columns packed with sub-2 μm particles [24,30,31]. The reduced column internal diameter (1.0 mm I.D. and 50–100 μm I.D.) and the relative low flow rate, were not considered as a possible cause of the low backpressure. In fact, taking into account the Darcy's law, the pressure is a function of the linear velocity of mobile phase and does not depend on internal diameter and flow rate in the column.

3.1. Effect of column internal diameter in nano-LC and comparison with μ-LC

For investigating the effect of the column internal diameter on the chromatographic separation (especially considering retention factor and efficiency), three capillary columns, with an I.D. of 50, 75 or 100 μm, were packed with hydride-based RP-C18 particles for a length of 50 mm.

In the optimized experimental chromatographic conditions for each class of drugs (steroids and NSAIDS), the flow rate was adjusted in order to obtain about the same linear velocity.

In the range of 300–1200 nL/min (depending on the I.D. of the column) for steroids and 250–1300 nL/min for NSAIDs, the highest back pressures observed were about 200 and 150 bar respectively.

Fig. 2 shows the NSAIDs analytical separation achieved by using the three different columns. As observed, retention time of all studied NSAIDs decreased with bigger capillary I.D.

As Fig. 3a shows that, in general, an increase of internal diameter led to a reduction of retention factors. This trend was more remarkable in NSAIDs separation, also leading to shorter analysis time, even 20% less, and loss of chromatographic resolution.

The reason was probably due to the higher flow rate applied to the 75 and 100 μm I.D. columns.

The packing procedure and the frit preparation have an important role in changing the flow resistance through the column.

During the columns preparation, frits were more fragile for bigger I.D. column. Using 150 μm I.D. capillary, the stationary phase could not be retained inside. Frits prepared on 50 μm I.D. columns were more robust and far less permeable than those of 75 and 100 μm I.D. The data reported in Fig. 3b clearly shows an increase of column performance decreasing the capillary internal diameter. This phenomenon was described and explained by Kennedy and Jorgenson [32] and Jorgenson et al. [18,33]. They reported that, decreasing the column I.D., the non-homogeneity of mobile phase flow paths was strongly reduced due to more uniform cross-sectional packing structure. Furthermore, this effect guaranteed low peak dispersion and reduced column band broadening.

In conclusion, an higher density of packing material could suggest better packing structure and clarify both increased retention factors value and better performance of 50 μm I.D. column.

Because of the satisfactory results, the 50 μm I.D. packed capillary column was selected for further experiments.

The laboratory-made chromatographic system, previously used for nano-LC experiments, was reassembled to perform experiments in micro flow mode, simply removing the passive split system.

This new chromatographic configuration showed an extra column volume not present in the previous one, due to the insertion of a PEEK tube between injector and column.

Working at mobile phase flow-rates in the range 50–100 μL/min, the dead volume was estimated to be about 700 nL. Therefore the contribution of this extra column volume to the band broadening was considered negligible.

The new instrumental configuration was applied to chromatographic separation of selected steroids and NSAIDs mixtures by using a 1 mm I.D. × 50 mm column packed with C_{18} 1.9 μm particles, Hypersil GOLD™ and the same mobile phase optimized for nano-LC experiments.

Injection volume of the standard mixtures was about 2 μL (1 s of injection time).

The reliability of the analytical instrumental design and the performance of micro bore column were compared to the one of the 50 μm I.D. column packed with a stationary phase with similar properties and with the same packed length.

In order to obtain the same linear velocity of about 1.8 μm/μs, used with the 50 μm I.D. column, the flow rate was set

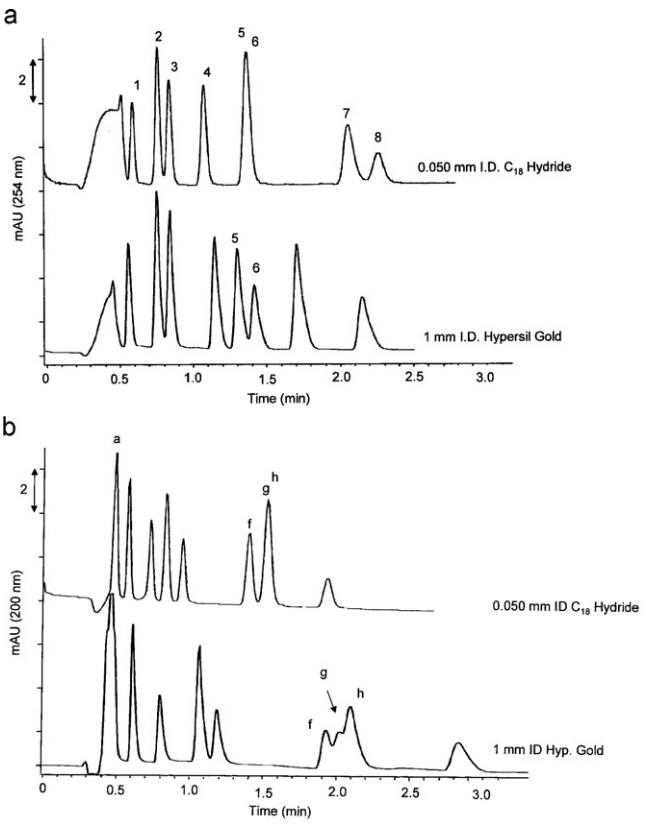

Fig. 4. (a) and (b) Comparison of nano-LC and μ-LC (1 mm I.D. Lpack = 5.0 cm with C18 Hypersil GOLD™) separation of mixture of studied compounds: (a) steroids and (b) NSAIDs. *Experimental conditions*: (a) mobile phase: 35:65 (v/v) ACN/water 0.1% HFo, detection wavelength: 254 nm, room temperature (25 °C); sample: 10 μg/mL diluted in water. (1 – triamcinolone, 2 – prednisolone, 3 – cortisone, 4 – dexamethasone, 5 – corticosterone, 6 – triamcinolone acetonide, 7 – (+)-11α-hydroxyprogesterone, 8 – cortisone 21-acetate.) For 1 mm I.D. column: inj volume, 8 μL, flow rate 100 μL/min. for 50 μm I.D. column: inj volume, 100 nL, flow rate 380 nL/min; and (b) mobile phase: 45:55 v/v ACN/water 0.1% HFo, detection wavelength: 200 nm, room temperature (25 °C); sample: 10 μg/mL diluted in water. For 1 mm I.D. column: inj volume, 8 μL, flow rate 100 μL/min. For 50 μm I.D. see Fig. 3.

at 100 μL/min resulting in a backpressure not higher than 215–230 bar.

Fig. 4a and b shows a comparison of the chromatograms obtained by nano-LC and μ-LC.

In such conditions the chromatographic separations were performed in less than 3 and 2.5 min for NSAIDs and steroids, respectively.

Although with the 50 μm I.D. column, 8 out of 9 NSAID drugs were separated in shorter analysis time (about 2 min), the C_{18} Hypersil GOLD™ showed higher selectivity for NSAIDs and steroids: baseline resolution of corticosterone (5) from triamcinolone acetonide (6), and cicloprofen (g) from flurbiprofen (h) were obtained. The higher selectivity can be explained considering both the larger amount of stationary phase in 1 mm I.D. column and the small differences of the packing material.

The peak efficiency of both columns was calculated and then compared. The data revealed that the 1 mm I.D. column provided higher efficiency with respect to the 50 μm I.D. one, for steroids separation. Calculated plates numbers/meter were in the range 54,000–108,000 and 36,000–60,000 for 1 mm and 50 μm I.D. column, respectively.

In contrast, in the analysis of NSAIDs, the performance obtained with 50 μm I.D. column was slightly higher; the plates counted up to 68,000–84,000 while the 1.0 mm I.D. column yielded 51,000–73,000 plates/m.

Fig. 5 shows the Van Deemter plots for two steroids and two NSAIDs obtained with 1.0 mm and 50 μm I.D. column.

In NSAIDs analyses, the Van Deemter fits for ketoprofen and fenoprofen (Fig. 5a) shows a minimum plate height of 12–14 μm at 0.9 μm/ms optimal linear velocity for 1.0 mm I.D. and 6 μm at 0.5 μm/ms for 50 μm I.D. column.

As reported before, although at optimal linear velocity, the 50 μm I.D. column showed an efficiency between 160,000 and 180,000 plates/m, the hydride-based RP-C18 phase exhibited poor selectivity for cicloprofen and flurbiprofen.

Above the linear velocity, the plate height for the test compounds increased with a slope (*C* term) between 3.6–3.9 and 2.8–3.2 ms for 50 μm and 1 mm I.D. column, respectively.

A different trend has been observed when the Van Deemter plots fitted the results of the tested steroids mixture.

The minimum plate height was between 7.0–7.9 and 8.0–10.6 μm for 50 μm and 1 mm I.D. column, respectively (Fig. 5b). These plate heights corresponded to 127,000–143,000 and 94,000–124,000 plates/m for nano and micro column respectively at the optimum velocity.

These results document the higher performance in terms of efficiency of the hydride-based RP-C18 in comparison with the Hypersil GOLD™ for both studied steroids, at the experimental conditions used. This behavior can be explained considering three aspects. First, the two SP differ in their pore size, being 100 Å for the hydride-based and 175 Å for Hypersil GOLD. It is well known that, for small molecules, small pore size reduces the *C* term of Van Deemter equation, leading to an increased efficiency. The chemistry of the studied SP is also different, and probably the mobile phase optimized for nano-LC studies is not the most appropriate for the Hypersil GOLD SP. Furthermore, it is worth considering the effect of column internal diameter on efficiency. For 50 μm I.D. column, the eddy diffusion (*A* term) was estimated to be in the range 3.3–4.7 μm instead of 6.8–9.2 μm for 1 mm I.D. column. This trend is in accordance with literature [33]. The *A* term becomes smaller as the inner diameter decreases suggesting a greater uniformity of packing bed as reported in Section 3.1.

The Van Deemter plots (see Fig. 5b) showed a quite different slope for each column above the optimal linear velocity, due to different mass transfer kinetics (*C* term).

The resistance to mass transfer is influenced by column diameter and largely depends on the analyte diffusion [33]. Estimated values of *C* term for steroids were between 8.3–9.7 and 3.2–5.2 ms for 50 μm and 1 mm I.D. column, respectively.

These results clearly show that the Hypersil GOLD™ column offers both a higher mass transfer kinetic and a similar chromatographic performance for studied compounds with respect to the hydride-based RP-C18 one.

In addition, at high flow rate, the Hypersil GOLD™ column allows steroids separation with higher efficiency than the hydride-based one, mainly because Van Deemter plot is less steep (see Fig. 5b).

3.2. Method validation

Validation of the method was performed using both chromatographic techniques employing the micro- and the 50 μm I.D. columns. The experimental conditions allowing the fastest analysis (total separation time <2.5 min) were applied. Therefore steroids mixture was analyzed at linear velocity of 1.7–1.8 μm/ms and parameters such as limit of detection (LOD), limit of quantification (LOQ), repeatability of retention time and peak areas, linearity were evaluated.

The use of columns with internal diameters in the millimeters diameter range shows a higher tolerance towards extra-column

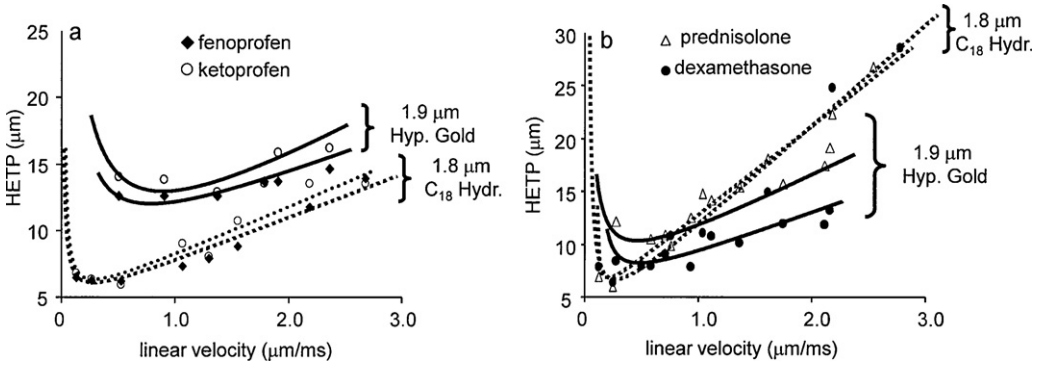

Fig. 5. Van Deemter measurements for 1.8 μm C_{18} Hydride Based (50 μm I.D. column) and 1.9 μm C_{18} Hypersil GOLD™ (1 mm I.D. column) in chromatographic separation of selected (a) NSAIDs and (b) steroids. For experimental conditions see Figs. 2 and 4.

band dispersion and allows a higher mass loading capability compared to capillary columns.

This aspect was evaluated comparing the LOD and LOQ values for all target compounds (steroids).

With injection sample volumes of 100 nL and 8 μL for 50 μm and 1.0 mm I.D. columns, respectively, usually applied in these cases, the sensitivity of the methods was in the order of ppm, e.g., LOD and LOQ were in the 0.3–1.5 μg/mL and 1.0–4.0 μg/mL ranges for commercial column, 0.4–1.4 μg/mL and 3.0–5.0 μg/mL for the 50 μm I.D. column, respectively.

Although the μ-LC system showed a slightly higher sensitivity than the capillary one, such LOD and LOQ values were not satisfactory compared with other results present in literature.

It is well known that the sensitivity of a certain chromatographic method can be improved applying a large-sample volume injection. However this approach has to be done selecting appropriate experimental conditions, avoiding sample overloading into the column and maintaining high chromatographic efficiency and resolution. Therefore on-column focusing technique was selected for next experiments [34,35].

The sample was diluted in a solvent with lower eluent strength than the one used as mobile phase. Consequently analytes were concentrated as a narrow band onto the top of the column.

The choice of the appropriate solvent was done dissolving the steroids sample in mixtures of ACN and water at different ratios.

Injecting a high sample volume (20 μL for 1.0 mm I.D. and 0.8 μL for 50 μm I.D.), pure water emerged as the most appropriate solvent, because it did not negatively affect column efficiency.

By controlling the flow rate and the injection time of the modified injector, the sample volume was increased in the range; 0.1–4.4 μL (15–660 s) for nano-LC and 8.0–75.0 μL (5–45 s) for μ-LC.

To find the maximum sample volume that could be injected without compromising the chromatographic performance of the separation system, the effect of the injected volume on peak height and width at half height was studied (see Fig. 6).

These results clearly show that the increase of the injected volume caused the raising of the ratio of peak height (H) and width at half height ($w_{1/2}$) of all studied compounds reaching a maximum at 500 and 30 s injection using capillary and micro-column, respectively.

Because a linear trend was observed, due to a constant value of the $w_{1/2}$ term in the considered range, any band broadening effect could be considered negligible.

A further increase of injection time produced a decrease of $w_{1/2}$ value, resulting in poor chromatographic separations.

The highest peak height and best efficiency was found at about 41.6 μL (25 s) and 3.6 μL (540 s) for 1.0 mm I.D. and 50 μm I.D. column respectively.

LOD values were in the range 0.010–0.040 μg/mL and 0.1–0.5 μg/mL, whereas LOQs were 0.06–0.2 μg/mL and 0.4–1.0 μg/mL for nano- and μ-LC, respectively.

The results clearly show that, although the 1 mm I.D. column had higher mass loadability, the on-column focusing was not so effective as for the capillary column.

The above described sample enrichment technique offered the capability to increase about 50 times the sensitivity using capillary columns. Only a small gain (3–4 times) was instead obtained for the 1 mm I.D. column.

The reason was likely due to the difference between injected volume with and without on-column focusing: the volume was 36 and 5 times higher for 50 μm and 1 mm I.D. column, respectively when on-column focusing was applied.

To explain these results it is worth noting that, using appropriate experimental conditions, on column-focusing is characterized by low longitudinal diffusion, that makes possible to obtain a narrow injection plug. Moreover a radial diffusion may also occur. Most probably, increasing the column I.D., the latter effect become more remarkable causing a distortion of injection plug and thus rising band broadening.

The analytical precision (RSD) was evaluated injecting the standard mixture of steroids (1 μg/mL) per six times (within-day precision) over two days (between-day precision) in terms of retention times and peak area.

The intra and inter days data for retention time were 0.1–1.0% and 2.3–3.5% for nano-LC, while 0.6–1.4% and 0.9–1.6% were calculated for μ-LC.

Acceptable results were also obtained for peak areas. The intra and inter-days RSD% values were in the 3.8–5.5% and 4.4–6.3% range for 50 μm I.D. capillary, 3.0–5.0% and 4.7–5.9% for 1.0 mm I.D. column.

The linearity was verified in both chromatographic systems at different concentration ranges. Six concentration levels of steroids mixtures were injected for three times. By using the internal standard method (11α-hydroxyprogesterone at 5.0 and 0.5 μg/mL for micro- and nano-LC respectively), the calibration curves were obtained plotting the peak area ratio as function of analytes concentration. The curve was estimated by means of the least-square method. All parameters are reported in Table 1.

4. Application

Once the μ- and nano-LC optimized methods were validated, they were applied to real sample analysis, exactly, for

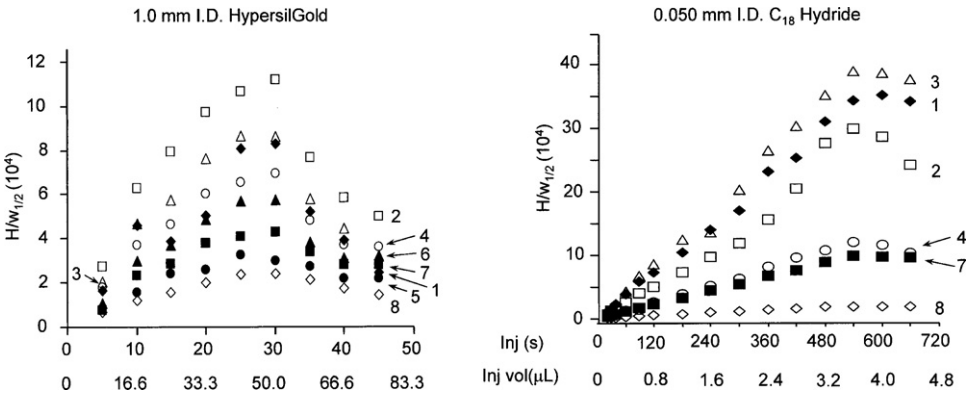

Fig. 6. The effect of the injection volume (injection time) on the peak height and peak width ratio of steroids. ♦ – triacinolone, □ – prednisolone, △ – cortisone, ○ – dexamethasone, ● – corticosterone, ▲ – triamcinolone acetonide, ■ – (+)-11α-hydroxyprogesterone, ◊ – cortisone 21-acetate. For experimental conditions see Figs. 2 and 4.

Table 1
Calibration data obtained for dexamethasone analysis.

	1.0 mm I.D. Hypersil GOLD[a]	0.050 mm I.D. C_{18} Hydride[a]
Points	6, $n = 3$	5, $n = 3$[b]
Linear regression Eq.	$y = 0.248x + 0.041$	$y = 1.248x + 0.029$
SD for the slope (Sa)	0.006	0.031
SD for the intercept (Sb)	0.051	0.032
Correlation coefficient (r^2)	0.998	0.997
Concentration range (μg/mL)	0.5–10.0	0.06–1.00

[a] Injection volume: 41.6 μL for 1 mm I.D. column; 3.6 μL for 50 μm I.D. column. (+)-11α-hydroxyprogesterone (S.I.): 5 μg/mL for 1 mm I.D., 0.5 μg/mL for 50 μm I.D., for experimental conditions see Figs. 2 and 4.
[b] $n = 3$, number of injection for each point.

the determination of dexamethasone content in a commercial pharmaceutical formulation (Decadron®).

Different extracting solvent mixtures, i.e. MeOH/water or ACN/water (50:50 v/v) and buffered mixture (50 mM NH_4Ac pH 4.5 in 25% (v/v) ACN) [36], were studied to gain the highest recovery of the drug from the pharmaceutical tablets. Pure methanol was considered the best extracting solvent.

The analysis of Decadron® tablets was performed in quadruplicate and the amount of dexamethasone was in agreement with the 0.5 mg labelled content, with a recovery of 97.5 ± 7% for μ-LC and 97.8 ± 9% for nano-LC method.

5. Conclusions

Fast chromatographic separations (less than 3 min) were obtained for two different model mixtures, nine NSAIDs and eight steroidal drugs, utilizing nano-LC as well as μ-LC. Analysis was carried out in columns with different I.D. (0.050, 0.075, 0.100 or 1.0 mm), packed for 50 mm with sub-2 μm particle size stationary phases.

Interestingly in both systems, no high backpressures were observed, contrary to what expected for so small particle size. This phenomenon, correlated to the short packing, to the splitting system in nano-LC, and to peculiar structure of the stationary phase, allowed the use conventional HPLC pump (6000 psi maximum tolerated pressure) to deliver mobile phase in the lab-made system for μ/nano-LC.

Data obtained in nano-LC were compared with those obtained in μ-LC working with the same mobile phase and at the same linear velocity. The methods developed for steroids analysis were also validated and applied for the determination of dexamethasone in a pharmaceutical formulation. Sensitivity of the μ- and nano-LC systems was increased by the on-column focusing technique, and good values of LOD and LOQ were achieved.

Both μ- and nano-LC systems showed satisfactory results, demonstrating once again, the important role of miniaturized chromatographic techniques in the analytical field, as alternative to conventional ones, i.e. HPLC or GC. Furthermore they demonstrated advantageous for fast analyses, because of the acceptable backpressures, the low amount of mobile phase and sample needed.

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