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Screening of rifamycin producing marine sponge bacteria by LC–MS–MS

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Abstract

HPLC–MS–MS has been used for the identification and characterisation of rifamycin B and rifamycin SV in various strains of the marine sponge-derived bacterium *Salinispora*. Gradient elution using acetonitrile/water/ammonium acetate was used to separate the rifamycins from the matrix and negative ion-electrospray mass spectrometry was used for detection and confirmation. The presence of rifamycin in bacterial extracts was confirmed by matching retention times, parent ion spectra and the fragmentated parent ion spectra of the standard compounds and the bacterial extracts. All strains of the marine sponge bacterium *Salinispora* tested were found to contain rifamycin thus an alternate actinobacterial source of rifamycin was established.

Keywords: HPLC–MS–MS; HPLC–MS; Tandem mass spectrometry; *Salinispora*; Rifamycin

1. Introduction

Rifamycins are a group of antibiotics of the ansamycin family produced by various actinomycetes which have marked antibacterial activity against Gram-positive bacteria [1,2]. Semi-synthetic rifamycin variants, such as rifampicin have been used clinically for the treatment of tuberculosis and other bacterial infections [3]. The naturally occurring variant rifamycin B is the basis for other biologically active rifamycin compounds. Rifamycin B is further processed either by natural enzymatic modification or by semi-synthetic mechanisms to produce biologically active rifamycins.

Rifamycins are known to be produced by the soil actinobacterial species *Amycolatopsis mediterranei* and rifamycin synthesis has been most intensively studied in this species [4–6]. In the present study a new potential marine actinobacterial source, strains of *Salinispora* obtained from the marine sponge *Pseudoceratina clavata*, were examined for the presence of rifamycins. The genetic/microbiological background and details of phylogenetic analysis predicting the hypothesis of rifamycin synthesis has been published elsewhere [7] and such details are beyond the scope of this paper. We report here the liquid chromatography/mass spectrometry analytical methodology used to test the hypothesis and to confirm the presence of rifamycin in the extracts of *Salinispora*.

In this study, we have applied the unique strengths of electrospray-based LC–MS–MS to test the hypothesis of rifamycin synthesis by a range of *Salinispora* strains. To our knowledge, LC–MS–MS has not been used previously for the analysis of rifamycins in natural products. The synthetic variant rifampicin has been the focus for analytical method development as it is the major form of rifamycin in clinical use. Various chromatographic (with UV–vis detection) and electrochemical methods have been developed for the analysis of rifampicin and its metabolites [8–10]. Vekey et al. [11] studied the mass spectral characteristics of rifampicin, rifapentin and rifamycin SV and evaluated the feasibility of direct liquid introduction (DLI) of these compounds by HPLC into the mass spectrometer by determining the effects of HPLC solvents and buffers on mass spectra. Their mass spectral studies were based on thermospray MS (TS-MS) in negative ion mode. Korfmacher et al. [12] used both TS-MS and electrospray MS (ESI-MS) to study the mass spectral characteristics of rifampicin, rifamycin B and rifamycin SV in positive ion mode. These studies focused on the potential of MS to characterise these compounds solely using pure standard rifamycins in both modes. LC was used to separate the three

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rifamycins but they were detected using only TS-MS rather than ESI-MS, and the LC–MS response was monitored using only the TIC mode. To date, there have been no reported investigations where HPLC with tandem mass spectroscopy (LC–MS–MS) has been used for rifamycin analysis or any studies where rifamycins have been analysed following extraction of real samples.

Here, we present LC with negative ion ESI-MS-total ion chromatographic (TIC) and tandem mass spectrometric (MS2) methods for the qualitative analysis (identification and confirmation) of rifamycin B and SV, and a multiple reaction monitoring (MRM) method for screening of bacterial extracts for the presence of rifamycins at picomole level.

2. Experimental

2.1. Instrumentation

The separation of compounds in *Salinispora* extracts was carried out using an Agilent binary HPLC system consisting of an Agilent 1100 LC pump, an Agilent 1100 well plate autosampler, and a Cogent Aclarity-C18 (2.0 mm × 50 mm, 5 μm) HPLC column (Microsolv Technology Corporation, Long Branch, NJ, USA). An API 3000 tandem mass spectrometer equipped with a turbo ion spray interface and supported by Analyst 1.4 software (Applied Biosystems, Foster City, CA, USA) was used to detect the separated compounds and process all data.

2.2. Materials

Rifamycin SV, sodium salt was purchased from Sigma (St. Louis, MO, USA) and Rifamycin B was kindly donated by Lupin Ltd. (Tarapur, Maharashtra, India). All solvents used were of HPLC grade.

2.3. Sample preparation

Salinispora spp. (M403, SW15, M102, M413, SW10, SW17, M414, M412, SW02, M101) were cultured on SYP agar media with artificial sea water (ASW) and *A. mediterranei* was cultured on SYP agar media without ASW at 28 °C for 4 weeks. The mycelial cell mass was harvested by scraping it off the growth medium using a glass slide and was weighed. Absolute ethanol was added in sufficient volume to submerge all the mycelial cell mass. The mycelial cell mass and ethanol mixture was incubated for 90 min at room temperature with rotation. The liquid phase was clarified by centrifugation and the extraction of the cell pellet with ethanol was repeated. The combined extracts were filtered (0.22 μm pore size) and stored at −20 °C.

2.4. HPLC separation

The ethanol extracts were evaporated to dryness and were reconstituted initially in an appropriate volume of methanol and then water to provide a 10-fold (5-fold for the MRM mode of MS) concentration of the original extract and a final solvent composition of 45% (v/v) methanol. The samples were filtered (0.22 μm) and a 100 μL aliquot (50 μL for the MRM mode) was injected onto the HPLC column using the autosampler. A blank extract was prepared in a similar manner using sterile culture medium which had been extracted using ethanol.

A binary solvent gradient consisting of a solution of ammonium acetate in water (5 mM; A) and a solution of ammonium acetate in acetonitrile (5 mM; B) was used for all separations The gradient was started after the column had been equilibrated using a mobile phase of 40% (v/v) A and 60% (v/v) B for 10 min. The composition of the mobile phase was changed from 60% (v/v) B to 100% (v/v) B over a period of 15 min and was then returned to the starting composition of 60% B over the next 8 min. The column was re-equilibrated using 60% (v/v) B for 10 min before the next sample was injected. The total run time was 35 min. The mobile phase flow rate through the column was 200 μL/min with 15 μL/min introduced into the ion spray-MS. A splitter and suitable lengths of tubing were used to split the mobile phase flow in the appropriate ratio.

The gradient used for MRM mode was slightly different and commenced after the column had been equilibrated with 45% (v/v) B for 10 min. The composition remained at the initial conditions for the first 5 min and was then changed linearly from 45% (v/v) B to 100% (v/v) B over the next 10 min, and was returned to 45% (v/v) B within the next 8 min. The composition remained at 45% (v/v) B for 10 min before injecting the next sample.

2.5. TIC negative ion mode mass spectrometry

To obtain the electrospray mass spectra of all peaks the TIC negative mode was employed. The mass spectrometric parameters used are listed in Table 1. The temperature of the ion spray was maintained at ambient. All chromatograms were obtained within the range 200–800 amu, at 0.3 s time interval. Resolution of Q1 was 1 amu.

2.6. MS2 negative ion mode mass spectrometry

To obtain the fragmentation pattern of each molecular ion the following conditions were employed: the fragmentation of 755 *m*/*z* ion (rifamycin B) was monitored for the first 3.2 min of the

Table 1
Mass spectrometric parameters used for TIC, MS2 and MRM (RB = Rifamycin B, RSV = Rifamycin SV) modes

	TIC	MS2	MRM (RB)	MRM (RSV)
IS (V)	−4200	−4500	−4500	−4500
DP (V)	−30	−125	−61	−91
FP (V)	−200	−200	−230	−330
EP (V)	−10	−10	−10	−10
CE (V)	–	−45	−68	−54
CXP (V)	–	−15	−5	−7
CUR (L/min)	8	7	7	7
NEB (L/min)	8	7	7	7
CAD (L/min)	–	8	8	8

Ion spray voltage (IS), orifice/declustering potential (DP), ring/focusing potential (FP), entrance potential (EP), collision energy (CE), collision exit potential (CXP), curtain gas flow (CUR), nebulizer gas flow (NEB) and the collision gas flow (CAD) used in each mode are shown.

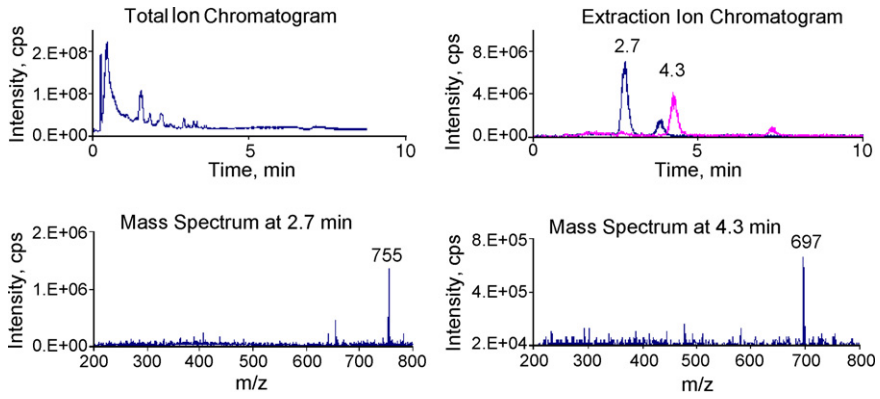

Fig. 1. Total ion chromatogram obtained for *Salinispora* extract, its extracted ion (*m/z* 755 and 697) chromatogram, and MS spectra of the rifamycin B and SV peaks at 2.7 min and 4.3 min, respectively.

chromatographic run and that of 697 *m/z* ion (Rifamycin SV) was monitored for the remainder of the chromatographic run. The mass spectrometric parameters used are listed in Table 1.

The temperature of the ion spray was maintained at ambient. All chromatograms were obtained within the range 200–800 amu, at 1 s time interval. Resolution of both Q1 and Q3 were 1 amu.

2.7. MRM negative ion mode mass spectrometry

The detector response in MRM is due to a specific transition of molecular ion → fragment. The fragmentation of 755 *m/z* ion → 273 *m/z* ion (rifamycin B) was monitored for the first 11 min, and that of 697 ion → 273 ion (Rifamycin SV) was monitored for the remainder of the chromatographic run. The mass spectrometric parameters optimized for rifamycin B and rifamycin SV to obtain the highest possible sensitivity for each are listed in Table 1.

Temperature of the ion spray was kept at ambient. Dwell time of 1 s was used in both transitions. Resolution of Q1 was low and Q3 was 1 amu.

3. Results and discussion

The ESI-MS method used in this study is well known for its "soft" ionisation characteristics and higher sensitivity compared to TS-MS which causes thermal degradation of some compounds [12]. We observed that the negative ion mode was much more suitable for this work since it produced molecular ions for both rifamycins of interest, with high abundance. The positive ion mode ESI-MS [12] produced only ions which were indicative of sodium adduct of rifamycin SV rather than the molecular ion (data not shown).

The chromatograms monitored using TIC mode showed that both the *Salinispora* and *A. mediterranei* extracts contained peaks which eluted with similar retention times to those of the standard compounds, rifamycin B and SV. These peaks were absent from the blank extracts of sterile culture medium. Fig. 1 shows the TIC and the extracted ion (*m/z* 755 and 697) chromatogram for *Salinispora* extract. The electrospray negative ion spectra of each peak were also shown in the same figure. Fig. 2 shows the chromatograms and the spectra of the standard rifamycin B and SV. Both the retention times and the spectra of the extracts and the standards are concordant, confirming the identity of the peaks in *Salinispora* and *A. mediterranei* extracts. When the *Salinispora* extract was spiked with standard rifamycin B and SV the chromatograms showed growths in peaks shown in Fig. 1, further confirming the identities.

For additional confirmation of the identity of the peaks present in extracts, the sample and standard chromatograms were monitored using MS2 (fragmentation monitored using the third quadrupole). As described in the experimental section the

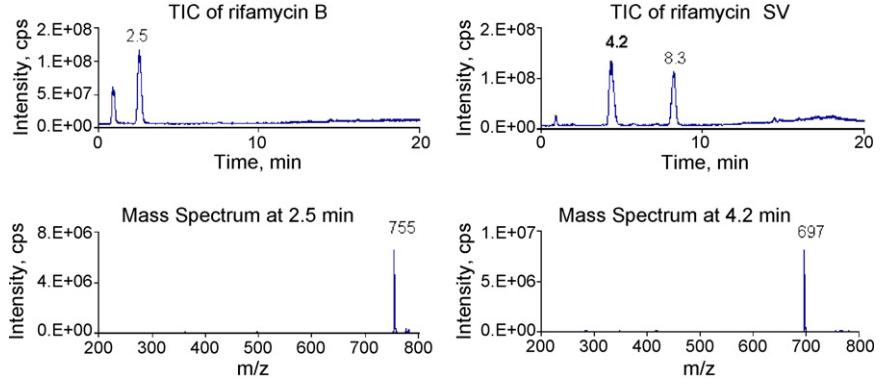

Fig. 2. Chromatograms and MS spectra obtained for the standard rifamycin B and those for the standard rifamycin SV. The degradation product rifamycin S is apparent at 8.3 min.

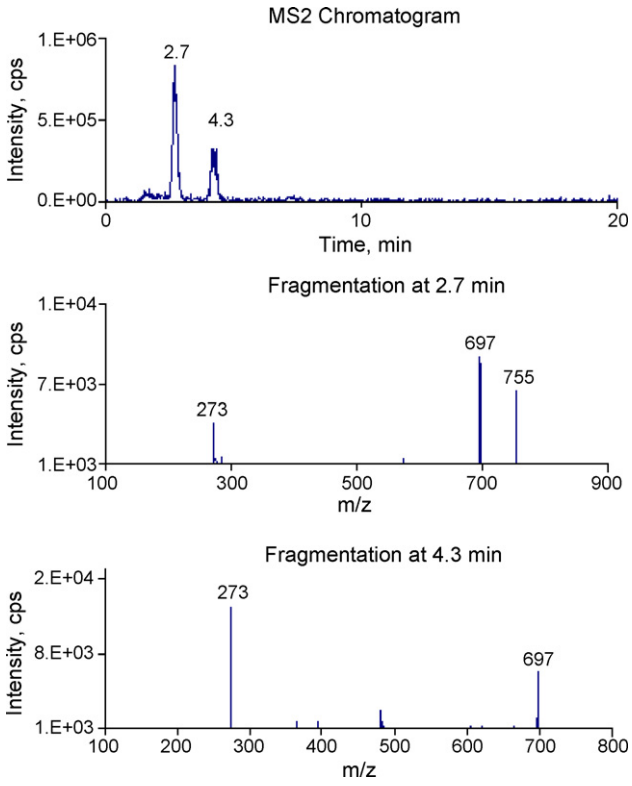

Fig. 3. Chromatogram and fragmentation patterns for the peaks rifamycin B and SV in *Salinispora* extract monitored using MS2 mode.

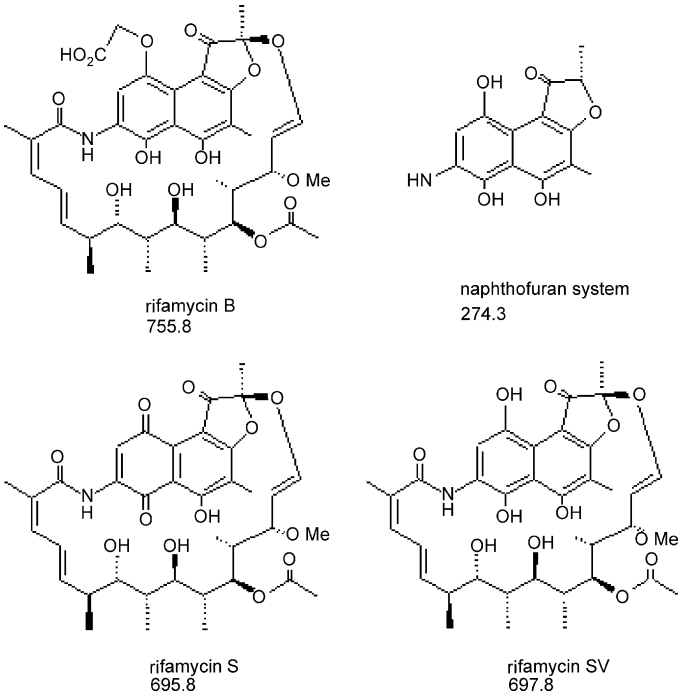

Fig. 4. Structures of rifamycin B, SV and S showing the fragmentation to naphthofuran (NF) observed at *m/z* 273 (figure modified on the basis of the structures provided in Ref. [11]).

fragmentation of the molecular ion *m/z* 755 (rifamycin B) was monitored for the first 3.2 min of the run and that of the molecular ion *m/z* 697 (rifamycin SV) was monitored for the remainder of the chromatographic run. The results for *Salinispora* extract are shown in Fig. 3. Both the chromatograms and the MS2 spectra of the standards and the extracts displayed marked similarity in terms of retention times and the fragmentation pattern, further confirming the identity of rifamycin B and SV in the samples.

The presence of *m/z* 273 (naphthofuran) fragment has been reported to be the structurally most useful ion indicative of rifamycins [11] and it reportedly provides clear evidence for the identification of rifamycins in the *Salinispora* extracts. The structure of this fragment is shown in Fig. 4. The fragmentation of Rifamycin B occurs initially by loss of an acetate group to produce rifamycin SV (*m/z* 697) and then subsequently by removal of the ansa group/fragmentation to produce the *m/z* 273 fragment which is observed for both rifamycin B and SV.

The HPLC–MS chromatograms and the mass spectra obtained for the ethanolic extract of *A. mediterranei* resemble those of the *Salinispora* extract. Since *A. mediterranei* is known to produce rifamycin B and SV [4–6] this resemblance is a further confirmation for the presence of these compounds in *Salinispora* spp.

All other extracts of strains of *Salinispora* were screened for the presence of rifamycins using an amended gradient to ensure complete resolution of all rifamycin peaks and using the MRM mode of detection with MS. The MRM mode was used to obtain a high specificity and sensitivity so that small amounts of rifamycins can be detected with confidence. As mentioned in the experimental section the samples were chromatographed after only a 5-fold concentration. The detector response in MRM was recorded for a specific transition of molecular ion → fragment. The fragmentation of *m/z* 755 ion → *m/z* 273 ion (rifamycin B) was monitored for the first 11 min and that of *m/z* 697 ion → *m/z* 273 ion (rifamycin SV) was monitored for the remainder of the chromatographic run. In this mode, a response would be detected only if both the molecular ion and the fragment are present. This mode therefore has the feature of adding specificity to the compound detected. The presence of *m/z* 273 fragment (naphthofuran) provides very strong evidence for the identification of rifamycins in extracts.

All strains showed three peaks at the retention times of rifamycin B, rifamycin SV and rifamycin S. As the MRM mode is inherently more sensitive than the TIC and MS2 modes used above, the rifamycin S peak is also visible in this mode. A typical chromatogram obtained for an extract is shown in Fig. 5. By an approximate estimation of the total amount of all three rifamycins by adding up peak areas in each strain the strains can

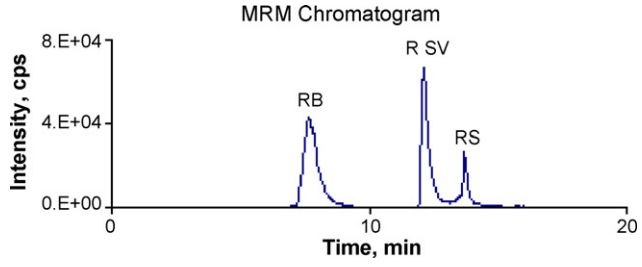

Fig. 5. Chromatogram of a typical extract of Salinispora sp. screened using MRM mode. RB, RSV and RS denote Rifamycins B, SV and S, respectively.

be arranged in the order of abundance of rifamycins as follows:

$$SW15 > M102 > M413 > SW10 > SW17 > M414 \gg M412 > SW02 > M101$$

We noted that the rifamycin B standard solution contained increasing amounts of rifamycin SV and rifamycin S with time indicating that it was likely to be subject to chemical change following laboratory exposure to air and light. Standard solutions of rifamycin B should therefore be freshly prepared and protected from light and exposure to air. Similar processes were observed for rifamycin SV: rifamycin S was observed in solutions of rifamycin SV which had been exposed to light and air, indicating possible oxidation of rifamycin SV. It is possible that although the sample processing was designed to minimize degradation similar processes may have occurred in sample extracts. Therefore, the proportions of rifamycin B, rifamycin SV and rifamycin S observed should be viewed as indicative and may not necessarily be representative of the ratios produced by *Salinispora*. This is particularly likely to be observed in those strains that were seen to contain smaller amounts of rifamycin B: it is possible that rifamycin B was chemically altered to other forms. It is likely that rifamycin B converts to rifamycin SV and then oxidises to rifamycin S (Fig. 4).

The on-column limits of detection for rifamycin B and rifamycin SV (estimated based on 3× baseline noise) were 1 pmol and 0.06 pmol (60 fmol), respectively. Considering the extent of extract concentration and the volume injected this equates to a rifamycin B and rifamycin SV concentrations in the bacterial extract of 4 nM (3 μg/L)and 0.24 nM (0.17 μg/L), respectively. This level of sensitivity was essential in the screening of bacteria, such as *Salinispora* for the production of natural products. The specificity of LC–MS–MS is crucial for the confirmation of the occurrence of the rifamycins in *Salinispora*. Compared to other methods available for characterisation of compounds in complex extracts, such as nuclear magnetic resonance (nmr) where isolation of the pure compound from the extract is required for confirmation, the method presented here is simple, more economical and rapid. Although it would have been possible to detect the rifamycins in extracts using nmr confirmation will require isolation of the compounds in relatively large amounts. With the method presented here all that was required was extraction with ethanol and concentration before injection. However, it is important to emphasis the fact that the comparison made here is not between nmr and MS2, rather nmr and LC–MS–MS. In order to confirm the presence of rifamycins in extracts comparisons were made between the reference standards and extracts in terms of the retention times as well as MS1 and MS2. Confirmation would not have been possible without the availability of the reference standards.

The emphasis of this work was to test the hypotheses that the marine sponge bacterium *Salinispora* produces rifamycin, with confidence, rather than to develop and validate a method for the quantitation of the rifamycins. The MRM method developed in this study is capable of screening bacterial extracts for the presence of rifamycins down to picomole levels, with confidence. It is capable of screening any biological fluid for the presence of rifamycin due to its inherent specificity and sensitivity. The same protocol can be used to screen any biological or natural extract for the presence of any compound of interest. However, as mentioned in the previous paragraph the availability of the reference standard of the compound of interest is essential. The HPLC method and the mass spectrometric conditions need to be optimised for the compound of interest using the reference standard, then the protocol described here for rifamycins can be used for the confirmation.

4. Conclusion

This is the first report of the LC–MS–MS technique being applied to demonstrate the presence of rifamycins in microbial extracts. The unique attributes of this technique enabled us to detect very small amounts of rifamycins in bacterial extracts with high specificity thereby demonstrating that *Salinispora* spp. do produce rifamycins. This method of identification and characterisation is simple and economical. The method can be easily applied to screen any other bacterial or biological extract for the presence of rifamycins, as a class of antibiotics. The protocol described here can be used to screen any compound of interest in biological/natural extracts.

References

[1] P.M. Dewick, Medicinal natural products: a biosynthetic approach, John Wiley, Chichester, 2002, p. 105.
[2] T.W. Yu, Y. Shen, Y. Doi-Katayama, L. Tang, C. Park, B.S. Moore, C.R. Hutchinson, H.G. Floss, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 9051.
[3] R.F. Service, Science 270 (1995) 724.
[4] P. Sensi, P. Margalith, M.T. Timbal, Farmaco [Prat] 14 (1959) 146.
[5] P.R. August, L. Tang, Y.J. Yoon, S. Ning, R. Muller, T.W. Yu, M. Taylor, D. Hoffmann, C.G. Kim, X. Zhang, C.R. Hutchinson, H.G. Floss, Chem. Biol. 5 (1998) 69.
[6] T. Schupp, C. Toupet, N. Engel, S. Goff, FEMS Microbiol. Lett. 159 (1998) 201.
[7] T.K. Kim, A.K. Hewavitharana, P.N. Shaw, J.A. Fuerst, Appl. Environ. Microbiol. 72 (2006) 2118.
[8] K.J. Swart, M. Papgis, J. Chromatogr. 593 (1992) 21.
[9] M. Ishii, H. Ogata, J. Chromatogr. 426 (1988) (1988) 412.
[10] Y. Hahn, S. Shin, Arch. Pharm. Res. 24 (2001) 100.
[11] K. Vekey, D.M. Edward, L.F. Zerilli, J. Chromatogr. 474 (1989) 317.
[12] W.A. Korfmacher, J. Bloom, M.I. Churchwell, T.A. Getek, E.B. Hansen, C.L. Holder, J. Chromatogr. Sci. 31 (1993) 498.