Journal of Chromatography, 631 (1993) 79–90
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2617

Characterization of a Chiral-AGP capillary column coupled to a micro sample-enrichment system with UV and electrospray mass spectrometric detection

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ABSTRACT

A Chiral-AGP capillary column (150 × 0.18 mm I.D.) was used for the separation of enantiomers of racemic drugs at flow-rates of 1–2 μl/min. Small sample volumes (60 nl) were injected directly on to the capillary column, whereas larger sample volumes were injected by enrichment of the sample on a capillary trapping column (45 × 0.25 mm I.D.). The trapping column and the Chiral-AGP capillary column were coupled together by a switching valve. A pH and solvent micro gradient was generated in the trapping system. The micro gradient could be used to compress the migrating bands on the Chiral-AGP capillary column. Thus, large sample volumes could be injected without a significant decrease in detection sensitivity. For some compounds, 45–50% higher detection sensitivity than with direct injection was obtained by injection of large sample volumes on to the trapping column. Sample volumes as high as 20 μl could be injected using the sample-enrichment technique. This sample volume is 330 times higher than the volume injected directly on to the column. The hydrophobicity of the trapping column influences the band broadening. The best detection sensitivity was obtained using the most hydrophilic trapping columns. The low flow-rate used for the Chiral-AGP capillary column allows coupling with a mass spectrometer through an electrospray interface (ESI). The combination of the capillary chiral column, the micro sample-enrichment and the ESI–MS technique permits the analysis of chiral compounds at low concentrations with high detection selectivity and high sensitivity.

INTRODUCTION

The biochemical reactions in living organisms occur on a three-dimensional basis as a result of the asymmetric nature of the macromolecules involved in such reactions. Enantiomers of drugs can be converted by the liver enzymes at different reaction rates or they can be converted into different metabolites [1–4]. As drugs also interfere or participate in the biochemical reactions in the organism, large differences in pharmacodynamic properties can also be obtained for drug enantiomers [5–8].

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In order to be able to study the disposition of drug enantiomers in the body, analytical methods are required that permit the analysis of enantiomers present at very low concentrations in a complex matrix such as plasma and urine. It is well known that all chiral columns give lower separation efficiencies than non-chiral columns. Another feature of chiral columns is that they tend to give low selectivity for structural analogues compared with non-chiral columns. Many drugs are tertiary amines and a common metabolic pathway for tertiary amines is N-dealkylation, which means that secondary and primary amines are formed by the liver enzymes. Owing to the lower selectivity for structural analogues obtained by chiral columns compared with non-chi-

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ral columns, it can sometimes be problematic to obtain chromatograms with sufficient selectivity between enantiomers of the drug and the metabolites. This type of problem can be solved as reported by Hermansson and co-workers [9,10] for the analysis of the enantiomers of dispyramide and its monodealkylated metabolite, by coupling a short non-chiral column in series with the chiral column. The metabolite and disopyramide are resolved on the non-chiral column and are transported to the chiral column for resolution of the enantiomers.

Another approach is to use a column-switching system where the bands of interest are cut out and transported to the chiral column for separation of the enantiomers [11]. A third way to overcome the problem of lower selectivity for structural analogues is to prepare simple enantiomeric derivatives, *e.g.*, by acetylation of secondary and primary amine metabolites by reaction with acetic anhydride or by esterification of acids. The preparation, of enantiomeric derivatives affects both the chiral selectivity and the selectivity between the enantiomers and the endogenous compounds. This approach has been utilized for the analysis of the enantiomers of atenolol present in human plasma [12].

A further way to overcome the problems with the relatively low selectivity for structural analogues by chiral columns is to use a detection technique, such as mass spectrometry (MS), which gives very high detection selectivity and high sensitivity. The development of soft ionization techniques coupled with MS has been a very important breakthrough in LC–MS. One of the most promising LC–MS techniques using soft ionization is the electrospray (ES) method [13,14]. This technique has revolutionized MS with its ability to ionize high molecular mass compounds with high efficiency. A few reports have also been published on the use of the electrospray for ionization of low molecular mass compounds [15–18]. However, the electrospray interface (ESI) is not compatible with the high flow-rates used for conventional columns. Flow-rates in the range 1–5 μl/min are required, which means that capillary columns of I.D. <350 μm can be used. The drawback with capillary columns is that only very small sample volumes can be injected on to such columns, normally in the range 50–200 nl. This means that low-concentration samples cannot be handled with this type of system. Therefore, in order to be able to utilize the ESI-MS detection, giving very high detection selectivity for low-concentration samples, the handling of highly dilute samples must be solved.

This paper reports the use of a Chiral-AGP capillary column together with a micro sample-enrichment technique that can be used in a capillary column system, making possible the introduction of large sample volumes on to capillary columns and the use of ESI-MS detection. The AGP column is based on the immobilization of α_1-acid glycoprotein on silica particles. Since the development of the conventional Chiral-AGP column in 1983 [19], it has been used for the resolution of large numbers of chiral drugs from many different compound classes [20–23, and references cited therein].

EXPERIMENTAL

Columns

A Chiral-AGP capillary column (ChromTech, Norsborg, Sweden) with the dimensions 150 × 0.18 mm I.D., packed with 5-μm particles in a fused-silica capillary tube with a polyimide coating, was used.

Five different trapping columns, TrapCap C_1, C_4, C_8 and C_{18} and TrapCap AGP (ChromTech) were used. The TrapCap columns were packed with 5-μm spherical particles. The dimensions of the columns were 45 × 0.25 mm I.D.

Apparatus

UV experiments. Two HPLC pumps were used, an LKB 2150 (Pharmacia LKB Biotechnology, Uppsala, Sweden) for the capillary column and a Kontron 420 (Tegimenta, Rotkreutz, Switzerland) for the trapping column. To split the flow from the pumps, HPLC columns (100 × 4.0 mm I.D.) packed with 5-μm particles were inserted between the pump and the capillary or the trapping column, using tee connectors. The capillary and the trapping columns were connected through a Valco C6W six-port valve (Vici Valco, Schenkon, Switzerland). In order to keep the dead volume low, polyetheretherketone (PEEK) tubing of 0.13 mm I.D. was used to connect the trapping column to the valve.

In the direct injection experiments, *i.e.*, when the sample was injected directly on to the capillary col-

umn without trapping, the injector was a Valco C14W internal sample injector equipped with a 60-nl internal loop (Vici Valco). When larger sample volumes were injected and trapped on the trapping column, a Rheodyne (Cotati, CA, USA) Model 7125 injector with different external loops between 4 and 20 μl was used.

The Kontron 433 UV detector (Tegimenta) was equipped with a 90 nl capillary cell, with a path length of 20 mm.

All experimental data were collected and analysed on a Kontron (Eching/Munich, Germany) 450 MT2, data system.

Electrospray MS experiments. The HPLC system was arranged in the same way as for the UV experiments, except that the following pumps were used: for flow delivery through the capillary column, an LKB 2150, and for flow delivery through the trapping column, a Waters 600-MS (Millipore–Waters, Milford, MA, USA). For automation of the analysis a CMA 240 sample injector (CMA Microdialys, Stockholm, Sweden) was attached to the system.

The MS system consisted of a Finnigan TSQ 700 mass spectrometer equipped with an electrospray interface (Analytica, Branford, CT, USA). The capillary HPLC column was attached to the central needle of the ESI unit with capillary PTFE tubing, drawn to minimize the dead volume. The central needle of the ESI unit was held at ground potential, and was made of stainless steel with I.D. 100 μm. The central needle was adjusted to end at a length of 85 mm from the inside of the flange and to be 0.4 mm longer than the sheath liquid tubing. A stream of pure nitrogen with a flow-rate of 4–4.5 l/min [pressure 30 p.s.i. (1 p.s.i. = 6894.76 Pa)] and with a temperature of 100°C was used for evaporation of the solvent. In some instances, *e.g.*, for disopyramide, methanol was pumped as sheath liquid at a flow-rate between 0.5 and 2 μl/min, to facilitate ionization. For infusion of the sheath liquid a syringe infusion pump (Model 22, Harvard Apparatus, South Natick, MA, USA) was used.

The potential in the ESI source was adjusted to give a stable signal and was held between 3.2 and 4.3 kV. The current was typically between 10^{-7} and 10^{-8} A.

The mass spectrometer was used as a single quadrupole system with scanning on the first quadrupole. The mass range was *ca.* 200 u with a scan rate of 2 s per scan, where the ions of interest, *e.g.*, MH^+ (protonated molecular ion) were focused under 1.8 s and the remainder of the time was spent on the scan. The reason for this was to check the performance of the ionization and the possiblity of detecting other compounds.

The electrometer gain was set to 10^{-8} A/V, the electron multiplier to 1200 V and the HV dynode to −15 kV. The pressure in the analyser was typically $1.2 \cdot 10^{-5}$ Torr (1 Torr = 133.322 Pa). Under automatic overnight operation the Waters 600-MS HPLC pump was controlled by the MS data system and the CMA autoinjector from a PC, with all systems linked together.

Chemicals

(*E*)-10-Hydroxynortriptyline was obtained as a gift from Dr. Leif Bertilsson (Huddinge University Hospital, Sweden). The other samples were obtained as gifts from the manufacturers. The structures of the compounds used are shown. 2-Propanol, acetonitrile and methanol were of HPLC grade (Lab-Scan, Dublin, Ireland).

Chromatographic conditions

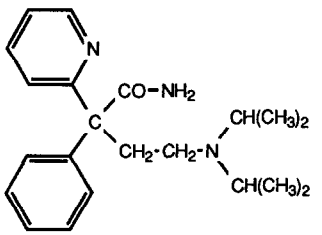
The mobile phases were prepared by adding appropriate concentrations of uncharged organic modifier to an ammonium acetate buffer or to an acetic acid solution. The buffer concentration in the mobile phases for the capillary column was 3 m*M* and that in the mobile phases used for sample enrichment on the trapping column was between 3 and 18 m*M*. The flow-rate through the capillary column was normally 1.3 μl/min and through the trapping column 10 μl/min. The flow-rate was determined using a microlitre syringe coupled to the outlet of the columns.

Injection technique

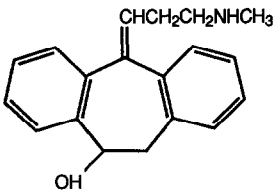
During trapping the sample is introduced into the chromatographic system in the following way.

(1) The six-port valve is in the trapping position, *i.e.*, the trapping and capillary columns are eluted with two different mobile phases (see Fig. 1). The sample is injected via the Rheodyne injector on to the trapping column. The trapping time is dependent on the loop volume and the flow-rate; when using a 4-μl loop, trapping times between 1 and 2 min were used.

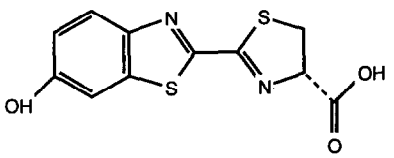
DISOPYRAMIDE



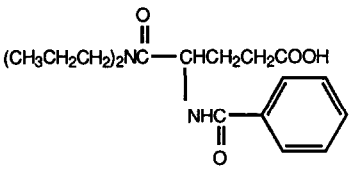
E-10-HYDROXYNORTRIPTYLINE



LUCIFERIN



PROGLUMIDE



(2) The six-port valve is switched to its elution position, *i.e.*, the trapping column and the capillary column are coupled on-line (see Fig. 1). The trapping column is back-flushed with the capillary column mobile phase and the sample is eluted on to the capillary column. The elution time is dependent on the type of sample and trapping column and on the elution strength of the mobile phase. Normally elution times between 10 and 13 min were used.

(3) The six-port valve is again switched to its trapping position for re-equilibration of the trapping column with the trapping mobile phase.

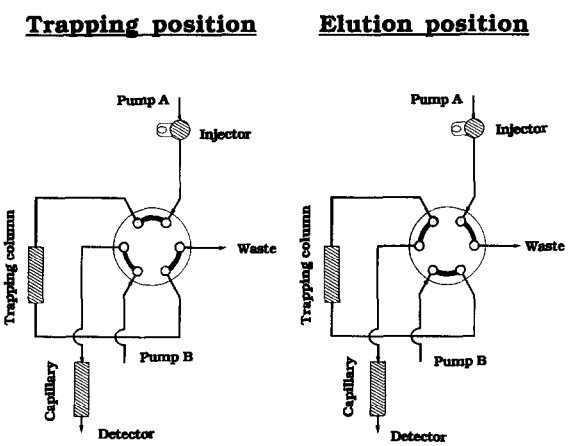


Fig. 1. Schematic illustration of the sample-enrichment system. In the trapping position the sample is loaded on to the trapping column. After switching the valve to the elution position, the trapping column is coupled on-line with the capillary column and back-flushed with the mobile phase from pump B.

Calculations

As a solvent and pH gradient is generated by the trapping system (see Results and Discussion), the band width of the peaks was not used for the calculation of the separation efficiency on the Chiral-AGP capillary column. The separation efficiency obtained with the direct injection system was compared with that with the trapping injection system by measuring the detection sensitivity obtained by the two systems. Also, the capacity factors were not calculated when using the trapping system, as this is not an isocratic elution of the compounds. Throughout the study the retention times were used.

However, the apparent plate number, N_{app}, and the apparent separation efficiency, H_{app}, were used in one experiment, calculated according to

$$N_{app} = 5.54(t_R/W_{1/2})^2$$

and

$$H_{app} = L/N_{app}$$

where L = column length (mm), and t_R = retention time (min), and $W_{1/2}$ = peak width at the half-height (min).

The resolution, R_s, is calculated according to

$$R_s = 2(t_{R_2} - t_{R_1})/(W_1 + W_2)$$

where W_1 and W_2 = base widths of the peaks (min).

RESULTS AND DISCUSSION

Sample-enrichment system

The Chiral-AGP column is based on the immobilization of α_1-acid glycoprotein on silica particles [21]. In this work we studied the Chiral-AGP (Cap-AGP) capillary column (150 × 0.18 mm I.D.) together with a new micro injection system, based on a capillary trapping column (45 × 0.25 mm I.D.), for enrichment of the sample. The capillary trapping system was studied using two types of detectors, a variable-wavelength UV detector equipped with a Z-shaped 90-nl capillary flow-cell, and the ESI-MS detector. The trapping column and the Cap-AGP column are coupled together by a six-port switching valve according to Fig. 1 and, as can be seen from Fig. 1, the sample is introduced by a conventional loop injector. The valve can be switched manually or automatically between the trapping and elution positions. The volume in the trapping column together with the tubes connecting the trapping column with the switching valve is about 5 μl, which is almost twice the volume in the Cap-AGP column. The mobile phase used for trapping is a pure buffer, without organic modifier, and with a pH that gives the sample a high affinity to the silica phase, giving a narrow sample band on top of the trapping column. When the valve is switched to the elution position, the mobile phase for the Cap-AGP column is back-flushed through the trapping column. The trapping mobile phase in the trapping column and the connecting tubes is pumped on to the Cap-AGP column. This column is equilibrated with a mobile phase, containing an organic modifier, and it has also a pH that gives a lower binding affinity of the solute to the packing material. Depending on the nature of the modifier, the modifier concentration and the hydrophobicity of the trapping column, the elution of the sample from the trapping column can be regulated.

The initial pH and the buffer concentration on the trapping column can also be used to regulate the elution of the sample from the trapping column. The pH changes slowly to the pH of the analytical mobile phase and the speed depends on the difference in the pH and the concentrations of the two buffers. When the trapping column has reached a certain degree of saturation with organic modifier and when the pH has changed to give the solute a lower affinity, the solute starts to migrate. Before the sample starts to migrate from the trapping column, the organic modifier adsorbed on the protein phase is partly stripped off by the trapping buffer and pushed in front of the mobile phase for the Cap-AGP column, which gives an enrichment of the sample on top of the Cap-AGP column. The pH of the Cap-AGP column has also changed towards the pH of the trapping buffer, also giving the solute a higher affinity.

By using this technique, we generated both a solvent gradient and a pH micro gradient that moves through the column system. The steepness of the gradient can be changed by varying the elution time of the trapping column. By switching the valve before the trapping column has been equilibrated with the Cap-AGP mobile phase, the gradient will be steeper. The pH and the solvent gradients generated by the trapping system can compress the bands on the column, wich means that a high detection sensitivity can be obtained despite the fact that very large sample volumes are injected compared with direct injection onto the Cap-AGP column.

Fig. 2 demonstrates the resolution of the enantiomers of luciferin using trapping injection of a 4-μl sample. This is a 67 times higher injection volume than the 60-nl sample volume that is normally injected on to the Cap-AGP column by direct injection.

Recovery of sample versus elution time

The recovery of the sample from the trapping column *versus* the elution time was studied using (*S*)-luciferin. The *S*-form of luciferin was enriched on a TrapCap C_8 column using a mobile phase of 3 m*M* ammonium acetate with a pH of 4.5. After the introduction of the sample on to the trapping column, the valve was switched to the elution position and the trapping column was eluted with a mobile phase of 4% 2-propanol in 3 m*M* ammonium acetate (pH

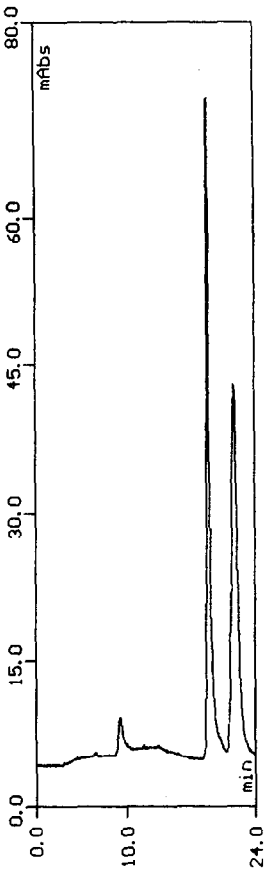


Fig. 2. Resolution of the enantiomers of luciferin. Column, Chiral-AGP capillary (150 × 0.180 mm I.D.); mobile phase, 4% 2-propanol in 3 m*M* ammonium acetate buffer (pH 6.0); flow-rate, 1.3 μl/min; trapping column, TrapCap C_8 (45 × 0.25 mm I.D.); mobile phase, 3 m*M* ammonium acetate (pH 4.5); flow-rate, 10 μl/min; sample concentration, 116 ng/ml of each enantiomer, injection volume, 4 μl; detection, UV (327 nm).

6.0). The elution time was varied between 5 and 13 min.

From Fig. 3 it can be seen that after 5 min. elution a recovery of about 40% was obtained. A 100% recovery was obtained after about 9–10 min with the mobile phases and the trapping column used in this study. This means that the sample leaves the trapping column as a broad sample zone. However, when the sample reaches the Cap-AGP column it is enriched on top of this column and the sample zone is compressed by the gradient during the migration on the column. This is demonstrated by measuring the band width of the sample zone

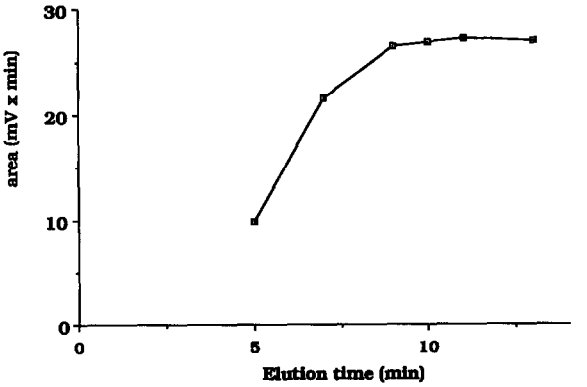


Fig. 3. Recovery of (*S*)-luciferin from the trapping column *versus* elution time. Column and mobile phases as in Fig. 2.

when it leaves the Cap-AGP column. A band width of 36 s was determined for (*S*)-luciferin presented in Fig. 3 (elution time 10 min) at a retention time of 18.5 min. The sample can be eluted faster from the trapping column by using a higher pH and higher organic modifier concentration, but the chiral selectivity decreases in general for acidic compounds with increasing pH and increasing organic modifier concentration [21,24].

Apparent efficiency versus flow-rate

The apparent plate height, H_{app}, was calculated from N_{app} as described under Experimental. In order to be able to compare the chromatograms obtained at different flow-rates on the Cap-AGP column, the samples were eluted from the trapping column for a constant time of 10 min using a flow-rate of 1.3 μl/min. In the calculation of N_{app}, this period of 10 min was subtracted from the retention time. After sample introduction, the flow-rate in the Cap-AGP column was varied in the range 0.47–1.8 μl/min, corresponding to linear velocities between 0.4 and 1.3 mm/s.

H_{app} *versus* flow-rate was studied using the same system as described above for luciferin. The results of this experiment are demonstrated in Fig. 4, where it can be seen that H_{app} increases as expected by increasing the flow-rate in this range. This is most likely caused by mass-transfer effects in the stationary phase [24]. It is interesting to note the large difference in separation efficiency for the two enantiomers. The first-eluted enantiomer gives H_{app}

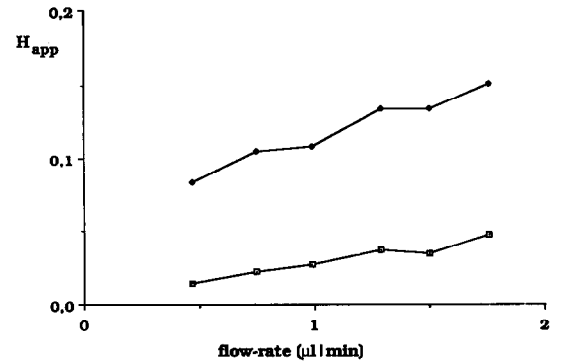


Fig. 4. Apparent separation efficiency of luciferin enantiomers *versus* mobile phase flow-rate. Columns and mobile phases as in Fig. 2. □ = *S*- and ● = *R*-enantiomers.

values between 0.015 and 0.048 and the corresponding values for the last-eluted enantiomer are 0.084–0.15. Obviously, the first enantiomer is compressed by the micro gradient, formed by the trapping system, to a greater extent than the more retarded enantiomer, which is only sligthly affected. By decreasing the flow-rate from 1.8 to 0.47 µl/min the resolution, R_s, increases from about 2.5 to 3.5. Throughout this study we used very low buffer concentrations in the mobile phases in order to make the chromatographic system compatible with the ES-MS technique. However, the low buffer concentration reduces the separation efficiency.

Influence of trapping buffer concentration on sensitivity

The concentration of the trapping buffer can be used to affect the compression effect on the migrating bands in this chromatographic system. A 40:60 mixture of the enantiomers of luciferin was used as a model compound system. The trapping buffer concentration was varied between 3 and 18 m*M* and the peak heights and the retention times were measured. The results are summarized in Table I together with the resolution, R_s. It is interesting to note the strong effect obtained on the first-eluted peak, which is highly compressed at the lowest trapping buffer concentration. A *ca.* four times higher peak was obtained at the lowest concentration of the trapping buffer, 3 m*M*, compared with that obtained at 18 m*M*. It can also be noted that the effect on the last-eluted peak was very limited. A reasonable explanation of the influence of the trapping buffer concentration on the peak height is that the gradient generated by the trapping system is not steep enough at the highest buffer concentration, owing to the resistance of this buffer to changes in pH from 4.5 to 6.0 with the weak (only 3 m*M*) mobile phase buffer. It can also be noted that a slightly higher retention was obtained using higher concentration of the trapping buffer.

Influence of large sample volumes on detection sensitivity

The trapping system allows the injection of large sample volumes on to the 180 µm I.D. Cap-AGP column. The detection sensitivity, expressed as the relative peak height obtained by direct injection of 60 nl of proglumide, was compared with the injection of a 4-µl sample by the trapping system. The sample amount injected was constant in both injections, which means that a 67 times lower concentration was injected with the trapping system. A

TABLE I

INFLUENCE OF TRAPPING BUFFER CONCENTRATION ON SENSITIVITY AND RESOLUTION

Column, Chiral-AGP capillary (150 × 0.180 mm I.D.); mobile phase, 4% 2-propanol in 3 m*M* ammonium acetate (pH 6.0), trapping column, TrapCap C_8 (45 × 0.25 mm I.D.); mobile phase, different concentrations of ammonium acetate buffer (pH 4.5); sample, (*R*)- and (*S*)-luciferin (40:60 mixture).

Buffer concentration (m*M*)	Peak height 1 (mV)	Peak height 2 (mV)	t_{R_1} (min)	t_{R_2} (min)	R_s
18	11.2	17.3	20.4	24.0	2.06
9	33.3	26.8	19.3	21.9	2.25
3	39.6	24.4	19.0	21.7	2.67

TABLE II

COMPARISON OF SENSITIVITIES USING DIRECT INJECTION AND TRAPPING

Column, Chiral-AGP capillary (150 × 0.180 mm I.D.); mobile phase, 8% acetonitrile in 3 m*M* ammonium acetate (pH 6.0); flow-rate, 1.4 μl/min; trapping column, TrapCap AGP (45 × 0.25 mm I.D.); mobile phase, 3 m*M* ammonium acetate buffer (pH 4.5); flow-rate, 11.0 μl/min; sample, proglumide.

Parameter	Direct injection	Trapping injection
Sample concentration (μg/ml)	188.0	2.8
Injection volume (nl)	60	4000
Relative peak height 1	1	0.82
Relative peak height 2	1	0.95
Peak area 1 (mV min)	19.8	20.2
Peak area 2 (mV min)	19.7	19.4

comparison of the peak heights obtained for the enantiomers in the two systems demonstrates that the detection sensitivity decreases to some extent on increasing the injection volume. The results are summarized in Table II. The first peak gives a detection sensitivity that is 82% of the sensitivity obtained by direct injection. However, the sensitivity for the last-eluted enantiomer was only slightly affected and the sensitivity for this peak was 95% compared with direct injection.

It is also possible to obtain a higher sensitivity with the trapping system compared with direct injection. This is demonstrated in Fig. 5, where the enantiomers of disopyramide were resolved after direct injection (60 nl) and by injection of a 4-μl sample by the trapping technique. The sample was enriched on a TrapCap C_4 column using a trapping mobile phase of distilled water. UV detection at 225 nm was used, which means that the disturbances of the equilibria on the Cap-AGP column can easily be detected. As can be seen from the chromatogram, the first-eluted enantiomer of disopyramide is highly compressed and is eluted as a very narrow band. This is due to the fact that the mobile phase in front of this zone retains the solute more strongly than the mobile phase behind the sample zone, therefore the zone is highly compressed. A 54% higher detection sensitivity was obtained for the first-eluted enantiomer using the trapping injection, whereas the peak height for the last-eluted enantiomer was

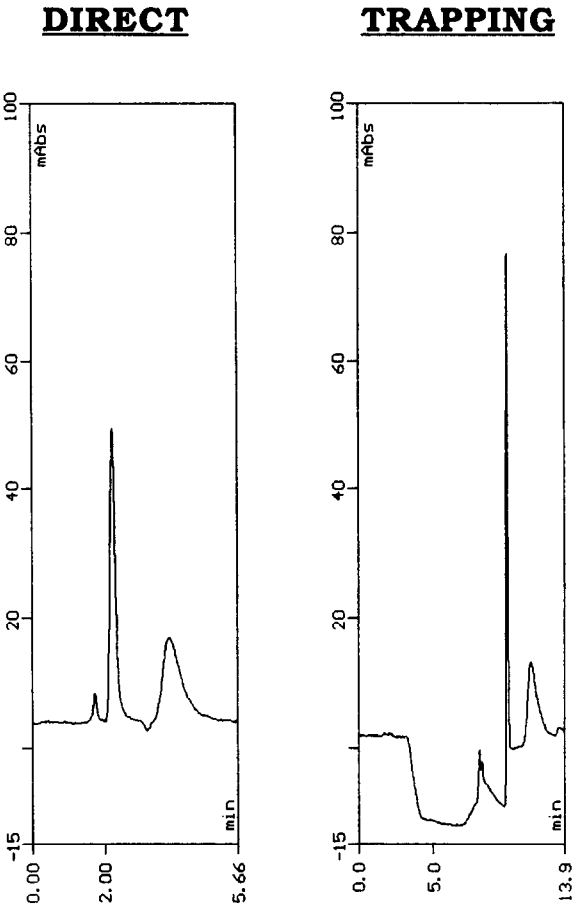


Fig. 5. Separation of disopyramide enantiomers by direct injection and by trapping. Column, Chiral-AGP capillary (150 × 0.180 mm I.D.); mobile phase, 1.2% 2-propanol in 3 m*M* acetic acid (pH 3.6); trapping column, TrapCap C_4 (45 × 0.25 mm I.D.); mobile phase, distilled water; sample concentration, direct injection 276 μg/ml (60 nl injected), trapping injection 4.2 μg/ml (4 μl injected); detection, UV (225 nm).

reduced to 83% of the that obtained by direct injection. The baseline disturbance detected by UV at 225 nm is not obtained using ESI–MS detection (see Fig. 8).

Fig. 6 shows two chromatograms for the resolution of the enantiomers of 10-hydroxynortriptyline where 4- and 12-μl samples were been injected by enrichment of the samples on a TrapCap C_{18} column. Comparison of the peak heights demonstrates that the band width is only slightly affected by the increase in injection volume from 4 to 12 μl, as relative peak heights of 0.90 and 1.02 was obtained for

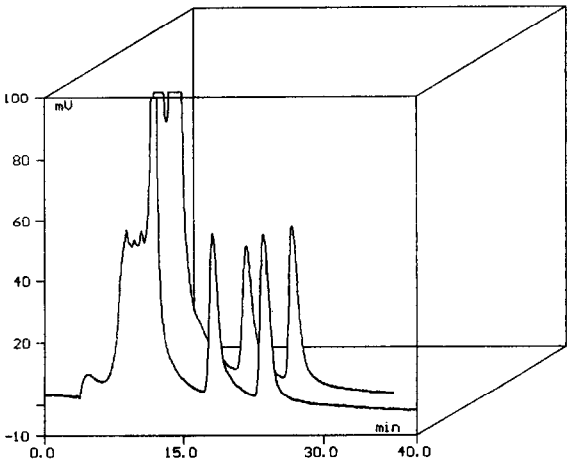


Fig. 6. Injection of large sample volumes using the trapping technique for (*E*)-10-hydroxynortriptyline. Column, Chiral-AGP capillary (150 × 0.180 mm I.D.); mobile phase, 25% 2-propanol in 10 m*M* phosphate buffer (pH 5.0); trapping column, TrapCap C_{18} (45 × 0.25 mm I.D.); mobile phase, 10 m*M* sodium phosphate buffer (pH 7.0); sample concentration, 4-μl injection (front chromatogram) of 2.5 μg/ml, 12-μl injection (back chromatogram) of 0.83 μg/ml; detection, UV (225 nm).

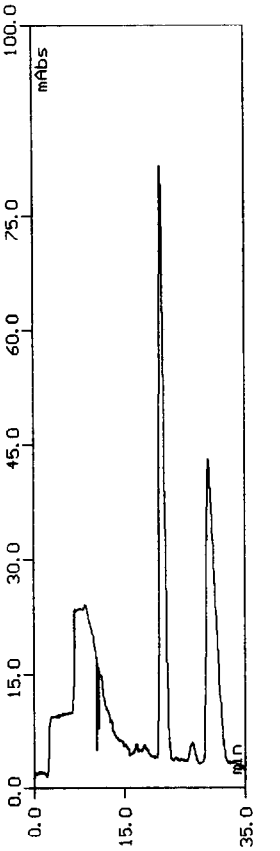


Fig. 7. Injection of large sample volumes using the trapping technique for proglumide. Column, Chiral-AGP capillary (150 × 0.180 mm I.D.); mobile phase, 8% acetonitrile in 3 m*M* ammonium acetate buffer (pH 6.0); trapping column, TrapCap AGP (45 × 0.25 mm I.D.); mobile phase, 3 m*M* ammonium acetate buffer (pH 4.5); injection volume, 20 μl; detection, UV (225 nm).

the first- and last-eluted enantiomer, respectively, on injection of 12 μl.

As has been reported above, large sample volumes can be injected by the trapping system with a slight decrease in detection sensitivity and in some instances also higher sensitivity could be obtained if the peaks are highly compressed by the micro gradient, generated by the trapping system. The possibility of injecting large sample volumes is demonstrated in Fig. 7, where a 20 μl sample of proglumide was injected by the trapping system. This injection volume is about 330 times larger than normally used in direct injection, which means that low-concentration samples could easily be analysed by this technique.

Nature of the trapping column versus chromatographic properties

The influence of the nature of the trapping column was studied using 10-hydroxynortriptyline as a model compound. Three different columns were used, TrapCap C_1, C_4 and AGP and the results were compared by direct injection of the same sample amount in 60 nl. Table III summarizes the relative peak heights, the retention times and the resolutions obtained in the different systems. The results clearly demonstrate that the nature of the trapping column influences the band broadening and the retention to a large extent. Using the most hydrophilic trapping column, TrapCap C_1, gives the lowest band broadening and thereby the highest detection sensitivity for both enantiomers of 10-hydroxynortriptyline. About a 45% higher detection sensitivity, compared with direct injection, could be obtained for both enantiomers of this compound using the most hydrophilic trapping column. The corresponding value for the C_4 and AGP trapping columns is about 20%. A higher hydrophobicity of the

trapping column gives a higher adsorption capacity of the organic modifier, which means that a less steep gradient will be generated. Hence, using a more hydrophobic trapping column gives less compression and a broader sample band. The higher affinity of the solute to the more hydrophobic column also contributes to this effect.

Quantification using the trapping system

The possibility of using the trapping injection system for quantitative work was studied by injection of (*S*)-luciferin at concentrations between 29 and 463 ng/ml. The system described in Fig. 1 was used for this study. The peak area were plotted against the sample concentration and a straight line was obtained. The linear regression equation was.

$$y = 0.2741 + 0.1248x$$

The correlation coefficient, r, was 0.9999.

Electrospray MS detection

The introduction of soft ionization techniques was a very important beakthrough for the LC–MS technique and one of the most promising technique is ESI. This technique has revolutionized mass spectrometry with its ability to ionize high molecular mass compounds with high efficiency, giving femtomole detection limits. The ESI-MS system has been utilized in very few studies for the detection of low-molecular-mass compounds [15–18]. However, previous results and this study demonstrate that this technique can be used for such solutes.

The fact that low flow-rates (1–2 μl/min) are used on the Cap-AGP column makes it possible to combine it with the ESI-MS system to give very high detection selectivity and sensitivity. Combination of the novel trapping system with the Cap-AGP column and the ESI-MS system gives the potential to determine low concentrations of enantiomers present in complex matrixes.

Fig. 8 shows a chromatogram of the enantiomers of disopyramide resolved on a 150 × 0.18 mm I.D. Cap-AGP column using ESI-MS detection. The 4-μl sample was enriched on a TrapCap C_4 column and the conditions were identical with those in Fig. 5. A comparison of the chromatograms obtained with UV detection at 225 nm and ESI-MS detection shows that disturbances of the baseline, caused by the distrubances of the equilibria on the Cap-AGP column by the micro gradient, are not observed using ESI-MS detection. Also, the first-eluted enantiomer is not compressed to the same extent as when using a UV detector. This is probably the result of band broadening due to extra-column effects obtained by transport of the sample from the column outlet to the interface. The sample is transported from the column into the ESI by a 210 mm × 100 μm I.D. stainless-steel capillary. This capillary is inserted in another capillary, forming a sheath between the two, where the sheath liquid is pumped. The sheath liquid is an organic solvent such as methanol, 2-propanol or acetonitrile and is pumped into the interface in order to give a better spray and thereby better ionization. For the diso-

TABLE III

INFLUENCE OF THE NATURE OF THE TRAPPING COLUMN ON THE CHROMATOGRAPHIC PROPERTIES

Column: Capillary Chiral-AGP (150 × 0.180 mm I.D.); mobile phase: 16% 2-propanol in 3 m*M* ammonium acetate buffer pH 6.0. Trapping columns: TrapCap C_1,-C_4 and -AGP (45 × 0.25 mm I.D.); mobile phase: distilled water. Sample: *E*-10-hydroxynortriptyline 28 μg/ml (direct injection 60 nl) and 420 ng/ml (trapping injection 4 μl).

	Direct injection (60 nl)	Trapping C_1 (4 μl)	Trapping C_4 (4 μl)	Trapping AGP (4 μl)
Rel. peak height$_1$	1	1.44	1.22	1.17
Rel. peak height$_2$	1	1.48	1.21	1.19
t_{R1} (min)	12.2	16.9	17.2	19.6
t_{R2} (min)	15.3	20.1	20.7	23.5
R_s	1.69	2.43	2.27	2.51

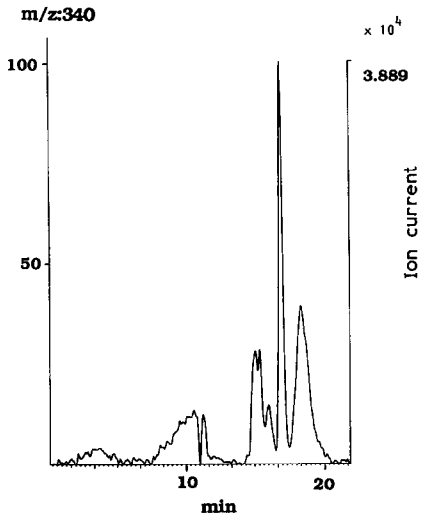


Fig. 8. Separation of disopyramide enantiomers by sample enrichment with ESI-ES detection. Column, Chiral-AGP capillary (150 × 0.180 mm I.D.); mobile phase, 1.2% 2-propanol in 3 m*M* acetic acid (pH 3.6); trapping column, TrapCap C_4 (45 × 0.25 mm I.D.); mobile phase, distilled water; sheath liquid, methanol containing 20% of the above capillary mobile phase; sheath liquid flow-rate, 1.5 μl/min; injection volume, 4 μl; sample concentration, 182 ng/ml.

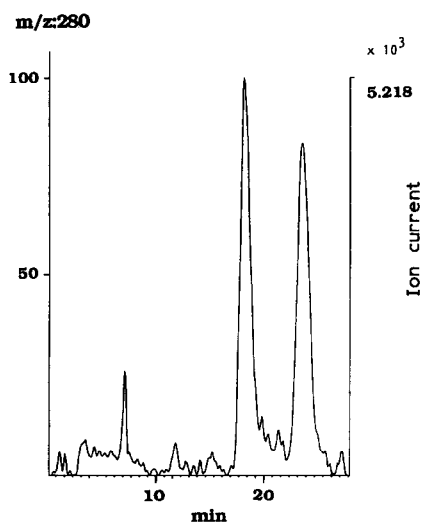


Fig. 9. Separation of (*E*)10-hydroxynortriptyline by sample enrichment with ESI-MS detection. Column, Chiral-AGP capillary (150 × 0.180 mm I.D.); mobile phase, 16% 2-propanol in 3 m*M* ammonium acetate buffer (pH 6.0); trapping column, TrapCap C_4 (45 × 0.25 mm I.D.); mobile phase, distilled water; injection volume, 4 μl; sample concentration, 200 ng/ml.

pyramide separation, methanol containing 20% of mobie phase was used as the sheath liquid, and it was pumped at a flow-rate of 1.5 μl/min.

Fig. 9 demonstrates the resolution of the enantiomers of 10-hydroxy nortriptyline using the trapping technique and ESI-MS detection. A 4-μl sample was injected and trapped on a TrapCap C_4 column. The mobile phase used for this separation contains a relatively high concentration of 2-propanol so it was not necessary to pump a sheath liquid in order to obtain ionization. However, the sensitivity could in many instances be optimized by changing the sheath liquid flow and the nature of the sheath liquid. The sheath liquid can also affect the baseline noise. By using ESI-MS detection low buffer concentrations were used to obtain ionization of the sample. The very low buffer concentration decreases the separation efficiency of the Cap-AGP column for both basic and acidic compounds. Despite this, a high detection sensitivity can be obtained.

CONCLUSIONS

A capillary sample-enrichment system was developed together with a Chiral-AGP capillary column, permitting the injection and enrichment of the sample from large injection volumes. A pH and solvent micro gradient is generated by the trapping column. The micro gradient can be used to compress the migrating bands on the Cap-AGP column, which means that high sample volumes could be injected and a higher detection sensitivity was obtained for many compounds, compared with direct injection, by injection of large volumes by the trapping system. The low flow-rates (1–2 μl/min) used on the Cap-AGP column make it possible to utilize ESI-MS for detection. A combination of the Cap-AGP column, the trapping system and the ESI-MS detector gives the possibility of analysing low-concentration samples with very high detection selectivity.

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