Journal of Chromatography, 474 (1989) 257–263
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 390

COUPLED COLUMN CHROMATOGRAPHY–MASS SPECTROMETRY

THERMOSPRAY LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC AND LIQUID CHROMATOGRAPHIC–TANDEM MASS SPECTROMETRIC ANALYSIS OF METOPROLOL ENANTIOMERS IN PLASMA USING PHASE-SYSTEM SWITCHING

A. WALHAGEN*
Technical Analytical Chemistry, University of Lund, P.O. Box 124, S-221 00 Lund (Sweden)
L.-E. EDHOLM
Bioanalytical Laboratory, AB Draco (Subsidiary of AB Astra), P.O. Box 24, S-221 00 Lund (Sweden)
and
C. E. M. HEEREMANS, R. A. M. VAN DER HOEVEN, W. M. A. NIESSEN, U. R. TJADEN and J. VAN DER GREEF
Division of Analytical Chemistry, Centre for Bio-Pharmaceutical Sciences, P.O. Box 9502, 2300 RA Leiden (The Netherlands)

SUMMARY

The applicability of phase-system switching using thermospray tandem mass spectrometry is demonstrated for the bioanalysis of the enantiomers of the betablocker metoprolol. Independent optimization of the chromatography, using an α_1-acid glycoprotein chiral stationary phase, and the mass spectrometric detection system is realized. By utilizing peak compression, 10 ng (37 pmol) of each enantiomer is easily detected in standard solutions. In plasma samples, with use of tandem mass spectrometry for additional selectivity, levels of 61 ng/ml (230 nmol/l) can be measured by using [$^2H_6$]metoprolol as internal standard.

INTRODUCTION

Stereochemical aspects are of growing importance in the development of drugs, since the pharmacological activity often resides in only one of the enantiomers[1]. As the awareness of the pharmacological and pharmacokinetic differences of enantiomers increases, there is a growing need for sensitive and selective bioanalytical methods for the determination of enantiomeric drugs in biological matrices[2]. The technique with the greatest potential for the separation of enantiomers is column liquid chromatography (LC).

Separation can be performed either directly or indirectly. Direct separation is to be preferred, and a number of chromatographic systems have been developed utilizing either chemically bonded chiral stationary phases or a chiral phases with a chiral mobile phase. Most of the systems, however, have only been applied to relatively

0021-9673/89/$03.50

uncomplicated samples at high concentrations. Application to bioanalytical work at low concentrations will not be straightforward and a number of problems can be foreseen.

In a recent paper[2], the use of coupled column chromatography (CCC) and liquid chromatography–mass spectrometry (LC–MS) has been discussed as a way to overcome some of these problems. With CCC, selective on-line sample work-up can be performed, and if combined with a heart-cutting technique, excellent selectivity can be obtained. Further work to explore the use of CCC in separation and determination of enantiomers has focussed on applications of immobilized protein phases[3], in order to extend their usage to bioanalytical work. It was shown that CCC can be used to increase the overall selectivity and the often low efficiency obtained on this type of phase. Examples were given of the separation and determination of some selected drugs in plasma, among them the beta-blocker metoprolol[4].

As was discussed earlier[2], it is often necessary to use an internal standard in bioanalytical work, but finding a suitable standard can be very difficult. When a mass spectrometer is used as a detector, this problem is less complicated because isotopically labelled internal standards can be used.

Chromatography with α_1-acid glycoprotein chiral stationary phases is usually performed with a mobile phase of aqueous phosphate buffer, which is not compatible with on-line LC–MS. Therefore, a special application of coupled column chromatography, called phase-system switching (PSS)[5], is used to perform the analysis. PSS has been developed to solve problems of mobile phase incompatibilities in LC–MS target compound analysis[6,7]. By using valve-switching techniques, enantiomers can be successively heart-cut from the chromatogram developed on the chiral column and trapped on two short trapping columns, which are washed with water to remove residual buffer constituents. In the next step the enantiomers are eluted from the trapping columns with a solvent suitable for the applied LC–MS interface, and mass analyzed.

In this paper metoprolol (Fig. 1) has been used as a model substance to demonstrate the potential of the PSS approach, in combination with chiral separation by thermospray LC–MS or LC–MS–MS. The analysis of metoprolol enantiomers in plasma was carried out using deuterium-labelled metoprolol as internal standard.

EXPERIMENTAL

Equipment

A schematic diagram of the chromatographic system is given in Fig. 2. The system consists of two type 2150 LC pumps (LKB, Bromma, Sweden), a Model

Metoprolol

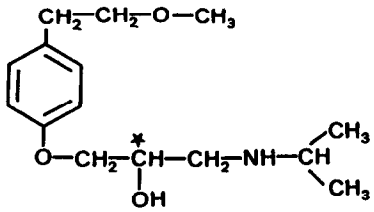

Fig. 1. Structural formula of metoprolol.

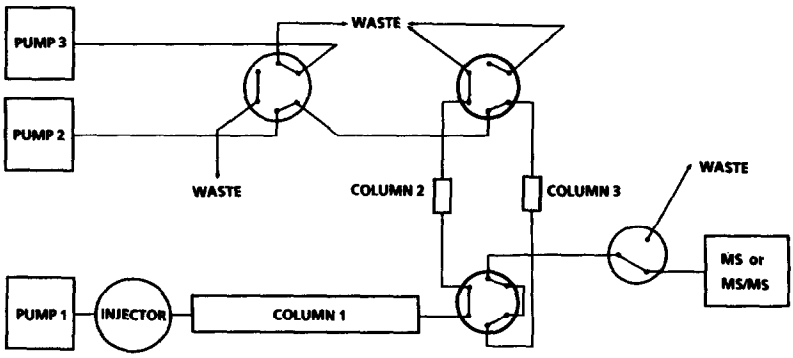

Fig. 2. Schematic diagram of the experimental setup.

SP-8700 LC pump (Spectra Physics, San José, CA, U.S.A.), a Model 7125 injection valve (Rheodyne, Berkeley, CA, U.S.A.), an MUST valve-switching unit (Spark Holland, Emmen, The Netherlands) and a Model 7010 switching valve (Rheodyne). UV detection at 254 nm was performed using a type 440 UV detector (Waters Assoc., Milford, MA, U.S.A.) coupled directly to the chiral column. Combined LC–MS was performed with a Finnigan MAT TSQ-70 triple quadrupole mass spectrometer equipped with a thermospray interface (Finnigan MAT, San José, CA, U.S.A.). Vaporizer temperature and repeller voltage were optimized under current conditions, resulting in values of 130°C and 50 V, respectively. The source block temperature was kept at 200°C. In the MS–MS experiments air was used as collision gas at a pressure of 0.05 Pa. The optimized collision energy was 30 V.

Materials

Racemic metoprolol {1-isopropylamino-3-[4-(2-methoxyethyl)phenoxy]-2-propanol}, $^2H_6$-labelled metoprolol and *S*-(−)-metoprolol were kindly supplied by Hässle AB (subsidiary of Astra, Gothenburg, Sweden). Sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dihydrate and 2-propanol were of analytical reagent grade and obtained from E. Merck (Darmstadt, F.R.G.). HPLC-grade methanol and acetonitrile were obtained from Rathburn Chemical (Walkerburn, U.K.). Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Bond Elut C_{18} was obtained from Analytichem International (Harbor City, CA, U.S.A.).

The α_1-acid glycoprotein column (100 mm × 4 mm I.D.) was obtained from ChromTech (Stockholm, Sweden). The trapping columns were Guard-Pak Bondapak C_{18} cartridges (4 mm × 5 mm I.D.) (Waters Assoc.).

Mobile phase 1, used in the chiral separation, was 0.25% 2-propanol in 20 m*M* phosphate buffer of pH 7 and the flow-rate was 0.8 ml/min. Mobile phase 2, used for desorption and thermospray LC–MS analysis, was 20% methanol in 50 m*M* ammonium acetate; the flow-rate was 1.5 ml/min. Washing of the trapping columns was performed with 1 ml/min of water.

Sample pre-treatment

Plasma samples were pre-treated using Bond Elut C_{18} columns (capacity 1 ml)

according to ref. 8. Each column was conditioned with 2 × 1 ml of acetonitrile and 2 × 1 ml of water. To 1 ml of plasma was added 100 μl of 1.8 μg/ml (6.4 μ*M*) internal standard solution. The sample was drawn through the column by suction. After washing the column with two 0.5 ml volumes of 10% acetonitrile in water the metoprolol-containing fraction was eluted with 500 μl of 50% acetonitrile in 0.1 *M* HCl into a test tube. After evaporating the eluate under a stream of nitrogen the residue was dissolved in 280 μl of mobile phase 1. The injection volume was 130 μl.

RESULTS AND DISCUSSION

System description

A schematic diagram of the PSS system is shown in Fig. 2. The procedure is as follows: the pre-treated samples are injected on to the α_1-acid glycoprotein column (column 1). The first-eluting enantiomer, (+)-metoprolol, is collected on the first trapping column (column 2) and the other enantiomer is collected on column 3. (The retention time windows, setting the volumes that must be switched to the two trapping columns, are determined daily in a separate experiment using a UV detector coupled directly to the outlet of the chiral column). The two trapping columns are then washed with water to remove residual buffer salts, and the metoprolol enantiomers are subsequently eluted and introduced into the mass spectrometer. A three-port valve is placed between the valve-switching unit and the thermospray interface in order to discard water containing the phosphate buffer from the washing step. If this latter step is omitted, the thermospray interface capillary is frequently clogged by buffer salts.

In the development of phase-system switching several aspects have to be dealt with. Baseline separation of the two enantiomers on the chiral column is essential in order to enable quantitative heart-cutting from the developing chromatogram. Trapping columns must be selected that have sufficient sample capacity and sufficiently high capacity ratios for the metoprolol enantiomers both in the mobile phase of the chiral column and in water, which is used for removal of the residual phosphate buffer in the washing step. Furthermore, the enantiomers should have low capacity ratios in the desorbing eluent. Ideally, the compound of interest is adsorbed on top of the trapping column and rapidly desorbed in the backflush mode in a sharp band. In the present case it appears that metoprolol is distributed over the trapping column during adsorption and washing. Thus the washing should be kept to a minimum. Without washing of the trapping columns, frequent clogging of the thermospray interface capillary occurs. When the trapping columns are washed with water for 30 s at a flow-rate of 1 ml/min, clogging of the thermospray interface capillary is no longer observed and stable ionization conditions can be achieved. The dimensions of the trapping columns are also important in optimizing the peak compression. Optimization of the system in this respect has not been studied here.

Liquid chromatography–mass spectrometry

Under the present conditions an intense peak at $m/z = 268$ for the protonated metoprolol molecule is observed in thermospray buffer ionization mode (Fig. 3). Hardly any fragmentation occurs. Full-scan mass spectra with good signal-to-noise ratios can be obtained with amounts as low as 4 ng of metoprolol, injected in the column-bypass mode.

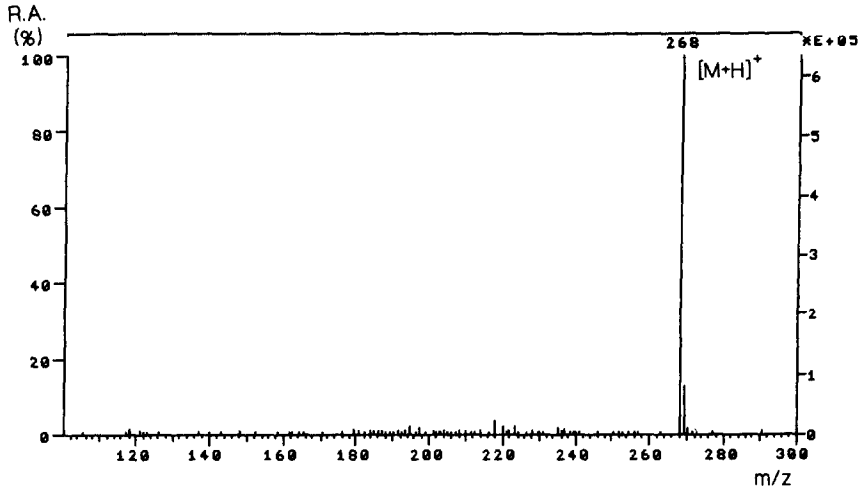

Fig. 3. Thermospray mass spectrum of metoprolol. For conditions see text. Right-hand side: absolute intensity in arbitrary units.

The successful performance of the valve-switching system is demonstrated in Fig. 4. The system shows good performance with respect to the separation of the two enantiomers, the quantitative collection of both enantiomers, and the mass analysis of them after phase-system switching from the LC–MS-incompatible mobile phase, containing phosphate buffer, to an ammonium acetate buffer system, as frequently used in thermospray LC–MS. Equal peak areas were obtained for the two enantiomers using the racemate standard solution containing 29 ng (110 pmol) of metoprolol [14.5 ng (55 pmol) of each enantiomer].

Considerable peak compression is evident upon comparison of the peak width obtained with UV detection directly after chiral separation on one hand and the peak

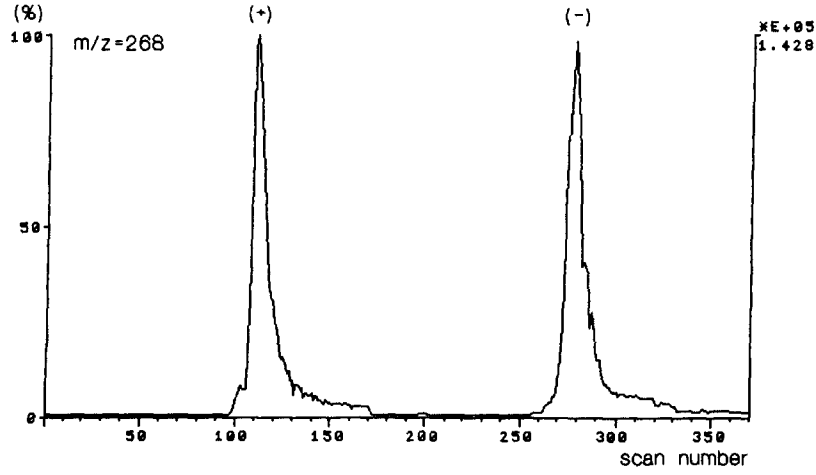

Fig. 4. Mass chromatogram after PSS for the racemic mixture; each peak corresponds to 18 ng. For conditions see text. Right-hand side: absolute intensity in arbitrary units.

width after PSS and LC–MS analysis at the other. The peak width is decreased by a factor of *ca.* 5 by applying the PSS approach.

A drawback of the PSS approach in combination with the thermospray interface in this case appears to be the increase in the background ion current which results from valve switching. Before switching the valves in the desorption mode no liquid is entering the thermospray interface. Upon switching, when introduction of liquid into the interface is resumed the thermospray conditions need some time to stabilize, resulting in baseline disturbances at all m/z ratios. As a result the mass chromatograms of blank samples show a signal. This is a problem for compounds eluting directly from the trapping column, which is in fact the most favourable situation with respect to peak compression. There are several ways to overcome this problem. By using either a more hydrophobic trapping column or a weaker eluting solvent (or both) some retention of the compound of interest will be obtained on the trapping column, resulting in some delay between the solvent front causing the baseline disturbance and the compound of interest. Another approach is the use of a second column between the trapping column and the LC–MS interface to achieve a similar effect. However, both approaches will influence the peak shape and result in less peak compression. These two approaches have therefore not been investigated extensively.

Liquid chromatography–tandem mass spectrometry

Another solution to the problem of baseline disturbance due to valve switching is the use of MS–MS. The signal in MS–MS will be less subject to disturbances due to changes in mobile phase composition and flow-rate occurring in the ion source, because a selective reaction in the collision chamber is monitored. Because the use of MS–MS circumvents the most significant disadvantages of the other solutions mentioned above, and even adds selectivity in the bioanalytical determination, its applicability has been investigated.

The daughter spectra of protonated metoprolol ($m/z=268$) show structurally informative fragmentation, *e.g.*, peaks at $m/z=72$, 98 and 116, corresponding to neutral losses of 196, 170 and 152 amu, respectively. Similar neutral losses are also observed in the daughter spectrum of protonated [$^2H_6$]metoprolol. Since the enantiomers and internal standards (the deuterium labels remain incorporated in the fragment ions) show an equal neutral loss, the neutral loss scan mode can be used to detect these compounds with enhanced selectivity. The neutral loss of 152 a.m.u. corresponding to the loss of 4-(2-methoxyethyl)phenol, is selected as a probe in the analysis of the metoprolol enantiomers in plasma. Fig. 5 shows the MS–MS mass chromatograms of both metoprolol and the deuterated internal standard as obtained after injection of a plasma sample spiked with 0.37 μg/ml (1.4 μmol/l) of racemic metoprolol and 0.18 μg/ml of internal standard, which corresponds to an injected amount of 86 ng (320 pmol) of each enantiomer (and 46 ng of each deuterated enantiomer). The ratio between the peak areas from the internal standard and metoprolol itself is the same for both enantiomers, even when their areas are not the same (probably due to either a less favourable setting of the retention time window in this particular experiment or differences in desorption characteristics). Thus, it is shown that the internal standard method compensates for variations in thermospray MS–MS conditions. Mass chromatograms of blank plasma samples are free of erroneous signals. The lowest detectable plasma level was 70 ng/ml of racemic metoprolol using an internal standard concentration of 100 ng/ml.

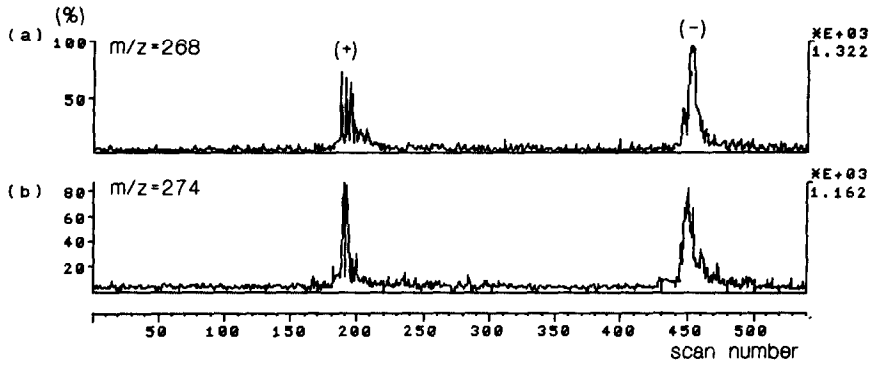

Fig. 5. MS–MS (neutral loss mode) mass chromatograms of (a) metoprolol enantiomers (following the reaction $m/z = 268 \rightarrow m/z = 116$) and (b) $^2H_6$-labelled internal standard ($m/z = 274 \rightarrow m/z = 122$) in plasma. Right-hand side: absolute intensity in arbitrary units.

CONCLUSION

The PSS approach coupled to MS, developed for target compound analysis, increases the possibility of determination of enantiomeric compounds, as shown by using metoprolol as an example. The benefit of using MS is that isotopically labelled internal standards can be used, selectivity can be tuned and, if necessary, identification can be performed. Enhanced selectivity in bioanalytical applications can be achieved by utilizing MS–MS.

REFERENCES

1 E. J. Ariens, *Eur. J. Clin. Pharmacol.*, 26 (1984) 663.
2 L.-E. Edholm, C. Lindberg, J. Paulson and A. Walhagen, *J. Chromatogr.*, 424 (1988) 61.
3 A. Walhagen and L.-E. Edholm, presented at *the 12th International Symposium on Column Liquid Chromatography, Washington, June 19–24, 1988.*
4 A. Walhagen and L.-E. Edholm, *J. Chromatogr.*, 473 (1989) 371.
5 J. van der Greef, W. M. A. Niessen and U. R. Tjaden, presented at *the International Symposium on Pharmaceutical and Biomedical Analysis, Barcelona, September 23–25, 1987.*
6 J. van der Greef, W. M. A. Niessen and U. R. Tjaden, *J. Pharm. Biomed. Anal.*, 6 (1988) 565.
7 E. R. Verheij, H. J. E. M. Reeuwijk, G. F LaVos, W. M. A. Niessen, U. R. Tjaden and J. van der Greef, *Biomed. Environ. Mass Spectrom.*, 16 (1988) 393.
8 P. M. Harrison, A. M. Tonkin and A. J. McLean, *J. Chromatogr.*, 339 (1985) 429.