Chiral liquid chromatography–mass spectrometry for high-throughput screening of enzymatic racemase activity

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Abstract

In finding suitable biocatalysts for processes in chemical industry, expression libraries are constructed containing typically >10 000 clones. Search for a desired activity is done by examination of all the clones in one or more libraries using a high-throughput screening assay. Here we describe a method for the screening of the enzymatic racemase activity of clones from an expression library on α-amino-ε-caprolactam (ACL) using a fast chiral LC separation and ionspray-MS as the detection technique. After substrate incubation with *S*-ACL, the 96-well microplates were centrifuged to remove cell material. The conversion of *S*-ACL to *R*-ACL was monitored by quantitation of the *R*-ACL enantiomer. Separation of the two ACL enantiomers was performed on a Crownpak CR+ column within 1 min. A Gilson 215 autosampler with a 889 multiple injection probe was used for injecting the samples into the LC system. The total analysis time for a 96-well microplate was 56 min. The MS was operated in the positive-ion mode using selected ion monitoring at m/z 129 [M+H]$^+$ of ACL. Using this method over 12 000 samples were analyzed without loss in performance of the system. The LC column remained stable without loss of resolution and the MS system did not show loss in sensitivity throughout the screening. Inter-day reproducibility was within 15%.
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Keywords: Enantiomer separation; Expression libraries; High-throughput screening; Aminocaprolactam; Enzymes

1. Introduction

There is a growing interest in biocatalytic processes in chemical industry. In finding suitable biocatalysts, expression libraries are constructed containing typi-

cally >10 000 clones per library. To be successful in finding (the few) positive clones, a high-throughput screening assay is essential. Although high-throughput screening assays of expression libraries for enantioselective enzymes is a relatively new area in analytical chemistry, several analytical techniques like UV–Vis [1], fluorescence, IR thermography, circular dichroism spectroscopy, mass spectrometry and several chromatographic techniques have already been described. A comprehensive review was given by Reetz [2]. In

recent years there has been an increasing interest in mass spectrometry as an attractive high-throughput technique in combinatorial chemistry, as for some applications it can be used without prior chromatographic separation [3–5] and it is a more or less generic technique. By co-injecting a suitable internal standard quantitative results can be obtained. Use of mass spectrometry for screening of racemase activity, however, is not straightforward, because enantiomers do not show any differences in their mass spectra.

In the case of screening for biocatalysts with racemase activity, a selective assay capable of distinguishing between the enantiomers is required. Chiral chromatography is a good candidate as it can easily be linked to a mass spectrometer.

Furthermore, detection of the enantiomers in a complex mixture (e.g. a biological matrix) requires a high degree of detector selectivity to distinguish the enantiomeric compounds of interest from endogenic compounds.

Recent review articles [2–4,6,7] show that the mass spectrometer has been recognized as an HPLC-coupled detector that is capable of supplying the desired selectivity in chiral analysis and in some cases may be employed for high-throughput analysis. At present, several commercial chiral stationary phases for HPLC are available for the enantiomeric separation of a broad range of chiral molecules. Often these separations take several minutes, which is not desirable in high-throughput analysis. Our aim was to develop a chiral separation for the enantiomers of α-amino-ε-caprolactam (ACL) within 1 min.

In this paper, we describe a novel assay for the high-throughput screening of enzymatic racemase activity of clones from an expression library using S-ACL as substrate (see Fig. 1). The assay is based on a fast chromatographic separation of the two enantiomers in combination with selective mass spectrometric detection and quantitation of the racemase product R-ACL.

2. Experimental

2.1. Chemicals and reagents

S-ACL with an enantiomeric excess (e.e.) of 99.0% and chemical purity of 97% from Fluka (Buchs, Switzerland) was used as obtained. R-ACL with an e.e. of 98.9% was synthesized at DSM Research (Geleen, The Netherlands). Water was purified with a Milli-Q (Millipore, Bedford, MA, USA) system. All other chemicals were of analytical reagent grade.

2.2. LC–MS equipment

The system consists of an HP1100 binary pump (Agilent Technologies, Waldbronn, Germany), a Gilson 215 sampler with an 889 multiple injection probe (Gilson, France) equipped with 5-μl injection loops and an API 150EX LC–MS system with a turbo ion interface (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The binary pump and the MS system are controlled by ANALYST software (Applied Biosystems). Between the pump and the sampler a 10-port Rheodyne valve is positioned to direct the total flow to the channel being sampled [8]. Samples were injected by switching the valves from the Gilson 889 multiple injection probe individually with a time interval of 30 s. The Rheodyne valve operating as a stream selector is switched simultaneously. The Gilson system and the Rheodyne valve were controlled by Gilson 735 sampler software.

2.3. LC conditions

For the separation of the two ACL enantiomers a Crownpak CR+ column (15 cm × 4 mm I.D.) from Chiral Technologies (Illkirch, France) was used. The column was operated at ambient temperature. The mobile phase was water containing 0.1% formic acid, at a flow-rate of 1.5 ml min^{-1}.

Before entering the ionization interface the eluent flow was split 1:4, e.g. 0.3 ml min^{-1} was going to the MS system.
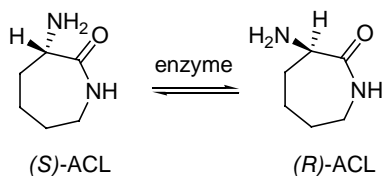

Fig. 1. Racemase reaction.

2.4. MS conditions

The turbo ionspray source was used as ionization interface in the selected ion mode, measuring the ion with m/z 129, with a dwell time of 100 ms. the capillary voltage was set at +5 kV. The source temperature was set at 400 °C.

The declustering potential (+15 V) and the focusing potential (+220 V) were optimized for best sensitivity for the ACL enantiomers.

2.5. Screening method for expression library

The expression library screening was performed as follows: 96-well shallow microplates, filled with 2∗TY medium (e.g. a cell growth medium with the composition: 10 g l^{-1} yeast extract, 16 g l^{-1} bacto trypton and 5 g l^{-1} sodium chloride) (200 μl per well) supplemented with kanamycin (35 μg ml^{-1}), were inoculated with clones from the expression library. After incubation for 48 h at 25 °C at a shaking speed of 600 rpm, two 100-μl samples from different cultures were transferred to one new well (in a wide well plate) containing 100 μl *S*-ACL solution ($60 \cdot 10^{-3}$ *M*). The *S*-ACL concentration in the pooled samples was $20 \cdot 10^{-3}$ *M* at the start of the substrate incubation of this screening. After incubation for 24 h at 37 °C, the microplates were centrifuged for 10 min at 4000 rpm. From the supernatant in each well, 100 μl was transferred to a shallow 96-well microplate. Next, 100 μl formic acid solution (0.1%, v/v in water), was transferred to all wells, using a Multidrop DW (Labsystems, Helsinki, Finland). After this two times dilution step (in which the sample was mixed simultaneously), a second dilution (25 times) was performed, by pipetting a 10-μl sample into 240 μl formic acid solution (0.1%, v/v), using a Gilson 215 multiprobe channel robot. By doing this, the samples in all wells were diluted 50 times after the substrate incubation. In the case no racemisation has taken place, the *S*-ACL concentration is expected to be 0.4 m*M* (after this 50 times dilution) and the *R*-ACL concentration is expected to be 0 m*M*.

2.6. Calibration

Calibration samples were prepared in the same way as real expression library samples, containing complementary amounts of the substrate *S*-ACL and the racemisation product *R*-ACL. All calibration samples contained the biological matrix as present in the expression library samples.

The system was calibrated every 24 h, doing four replicate injections of all calibration levels (0,10, 20, 30, 40 and 50% conversion).

3. Results and discussion

Racemase activity of the clones from an expression library is measured by analyzing the amount of *R*-ACL that is present in the samples. The assay is based on the chiral separation of the two ACL enantiomers and quantitative analysis of the reaction product *R*-ACL by MS detection. With respect to the separation of the ACL enantiomers, a good separation factor was obtained on a Crownpack CR+ column using an aqueous perchloric acid solution as mobile phase. This type of mobile phase modifier is usually employed on Crownpak CR+ columns, however, is not suited for interfacing with mass spectrometry because of its nonvolatile nature.

The use of volatile organic acids, such as acetic acid and trifluoroacetic acid, as mobile phase additives on a Crownpak column has been described [9–12]. A prerequisite is that the ACL enantiomers (pK_a of approximately 7.8), are fully protonated, which is theoretically achieved for this compound at a pH < 5. Because of its acid strength, formic acid proved to be a good volatile substitute for perchloric acid in our application. Using a 0.1% aqueous solution (measured pH 2.6), the selectivity factor and resolution of the ACL enantiomers were comparable with the chromatographic parameters obtained with the perchloric acid mobile phase. Taking into account the pressure limit of the column, the flow-rate was maximized to 1.5 ml min^{-1}, resulting in a run time of 1 min. To obtain a desired run time of <1 h per 96-well microplate overlapping injections were performed, resulting in a total analysis time of 56 min per microplate. For coupling to the mass spectrometer both the turbo ionspray interface and the heated nebulizer interface were tested for use as the ionization technique. Sensitivity for ACL was of the same order of magnitude for both interfaces. The decisive factor, however, was the peak broadening. In the heated nebulizer interface severe peak broadening occurred to such an extent that the

two enantiomers could not be baseline resolved at a flow of 1.5 ml min^{-1}. Flow splitting was tried, as peak broadening in this interface is flow dependent. Peak broadening did improve at lower flow-rates, but sensitivity was lost as the heated nebulizer interface is a mass sensitive interface. Peak broadening in the turbo ionspray interface was minimal and peak separation obtained on the LC column could be maintained in this interface. So, ionspray MS in the positive ion mode was used as a selective detection technique by monitoring the [M+H]$^{+}$ ion of the ACL enantiomers at m/z 129. *R*-ACL elutes practically unretained by the column (i.e. at t_0) and co-elutes with components of the biological matrix, giving rise to some ionization suppression on the *R*-ACL enantiomer. On the other hand, the biological matrix did not give a contribution on the m/z 129 signal. Selected ion chromatograms of m/z 129 of a racemic mixture of ACL enantiomers with and without biological matrix are shown in Fig. 2. The signal intensity for the *R*-enantiomer is suppressed by about 20%, while the *S*-enantiomer is hardly influenced by the matrix. As we do not intend to determine e.e. in this assay, but merely focus on analyzing *R*-ACL, this difference in suppression is not a problem. For the calibration standards we mimicked a racemase reaction in the same biological matrix as
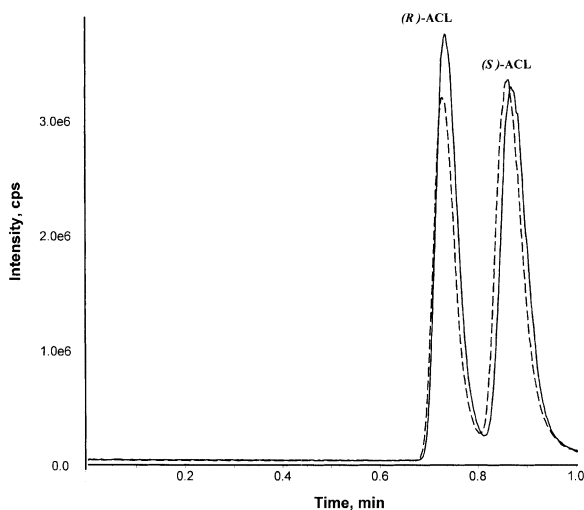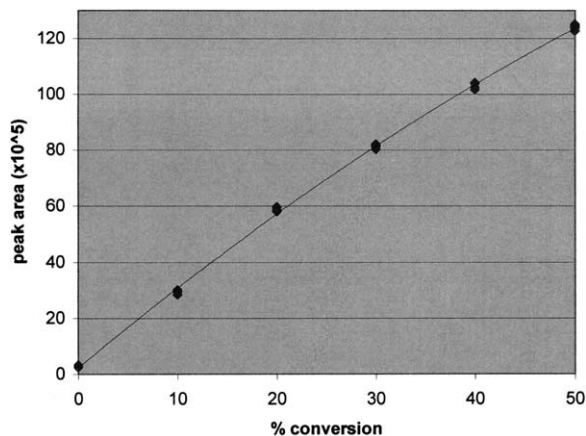

Fig. 3. Typical intra-day calibration graph for *R*-ACL, showing the four replicate data points per calibration level.

present in the expression library samples. The concentration range of 0–50% conversion of *S*-ACL to *R*-ACL is covered, 50% conversion being full racemization. A typical intra-day calibration graph which was recorded in 4-fold every day during the screening is given in Fig. 3. A nonlinear calibration curve is found with a quadratic fit $y = -0.0106x^2 + 2.9546x + 2.3098$. The coefficient of regression is 0.9992. This nonlinearity is due to a limited dynamic range in ionspray ionization. Ion signal saturation and ionization suppression account for this effect [13]. The intra-day relative standard deviation (RSD) (four replicate injections of the calibration samples) was typically around 8% for the calibrated concentration range. The inter-day RSD was about 15%. Theoretically 0.1% conversion of *S*-ACL could be measured. However, the substrate used contained 0.5% of the *R*-ACL enantiomer. Due to this impurity level the detection limit for the actual screening was set to 1% conversion. In total, almost 12 000 clones of an expression library were analyzed using this assay. Fig. 4 shows the chromatogram of a microplate containing positive control samples in the first three rows of the microplate. The positive control samples contained racemic ACL in biological matrix, equivalent to 50% conversion. The first injection of each series of eight shows a distorted peak shape which can be described to a little dead volume in the corresponding valve of the Gilson sampler.

In total more than 12 000 injections were made on the Crownpak CR+ column. No significant increase

Fig. 2. Selected ion chromatograms (m/z 129) showing the influence of the biological matrix on the signal intensity of the both ACL enantiomers. Chromatogram (———) showing the analysis of a racemic mixture of ACL without biological matrix and chromatogram (- - -) with biological matrix.
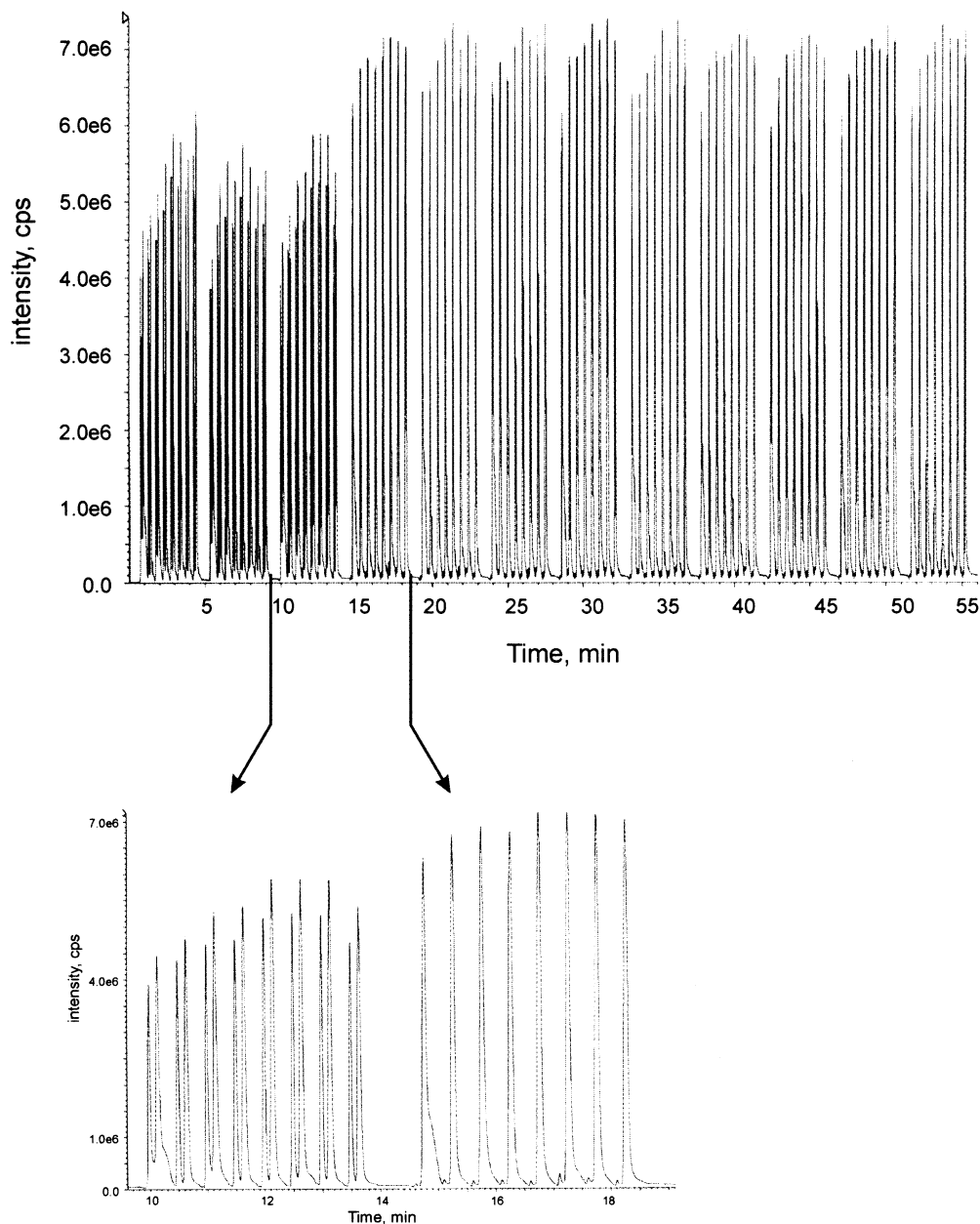
Fig. 4. Chromatogram showing the analysis of a 96-well plate, containing positive control samples, 1A–1H, 2A–2H and 3A–3H, with the other positions all containing negative samples from the expression library screening.

in pressure drop over the column was observed. As Fig. 5 shows there is still baseline separation of the two enantiomers after screening of the total expression library. The chromatographic resolution of the two ACL enantiomers did not change drastically: 2.1 before the screening and 1.9 after the screening. The mass spectrometer was used without intermediate cleaning throughout the screening of the expression library. No
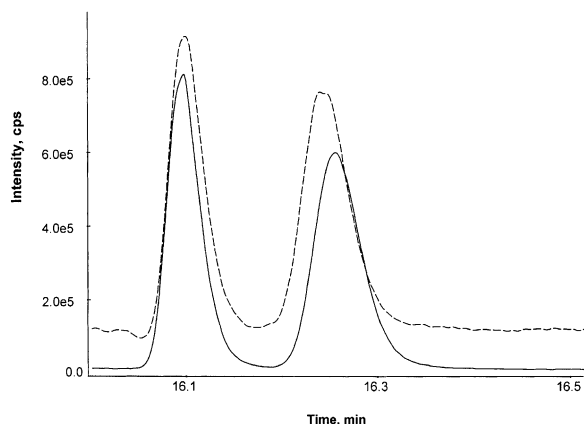
Fig. 5. Part of chromatograms showing separation of *R*-ACL and *S*-ACL before (———) and after (- - -) screening of the expression library (120 microplates).

significant sensitivity changes were observed, as the inter-day RSD showed.

4. Conclusions

A novel high-throughput screening assay has been demonstrated for the determination of racemase activity with *S*-ACL as a substrate and *R*-ACL as the corresponding product using a chiral Crownpak CR+ column in combination with ionspray mass spectrometry as selective detection technique. The applied LC–MS screening assay appeared to be very robust, e.g. hardly any loss in chromatographic resolution was observed after 12 000 injections and mass spectrometric sensitivity could be maintained through-out the screening, without intermediate cleaning of the system.

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