ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 967–975

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

The unusual gradient elution for reversed phase HPLC of a strong chelator as an active drug substance

T. Kalendarev, G. Zupkovitz, V. Ioffe *

D-Pharm Ltd., Kiryat Weizmann Science Park, P.O. Box 2313, 76123 Rehovot, Israel

Received 16 May 2000; received in revised form 31 October 2000; accepted 3 November 2000

Abstract

The new approach to drug development, especially for cardiovascular and brain diseases, brought to synthesis of new lipophillic derivatives of strong calcium chelator BAPTA — DP-b99 and DP-109. Due to their chelating ability, these compounds require metal-free stationary phases, and their high hydrophobicity resulted in unusually steep gradient elution. Novel HPLC methods for analysis of these two compounds were developed. Purospher® RP-C18, 5 μm, 125 × 3.0 mm and XTerra™ RP18, 3.5 μm, 100 × 4.6 mm columns with a steep gradient from: 1% acetic acid to acetonitrile were used for DP-b99, and Hypersil HyPurity™ C4, 5 μm, 100 × 4.6 mm column with a steep gradient from 1% Acetic acid to 5% THF in methanol — for DP-109. Versatile detection techniques could be used with these LC procedures. The methods appeared to be sensitive, selective, reproducible and stability indicating. They could be easily upgraded to bioanalytical methods with LC-MS technique.

Keywords: HPLC; Gradient elution; Lipophillic compounds; Chelating agents; Brain diseases; BAPTA

1. Introduction

The well known calcium chelator BAPTA (*Bis*(*AnminoPhenyloxythane*) *–N,N,N′,N′-Tetra-Acetic acid*) is known to be effective in reducing the damage of heart and brain ischaemia [1]. To enhance membrane penetration, various long chain aliphatic groups were attached to its four carboxyls making the molecule very lipophillic (Fig. 1), and still potent chelating agent for various bivalent metal ions [2].

2. Experimental

2.1. Reagents and materials

BAPTA was purchased from Teflabs (Austin, TX, USA). DP-b99 and DP-109 were synthesized by the Chemical dept., D-Pharm Ltd. (Rehovot, Israel). Water, acetonitrile and methanol HPLC grade were obtained from BDH (Poole, England). Tetrahydrofuran (THF) HPLC grade was purchased from J.T. Baker (Phillipsburg, NJ, USA). Glacial Acetic Acid 99 + % was provided by Sigma (St. Louis, MO, USA). *N,N*-Dimethylformamide (DMF) was purchased from Bio Lab Ltd. (Jerusalem, Israel). Ammonia solution, 32%

* Corresponding author. Tel.: + 972-8-9300794; fax: + 972-8-9300795.
E-mail address: vioffe@dpharm.com (V. Ioffe).

0731-7085/01/$ - see front matter
PII: S0731-7085(00)00567-7

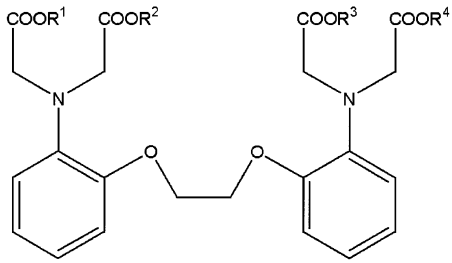

Fig. 1. $R^1 = R^2 = R^3 = R^4 = H = H -$ BAPTA; $R^1 = R^3 = H$; $R^2 = R^4 = CH_2CH_2OC_8H_{17} -$ DP-b99; $R^1 = R^3 = H$; $R^2 = R^4 = CH_2CH_2OC_{18}H_{37} -$ DP-109.

extra pure was obtained from Merck (Darmstadt, Germany).

2.2. Preparation of solvents and standards

BAPTA, DP-b99 and DP-109 free acids are practically insoluble in water and polar organic solvents. Therefore, BAPTA and DP-b99 free acids were dissolved in small amounts of DMF on sonication, and then diluted with either methanol or acetonitrile to obtain final working concentration of 1 mg/ml. In order to dissolve DP-109 free acid, few drops of aqueous ammonia were added to methanol to obtain the solution at the concentration of 1mg/ml. BAPTA as tetrasodium salt is soluble in water and DP-b99 and DP-109 disodium salts are readily soluble in methanol and acetonitrile at concentrations of 1 mg/ml.

2.3. Chromatographic instrumentation and conditions

LaChrom automatic HPLC system (Merck -Hitachi) consisting of L-7100 solvent delivery system, L-7200 autosampler, L-7400 multi-wavelength UV/Vis detector set at 250 nm and L-7000 interface, combined with additional light scattering detector (LSD) Sedex 55 (SEDERE, France).

Alliance 2690 Separation module automatic HPLC system with UV/Vis Photodiode Array detector (PDA) 996 (Waters, Milford, MA, USA)

HPLC columns: Purospher® RP-C18, 5 μm, 125 × 3.0 mm (Merck, Darmstadt, Germany), Xterra™ RP18, 3.5 μm, 100 × 4.6 mm (Waters, Milford, MA, USA), and Hypersil HyPurity™ C4, 5 μm, 100 × 4.6 mm (ThermoQuest, NJ, USA).

Table 1
Gradient profile for DP-b99 analysis

Time (min)	1% Aqueous acetic 0acid (%, v/v)	Acetonitrile (%, v/v)	Flow rate (ml/min)
0	100	0	1.00
1	100	0	1.00
5	0	100	1.00
15	0	100	1.00
20	100	0	1.00
25	100	0	1.00

Table 2
Gradient profile for DP-109 analysis

Time (min)	1% Aqueous acetic acid (%, v/v)	5% THF in methanol (%, v/v)	Flow rate (ml/min)
0	100	0	1.00
1	100	0	1.00
5	0	100	1.00
20	0	100	1.00
22	100	0	1.00
25	100	0	1.00

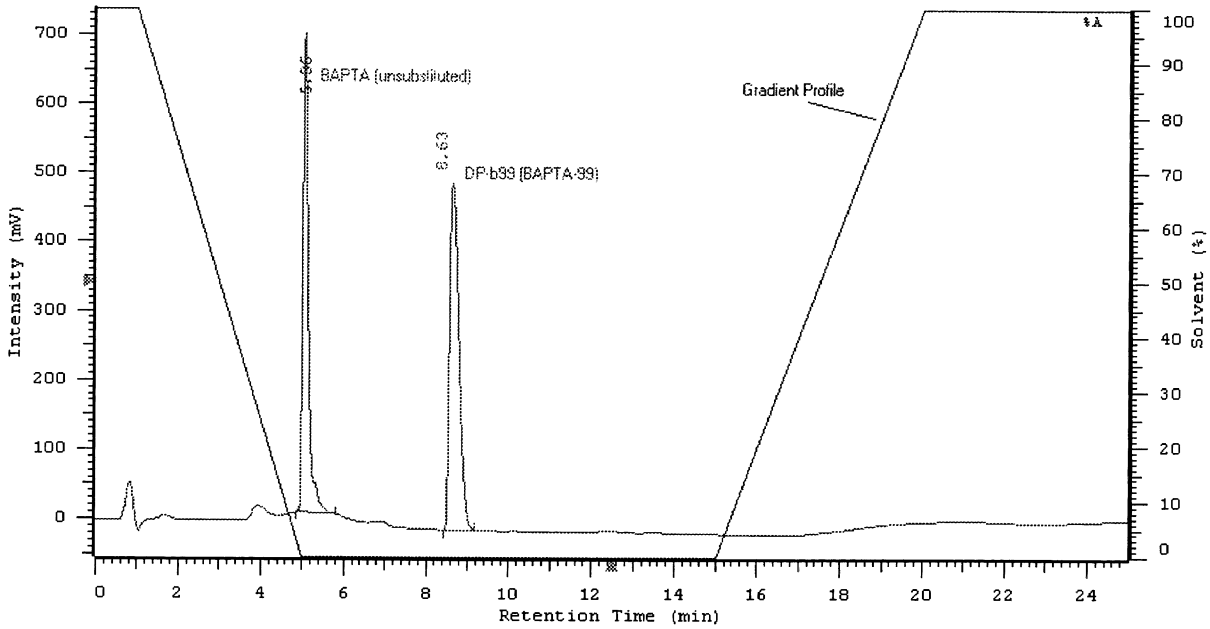

Fig. 3. Mixture of BAPTA and DP-b99 (0.5 mg/ml each). (Additional line — the gradient profile.) Detection — UV at λ 250 nm.

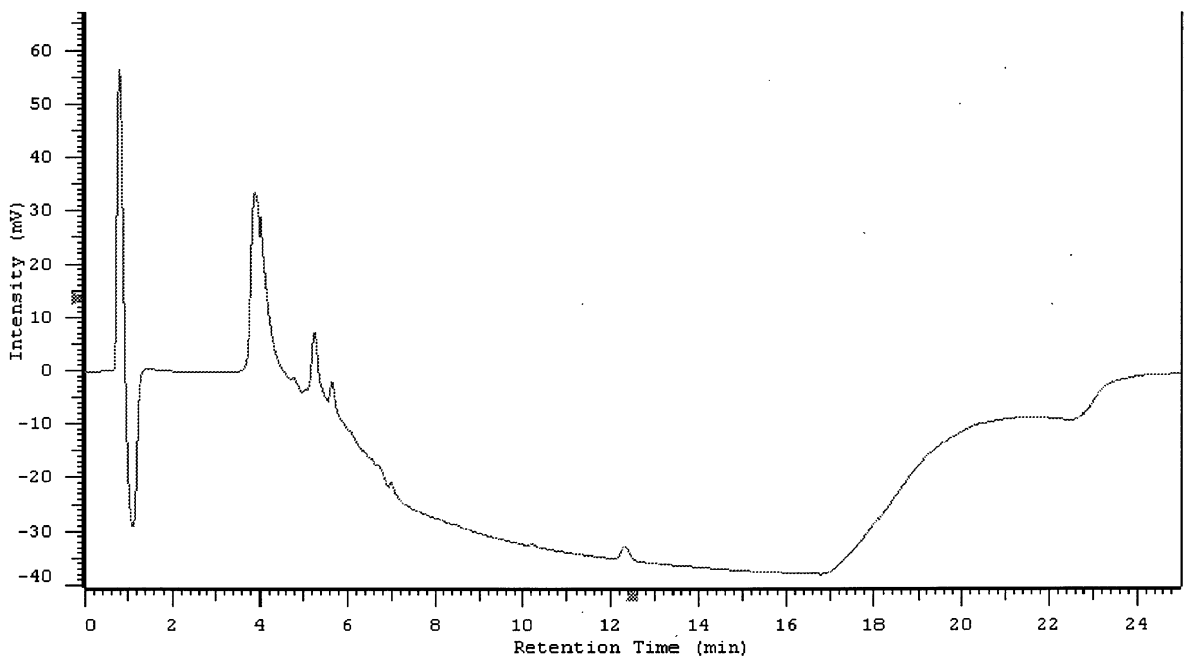

Fig. 2. Blank chromatographic run — the gradient profile. Detection — UV at λ 250 nm.

For DP-b99 analysis the gradient from 1% acetic acid to acetonitrile was used as an eluent (Table 1). For DP-109 — the gradient from 1% acetic acid to 5% THF in methanol (Table 2). In both methods the flow rate was 1 ml/min.

3. Results and discussion

3.1. Development of HPLC method for DP-b99

The starting point for HPLC method development for BAPTA and its derivatives was the attempt to use conventional C18 RP columns (Spherisorb 10 μm, 4.6 × 250 mm and 5 μm, 4.6 × 150 mm, and LiChrospher 10 μm, 4.0 × 200 mm and 5 μm, 4.0 × 125 mm) and various mixtures water — acetonitrile, including 'first choice' gradients (such as 20%water to pure acetonitrile in 30 min), at various pH. Neither BAPTA, nor its derivatives were eluted under these conditions.

The problem could be caused by various reasons, most critical was considered to be high chelating potency of BAPTA molecule, and relatively low hydrophobicity (as compared to usually analyzed by RP-HPLC compounds). Attempts to perform chromatography using C8 and C5 columns (Luna), as well as variation of carbon loading of C18 columns also failed. The additional problem — high activity of carboxyl groups, which could cause a strong interaction with non-endcapped or non-masked residual silanol hydroxyls.

The attempt was performed to run HPLC on an anion exchange polymer based column (IC-Pak HR of Waters), using diluted nitric acid as an eluent, but no positive results were obtained.

Analysis of main features of RP-chromatography, in connection with problems of separation of BAPTA and its derivatives, lead us to the following criteria of HPLC column choice:

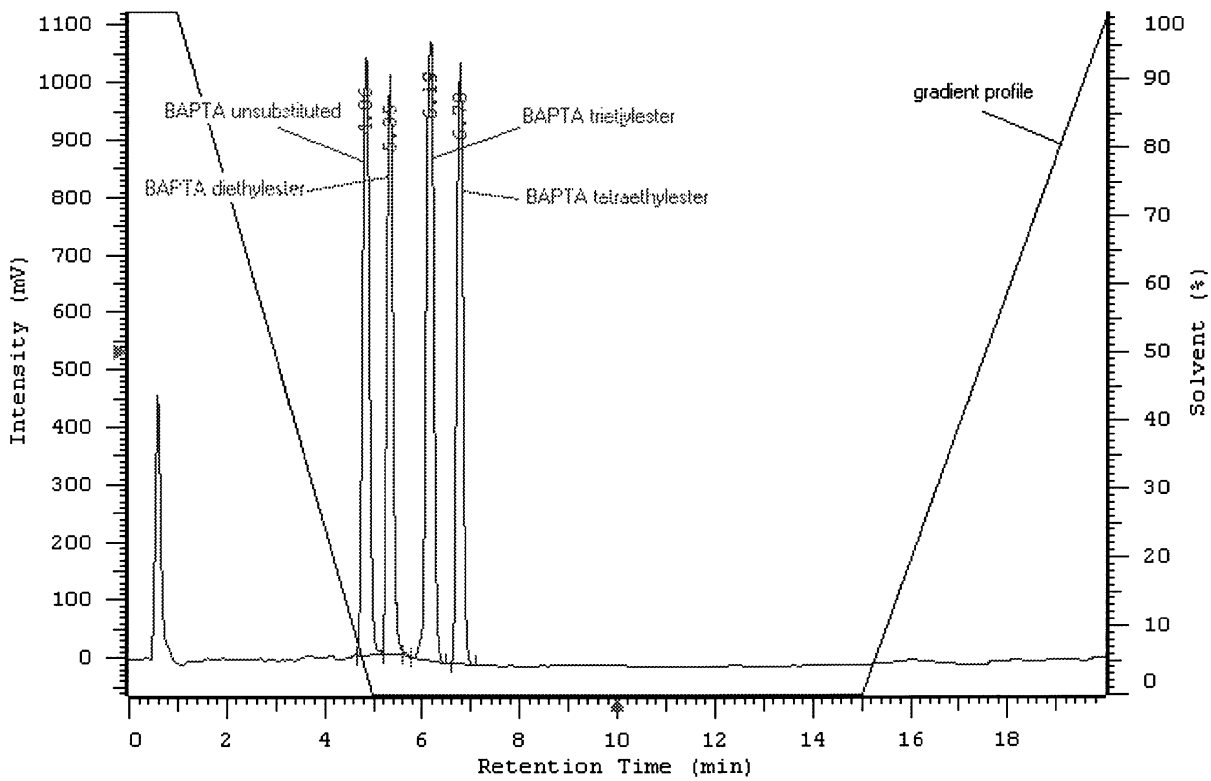

Fig. 4. Mixture of BAPTA and its di- tri- and tetraethyl esters, named in the order of elution. (0.25 mg/ml each). Detection — LSD.

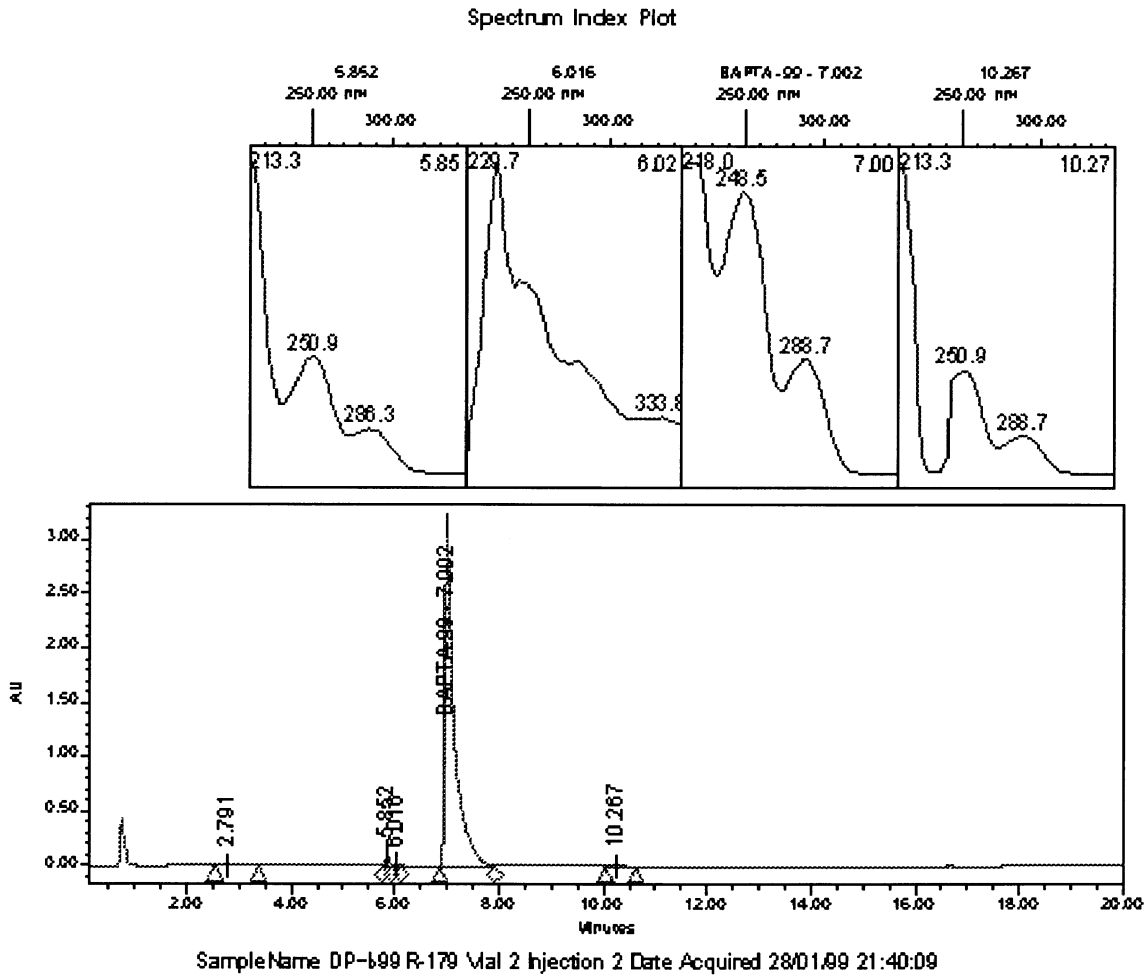

Fig. 5. DP-BAPTA-99 (0.5 mg/ml). UV-spectral identification of impurities. Detection — PDA detector in the range λ 220–400 nm.

Table 3
Suggested gradient profile during the developmentof HPLC method for DP-109

Time (min)	1% Aqueous acetic acid (%, v/v)	Methanol (%, v/v)	Flow rate (ml/min)
0	100	0	1.00
1	100	0	1.00
5	0	100	1.00
15	0	100	1.00
20	100	0	1.00
25	100	0	1.00

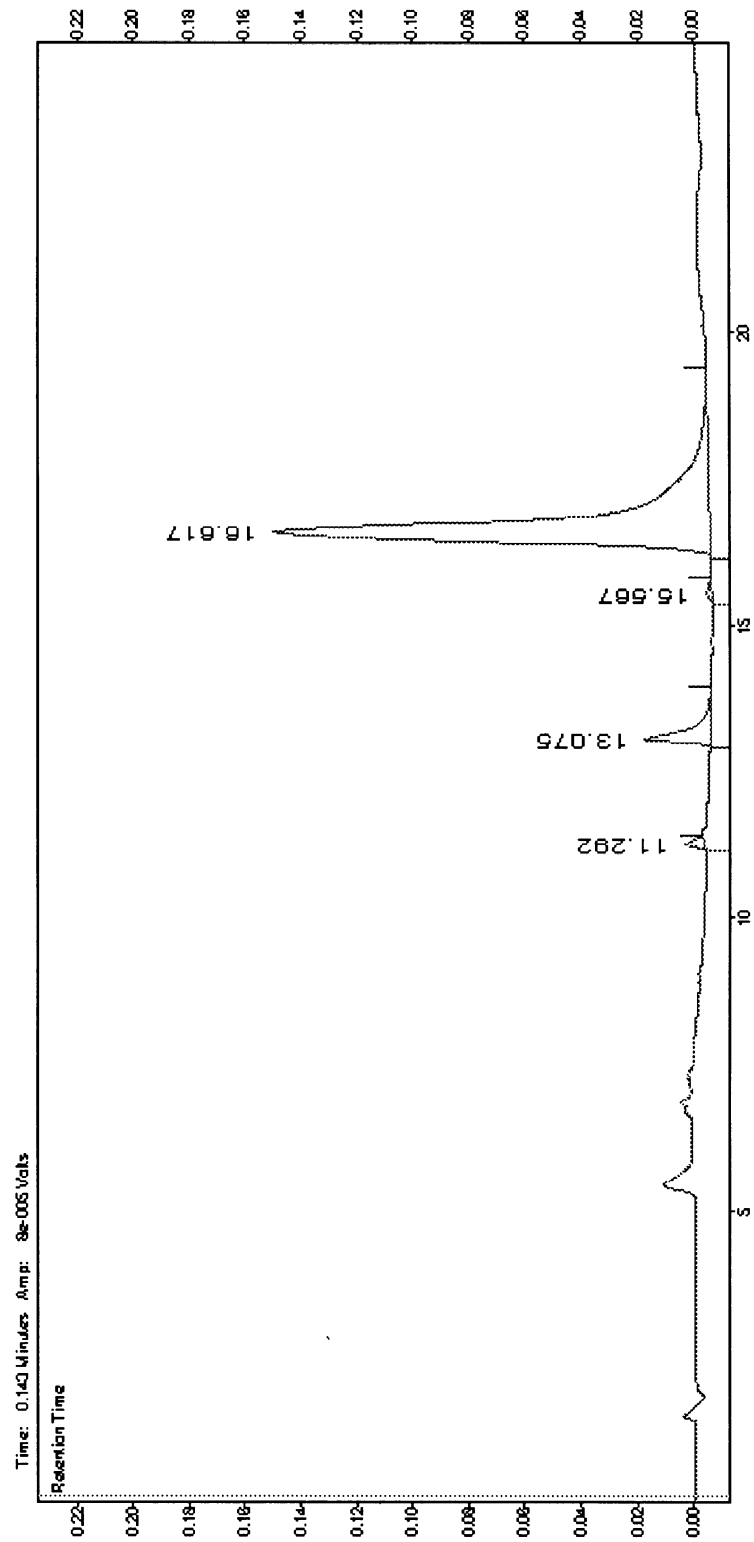

Fig. 6. Analysis of DP-109 using column C4 under 'Generic' gradient conditions.

1. Column should be based on polymer or, preferentially, on highly purified synthetic silica — the stationary phase should be metal free. This condition takes into account the high chelating potency of BAPTA.
2. Reversed phase column should be endcapped to avoid any residual silanol activity, as well as to ensure its comparatively high hydrophobicity even for moderate to low carbon loading. This takes into account both the basic nature of BAPTA amino-groups, which could interact with acidic silanol hydroxyls, and the acidic nature of BAPTA carboxyl groups, which have a tendency to bind to hydrophilic sites.
3. Chromatography should be carried out using comparatively low polar eluent — such as pure acetonitrile. But there should be initial very short 'push' of highly polar mobile phase at low pH to turn the analyte to free acid in non-ionized form. This could be reached by very steep gradient, which is possible to perform only with the low volume columns. Therefore, the HPLC column should be short and its bore should be comparatively narrow.

The column that could answer to all the above requirements — Purospher® RP-C18e, 5 μm, 125 × 3.0 mm (Merck).

Using this column together with steep gradient elution (4 min from 100% aqueous to 100% organic phase) allowed to obtain chromatograms of BAPTA and its several derivatives providing well-shaped peaks with high efficiency and symmetry.

The following graphs are to illustrate the developed chromatographic method and to display some of its possibilities.

Fig. 3 illustrates a typical picture of a Resolution Solution run (a mixture of BAPTA and DP-b99, 0.5 mg/ml each) as a system suitability test. Providing no interference with gradient system peaks (Fig. 2), high resolution of peaks is established. This makes evidence that the main possible synthetic impurity and/or degradation compound — the corresponding BAPTA monoester — which usually elutes halfway between BAPTA and DP-b99, will be well distinguished and at least baseline separated from the other peaks. This is the most critical requirement for an HPLC method pretending to be stability indicating, and this is what happens actually with the developed analytical procedure.

Separation of mixture of BAPTA and its ethyl esters (Fig. 4) (prepared individually) illustrates the selectivity of the HPLC analytical procedure. Structurally very close compounds are baseline

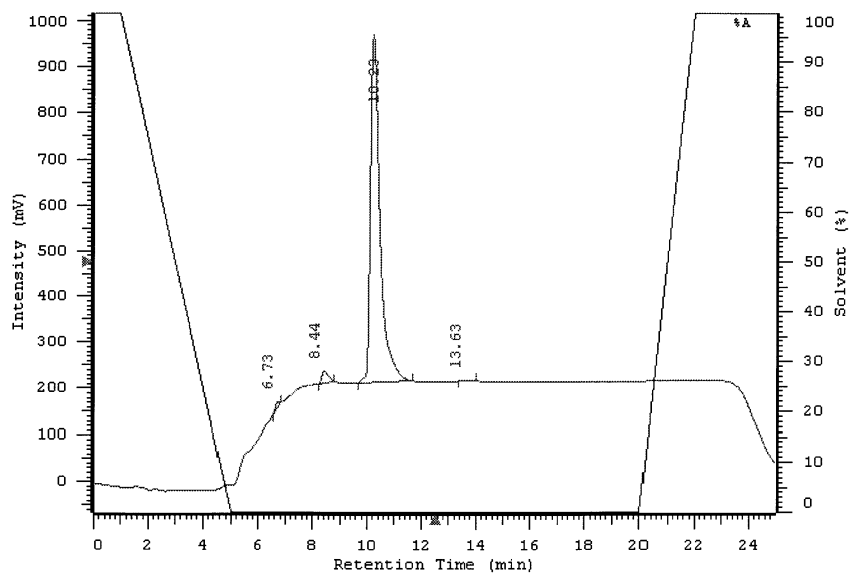

Fig. 7. Analysis of DP-109 on C4 column using 5% THF solution in methanol.

separated and could be identified by their retention time and relative retention time. Additional peak eluting at solvent front could be attributed to counter ion (Na), visible on the light scattering detector.

The data illustrated by Fig. 5. provide an example of spectral identification of minor peaks of related compounds, namely, synthetic impurities and degradation products. As could be seen, the minor peaks have the same chromophore as the main compound — DP-b99. Slight aberrations could be attributed to the influence of the mobile phase gradient. Its input is higher for lower concentrations of analyte, besides this, there are peaks eluting with various composition of eluent due to the steep gradient. The peak eluting at 5.9 min was identified as 'Monoester', the one at 10.3 min — as 'Triester'. The applied gradient elution conditions (Table 1) were attended to be used as the 'Generic' method for various BAPTA diesters.

3.2. Attempt of analysis of DP-109 by 'Generic' HPLC method for BAPTA diesters analysis

The starting point for the developing of the HPLC method for DP-109 was the analysis of DP-109 by use of the 'Generic' HPLC method for BAPTA diesters (Section 3.1).

As expected, the strong hydrophobic interaction between RP stationary phase and the C18 aliphatic chains of diester prevented the elution of DP-109 from the column. Therefore it was decided to adjust this method for DP-109 by choosing the alternative the chromatographic column and/or adjustment other parameters if necessary.

3.3. Adjustment of the 'Generic' HPLC method for BAPTA diesters for analysis of DP-109

3.3.1. Alternative column choice

Due to the strong hydrophobic interactions as mentioned above, it was decided to replace more hydrophobic C18 column with less hydrophobic C8 column with no success. Then the CN column was tried, the peak was eluted, but could not be properly shaped. Finally, the C4 column with the 'Generic' gradient conditions (Table 1) succeeded to elute better-shaped peak of DP-109 (Fig. 6).

The peak of DP-109 has a retention time (RT) of 16.62 min, this peak was absent in chromatographic run of blank (solvent) sample.

This peak is characterized by high tailing factor and low repeatability of peak area. The RSD for five replicate injections of the same sample was about 7%, while constant decrease in peak area was observed.

All this could be attributed to low solubility of DP-109 in acetonitrile. Therefore it was decided to substitute acetonitrile with methanol in gradient elution.

3.3.2. Analysis of DP-109 using gradient elution 1% acetic acid–methanol

The analysis of DP-109 was performed using the column C4 and methanol instead of acetonitrile. Other parameters of 'Generic' gradient profile remained unchanged.

These new conditions provided shorter RT (14.78 min) and a slight improvement in peak shape and repeatability. The fact, that the RT of DP-109 was closer to starting point of gradient return (< 15 min), could probably affect the peak shape due to the changes in mobile phase hydrophobicity. The additional factor influencing the peak shape could be the closeness of the peak to the gradient slope (return from 100% methanol). To achieve further improvement of the peak shape, the next step was to prolong the isocratic period of 100% methanol in the gradient (Table 3).

3.3.3. Analysis of DP-109 applying a prolonged 100% methanol period in gradient profile

The HPLC procedure as described in previous section was applied with the only change — the prolongation of isocratic period of 100% methanol to 20 min.

New gradient profile is presented in the Table 3.

No changes in RT of the main peak were observed. In comparison with the previous chromatogram, the slight improvement in peak shape could be seen. But the problem of tailing was still not solved.

The addition of THF to the mobile phase usually decreases the hydrophobic interactions between analyte and RP sorbent, and supports its

affinity towards the mobile phase. This could lead to reduction of peak tailing. Therefore, the addition of 3–5% of THF to methanol (instead of pure methanol for gradient) was made as attempt to improve the peak shape.

3.3.4. Analysis of DP-109 by gradient elution using addition of 3–5% of THF in methanol

As mentioned above, it was decided to replace the pure methanol with 3–5% solutions of THF in methanol to decrease the polarity of the organic part of mobile phase. All the other parameters remained unchanged (Section 3.3.3). The gradient is presented in Table 2, and the chromatography using these conditions is illustrated by Fig. 7.

A significant improvement of peak shape can be observed. The higher is THF content, the narrower is the peak.

4. Conclusions

Chromatographic methods for analysis of a number of BAPTA derivatives have been developed.

The HPLC methods use endcapped metal-free silica based reversed phase columns of low volume, that allows steep gradient from aqueous to organic mobile phase.

No interference of main analyte and its related compounds with gradient system peaks achieved. All the peaks are well-shaped and baseline separated.

Main impurities — the corresponding monoesters of BAPTA — elute between BAPTA and DP-b99 (109).

Versatile detection techniques could be used with the LC procedures, that allows to identify the related compounds.

The developed chromatographic methods appeared to be sensitive, selective, accurate and precise, providing evidence of stability indicating analytical procedure.

References

[1] M. Krakovsky, M. Polyak, A. Kozak, Soc. Neurosci. Abstr. 24 (1) (1998) 215.
[2] Lipophillic diesters of chelating agents, International patent publication WO 99/16741.