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Reversed-phase liquid chromatography of biologically active lipophilic chelators
II. Improvement of chromatographic performance and selected applications in biochemical analysis☆

V. Ioffe*, T. Kalendarev, I. Rubinstein, E. Mizerikhin, E. Tsibulsky
D-Pharm Ltd, Kiryat Weizmann Science Park, PO Box 2313, 76123 Rehovot, Israel

Abstract

Lipophillic derivatives of strong calcium chelator BAPTA—DP-b99 and DP-109—are potential drug substances for cerebrovascular and neurodegenerative diseases. The previously published reversed phase HPLC methods for these compounds [6,7] suffered from integration problems due to gradient dip, insufficient repeatability and peak shape. A C_4 column rapidly aged. The addition of acetic acid to the organic part and of ammonium acetate to the aqueous part results in more symmetric peaks, improves method precision and solves integration problems. Washing the column in both directions with a combination of methanol, tetrahydrofuran and water extends its use. The improved methods are sensitive, selective, reproducible, and stability indicating. Impurities and degradation products were identified by LC–MS. Versatile detection techniques can be used with these HPLC methods, allowing performance of bioanalysis with mass spectrometric or flow scintillation detectors. The bioanalytical application of these methods is illustrated by examples of pharmacokinetic and metabolic studies with the labeled compounds.

Keywords: Gradient elution; Chelation; Lipophilicity; BAPTA; DP-b99; DP-109; Bis(aminophenyloxyethane)tetra acetic acid

1. Introduction

Long-chain aliphatic esters of the calcium chelator BAPTA (1,2-bis(2-amino phenyloxyethane)-*N*,*N*,*N′*,*N′*-tetra acetic acid), have demonstrated efficacy in several animal models of cerebral ischaemia [1–3]. These compounds were designed in order to enhance membrane penetration. This lipid modification makes the molecule lipophilic, whilst retaining its chelating potency for various bivalent metal ions [2]. Due to the high lipophilicity of the BAPTA esters, they can restrict their chelating capability to lipophillic environments, such as cell membranes and control transport of metal ions across cell membranes (membrane-activated chelators (MAC) technology [3]). Preclinical studies with DP-b99 have shown a significant reduction in infarct volume in several animal models of cerebral ischaemia, thereby indicating its therapeutic potential [4,5].

☆For Part I, see Ref. [6].

*Corresponding author. Tel.: +972-8-930-0794x137; fax: +972-8-930-0795.
E-mail address: vioffe@dpharm.com (V. Ioffe).

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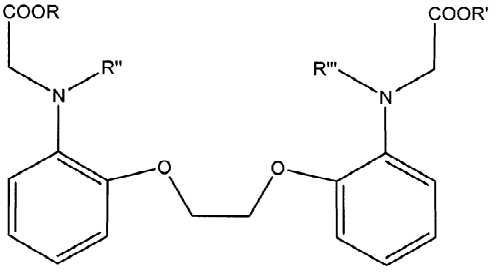
In our previous paper [6] we described the development of HPLC methods for two of the lipophillic BAPTA esters: DP-b99 (Fig. 1, Ib), which is currently being pursued in a Phase II clinical study and DP-109 (Fig. 1, Il), which is in the preclinical stages. In this paper we discuss the means to improve the performance of these methods and their adjustment for bioanalytical applications.

2. Experimental

2.1. Reagents and materials

BAPTA was purchased from Teflabs (Austin, TX, USA) and BioLab (Jerusalem, Israel). DP-b99, DP-109 and their tritium-labelled analogues were synthesized by the Chemistry Department, D-Pharm



I a-m

Code	Name of Compound	R	R'	R'''	R''
Ia	**BAPTA**	H	H	CH_2COOH	CH_2COOH
Ib	**DP-b99**	$CH_2CH_2OC_8H_{17}$	$CH_2CH_2OC_8H_{17}$	CH_2COOH	CH_2COOH
Ic	Monoester related to DP-b99	H	$CH_2CH_2OC_8H_{17}$	CH_2COOH	CH_2COOH
Id	Monoester-monoethylester	CH_2CH_3	$CH_2CH_2OC_8H_{17}$	CH_2COOH	CH_2COOH
Ie	Monoester with 2 eliminated acetic acid moieties	H	$CH_2CH_2OC_8H_{17}$	H	H
If	DP-b99 with eliminated acetic acid moiety	$CH_2CH_2OC_8H_{17}$	$CH_2CH_2OC_8H_{17}$	H	CH_2COOH
Ig	DP-b99 decarboxylated	$CH_2CH_2OC_8H_{17}$	$CH_2CH_2OC_8H_{17}$	CH_3	CH_2COOH
Ih	DP-b99 twice decarboxylated	$CH_2CH_2OC_8H_{17}$	$CH_2CH_2OC_8H_{17}$	CH_3	CH_3
Ii	Triester related to DP-b99	$CH_2CH_2OC_8H_{17}$	$CH_2CH_2OC_8H_{17}$	CH_2COOH	$CH_2COOCH_2CH_2OC_8H_{17}$
Ij	BAPTA dianhydride	R + R'' = R' + R''' = $C(O)CH_2$			
Ik	BAPTA monoanhydride	H	R' + R''' = $C(O)CH_2$		CH_2COOH
Il	**DP-109**	$CH_2CH_2OC_{18}H_{35}$	$CH_2CH_2OC_{18}H_{35}$	CH_2COOH	CH_2COOH
Im	Monoester related to DP-109	H	$CH_2CH_2OC_{18}H_{35}$	CH_2COOH	CH_2COOH
In	Triester related to DP-109	$CH_2CH_2OC_{18}H_{35}$	$CH_2CH_2OC_{18}H_{35}$	CH_2COOH	$CH_2COOCH_2CH_2OC_{18}H_{35}$

Fig. 1. Lipophilic chelators and their related compounds.

(Rehovot, Israel). Water, acetonitrile and methanol HPLC grade were obtained from Merck (Darmstadt, Germany) or BDH (Poole, UK). Tetrahydrofuran (THF), HPLC grade, was purchased from J.T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid 99+%, analytical grade, was provided by Sigma (St. Louis, MO, USA). *N*,*N*-Dimethylformamide (DMF) was purchased from BioLab. Ammonium acetate HiPerSolv for HPLC was purchased from BDH. Scintillation fluid Ultima-Flo M was purchased from Packard Bioscience (Groningen, The Netherlands)

2.2. Preparation of solvents and standards

BAPTA, DP-b99 and DP-109 free acids are practically insoluble both in water and in 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 a final working concentration of ~1 mg/ml. DP-109 free acid was dissolved in a mixture of methanol—THF (1:1) to obtain a solution at a concentration of ~1 mg/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

A LaChrom automatic HPLC system (Merck–Hitachi, Darmstadt, Germany/Tokyo, Japan) consisting of L-7100 solvent delivery system, L-7200 autosampler, L-7400 multi-wavelength UV–Vis detector and L-7000 interface, combined with auxiliary flow scintillation analyzer Radiomatic 150TR (Packard, Meriden, CT, USA), was used. An Alliance HT 2790 separation module automatic HPLC system with UV–Vis photodiode array detector 996 (Waters, Milford, MA, USA), was used, equipped with an auxiliary MS detector LC Platform (Micromass, Manchester, UK). A TSP automatic HPLC system (ThermoFinnigan, San Jose, CA, USA), consisting of P-4000 solvent delivery system, AS-3000 autosampler, UV-6000LP diode array UV–Vis detector and SN-4000 interface, was used. The HPLC column for DP-b99 was Purospher RP-C_{18}, 5 μm, 125×3.0 mm (Merck), while the HPLC column for DP-109 was Hypersil HyPurity™ C_4, 5 μm, 100×4.6 mm (ThermoHypersil, Bellefonte, PA, USA). Column temperature was 25±3 °C.

The initial mobile phase compositions was a gradient from 1% aqueous acetic acid (solvent A) to acetonitrile (solvent B) for analysis of DP-b99 and a gradient from 1% aqueous acetic acid (solvent A) to 5% THF in methanol (solvent B) for DP-109. In both methods the flow-rate was 1.0 ml/min, and the gradient profile was: pure solvent A for 1 min, then linear gradient to pure solvent B in 4 min, followed by return to pure solvent A after 22 min. The working concentration was 1.0 mg/ml. The injection volume was 20 μl. For UV detection the detectors were set at 250 nm.

MS detection is performed using the electrospray ionisation (ESI) ion source, after splitting the flow between the UV and MS detectors. Conditions were as follows: flow rate before the MS detector 0.3 ml/min; nebulizer gas, nitrogen, 350 l/h; probe temperature 100 °C; capillary voltage 3.3 kV; cone voltage +20 V for the positive ions mode and −30 V for the negative ions mode. The flow rate of scintillation fluid for flow scintillation detection was 1.0 ml/min.

3. Results and discussion

3.1. Further development of HPLC method for DP-b99

The analytical HPLC method used for testing DP-b99 [6,7] had at least two problems. (i) The problem of analytical technology transfer to outsourcers/subcontractors. Software used by some of the HPLC systems has a small negative cut-off (Fig. 2), which does not allow reliable quantitation and detection of peaks appearing on the gradient ''dip'' (negative absorption of the gradient baseline due to different transparency of solvents in UV). (ii) Non-symmetrical shape (with substantial tailing) of the peaks of some of the derivatives of DP-b99 caused unreliable results for quantitation of related compounds of DP-b99 in samples. This is particularly

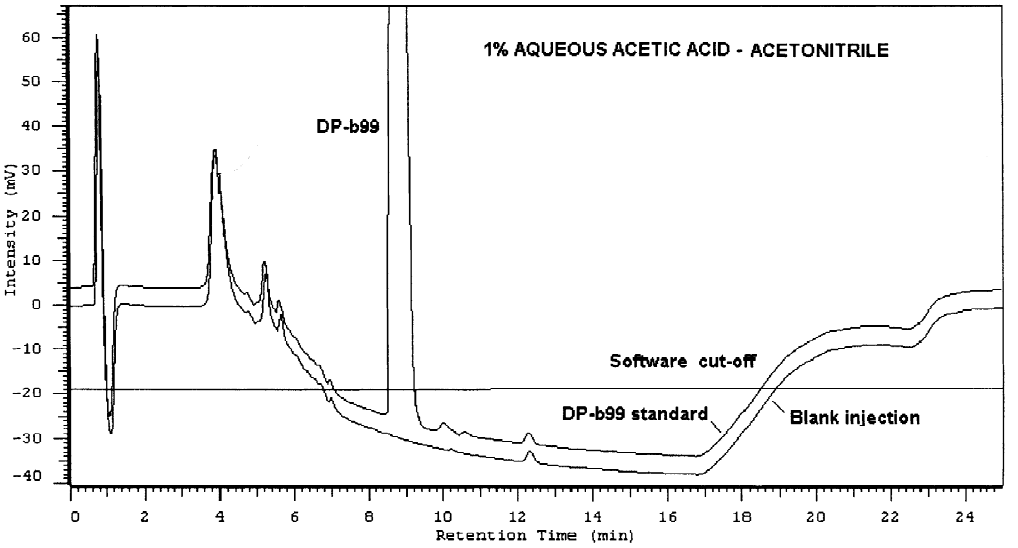


Fig. 2. Gradient dip and integration problems.

important for stability studies and in the analysis of DP-b99 metabolism/digestion in biological samples.

The quality of chromatographic separation between the peaks of DP-b99 and its synthetic impurities in the initial method was satisfactory. At least baseline separation was achieved for any two neighbouring peaks. Therefore, there was no basis for substitution of the chromatographic column or for drastically changing the chromatographic conditions, which provided sufficient chromatographic performance.

3.1.1. Gradient ''dip'' problems: experiments with acetic acid

When using the initial mobile phase composition together with the software having a small negative cut-off (for instance, the software of Perkin-Elmer), the gradient ''dip'' does not allow integration of the whole DP-b99 peak (Fig. 2). This may also result in some impurity peaks being missed. After the addition of acetic acid to solvent B (in the same concentration as used in solvent A), the increase of its UV absorption resulted in relief of the gradient ''dip'' thus solving the integration problem (Fig. 3).

3.1.2. Peak shape and resolution: experiments with ammonium acetate

In the course of experiments aimed at determining the structure of the impurities of DP-b99 using LC–MS, a small amount of ammonium acetate (0.07%, w/v) was added to the aqueous part of the mobile phase in order to enhance the ionisation by means of the ESI ion source.

When comparing any two UV chromatograms obtained with or without the addition of ammonium acetate, the following observation was made: the height and the area of the peak of the first eluting impurity (the corresponding BAPTA monoester, see Fig. 1, Ic) in the presence of ammonium acetate were substantially higher than in its absence (Fig. 4A,B). No influence on shape or retention of other peaks was observed. It was also proven that the addition of ammonium acetate to the mobile phase does not cause any degradation of DP-b99.

The analysis of the chemical structure of the monoester brought us to the following assumption. The monoester is the only related compound of DP-b99 bearing more carboxyl groups that the main compound (three carboxyl groups, as compared to one or two of all the other substances). Hence, its polarity, hydrophilicity and capability of binding to the stationary phase should be much higher than that of the others. This assumption is also supported by the fact, that in the aged columns the retention time of the monoester could become a little higher, and it can result even in coelution with the next eluting

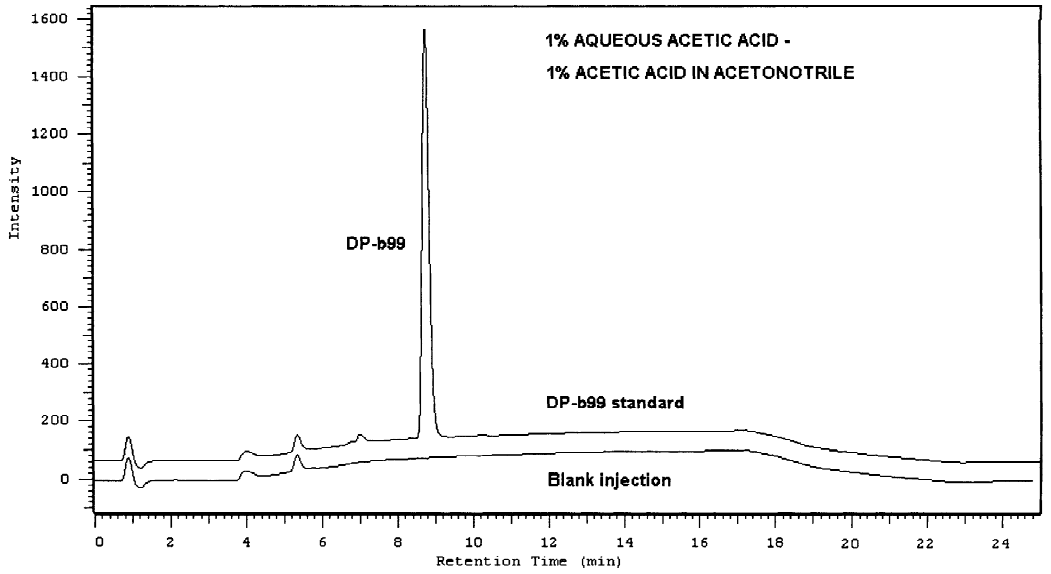


Fig. 3. Modified mobile phase: the solution of gradient dip problems.

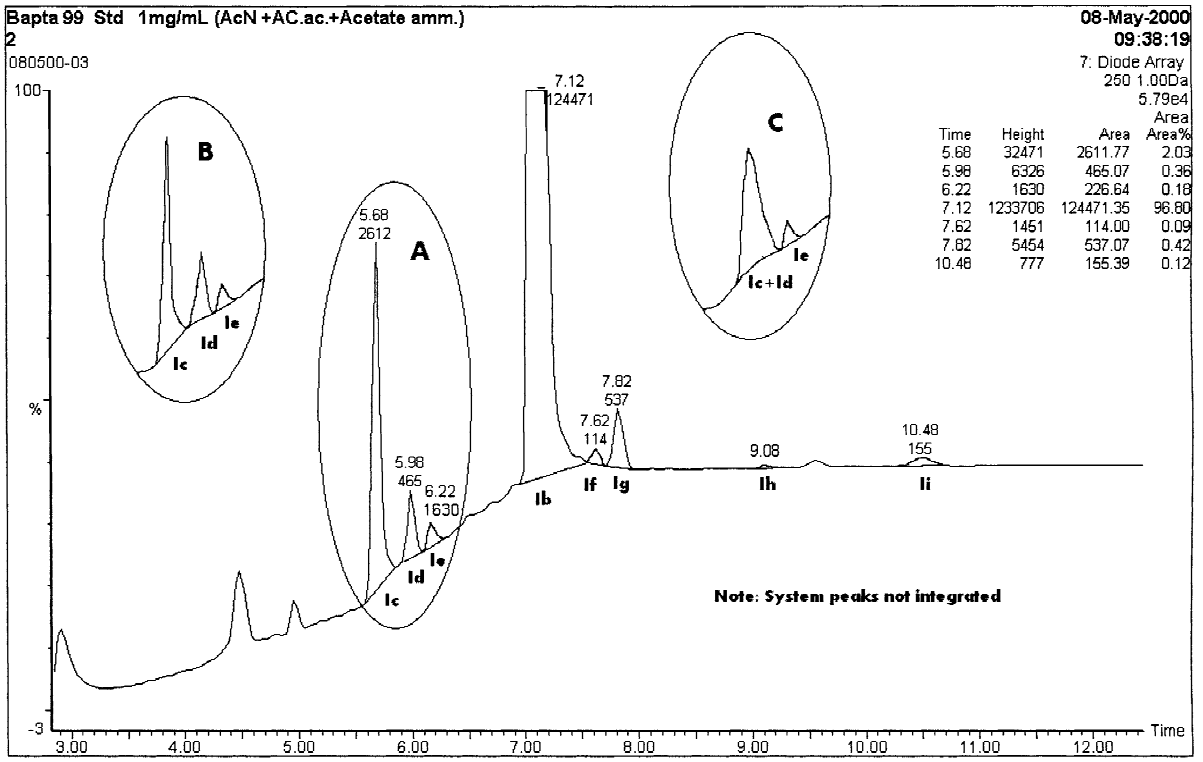


Fig. 4. Chromatographic profile of DP-b99: separation and identification of related compounds. A, mobile phase with added ammonium acetate; B, mobile phase without ammonium acetate, new column; C, mobile phase without ammonium acetate, aged column.

peak (Fig. 4C), i.e. monoester-monoethylester (Fig. 1, Id). The retention time of other impurities, bearing less carboxyl groups, is not affected.

Addition of small amounts of a strong electrolyte—ammonium acetate—results in a greater tendency of the monoester towards the mobile phase, most probably due to both higher ion strength and pH and, therefore, more efficient elution from the stationary phase. All this improves the peak shape (sharp instead of diffused) and, consequently, the accuracy of the integration. The problem of coelution of the two first impurity peaks is also solved—in the presence of ammonium acetate these peaks are baseline separated even when running the procedure on substantially aged columns.

3.2. Structure elucidation of related compounds of DP-b99

3.2.1. Synthetic pathway: estimation of possible synthetic impurities

Preparation of DP-b99 (Fig. 1, Ib) is a multistage synthesis [2]. The starting materials of this synthesis are BAPTA (Ia) and 2-(octyloxy)ethanol, which can be purchased or prepared, for instance, from 1-bromooctane and 1,2-ethanediol. In the first stage BAPTA (Ia) is converted into its dianhydride (Ij) by means of refluxing with an excess of acetic anhydride. Then BAPTA dianhydride (Ij) reacts with 2-(octyloxy)ethanol to produce DP-b99 (Ib) in the form of free acid. The final stage is the conversion of DP-b99 acid into its disodium salt by reacting with sodium methylate, ethylate, or hydroxide.

The most critical stage is the purification process of the final product. It involves the successive crystallization of DP-b99 disodium salt from ethanol, diethyl ether, and hexane, followed by freeze-drying in vacuum. As a result of the chemical synthesis and the purification process, possible impurities could be: monoester (Ic) (probably resulting from disproportionation of Ib or from esterification of monoanhydride Ik impurity of Ij); triester (Ii) (which possibly results from disproportionation of Ib); and monoester-monoethylester (Id) (most likely a product of further ethanolic esterification of Ic).

3.2.2. Stability studies: estimation of possible degradation products

DP-b99 disodium salt is a highly hygroscopic compound, displaying substantial sensitivity to light and heat. Stability testing and forced degradation studies of DP-b99 disodium salt also highlight its strong tendency to alkaline hydrolysis (saponification) and destructive processes following autooxidation. Acidic hydrolysis converts the ester groups into free carboxylic, which can then easily decarboxylate, especially upon heating.

Therefore, the most likely products to be subject to degradation could be the compounds obtained by desalkylation, decarboxylation, or elimination of acetic acid moiety (most probably as beta-split of initial carbonyl radical originated in homolytic oxidation), and the combinations thereof. These could be: monoester (Ic), monoester with eliminated one or two acetic acid groups (Ie,f), and mono- and twice decarboxylated DP-b99 (Ig,h).

3.2.3. Analysis of minor peaks: structure elucidation of related compounds of DP-b99

The criteria for structure elucidation of the related compounds are: the similarity of chromophore for all the peaks (using LC–diode array detector), molecular masses (LC–MS) for all the peaks and order of elution of peaks (pointing at the relative polarity and lipophilicity), which also supports structure elucidation.

The characteristic UV spectrum of DP-b99 includes three maxima at about 210, 250, and 288 nm (Fig. 5). The same chromophore should remain for the related compounds, bearing the same core molecular structure. Therefore, for all the minor peaks appearing in the chromatogram of DP-b99 (Fig. 4A), the UV spectra were examined. Taking into account the baseline noise and drift (caused by the gradient), all the observed UV spectra provided evidence of similar chromophore, as compared to the main peak of DP-b99, as well as to unsubstantiated BAPTA injected separately.

This important information suggesting the chemical structures of the impurities was the result of molecular mass determination using the MS detector attached to the HPLC instrument. The decision to consider the found mass to be a protonated molecule, $[M+H]^+$, having the $(M+1)$ mass (as compared to the impurity molecule), was based on the ability of the molecules (and not their fragments) to form adduct cations under the applied ionisation conditions. The confirmation of the molecular ions was

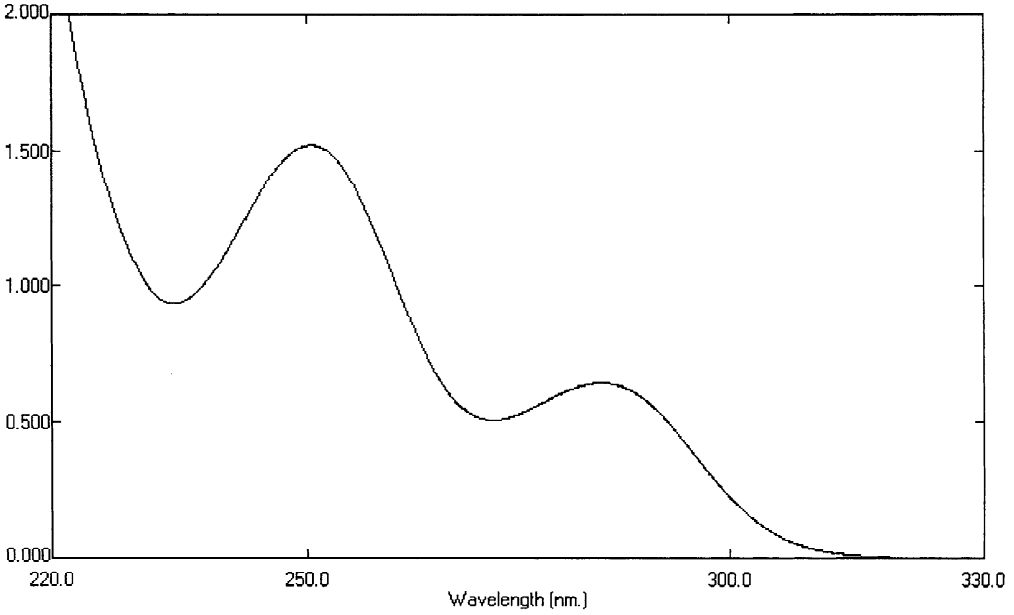


Fig. 5. UV-spectrum of BAPTA and its derivatives.

the formation of their sodium adducts, $[M+Na]^+$, having the (M+23) mass, and the difference of 22 u from the mass of the protonated molecule. All the obtained masses were checked to match the calculated values, using the molecular mass calculating program (part of MassLynx software for LC–MS) in the single isotope calculating mode.

The order of elution served as an additional means to confirm the structure. The main factors, determining the retention on the reversed-phase column, are the polarity and lipophilicity of the compound. Therefore, the compounds with eliminated carboxyl groups or acetic acid moieties elute after the parent compound even with substantially lower molecular masses, because they lose very polar groups and hence become more lipophilic.

The identification of related compounds is summarized in Table 1.

Table 1
Mass-spectral identification of related compounds of DP-b99

No.	Code	Identification	MS data (rounded)		HPLC data	
			$[M+H]^+$	$[M+Na]^+$	RT, min	RRT
1	Ic	BAPTA monoester	633	655	5.68	0.80
2	Id	BAPTA monoester monoethylester	661	683	5.98	0.84
3	Ie	BAPTA monoester with two eliminated acetic acid moieties	517	539	6.22	0.87
4	Ib	DP-b99 (BAPTA diester), main	789	811	7.12	1
5	If	DP-b99 (BAPTA diester) with one eliminated acetic acid moiety	731	753	7.62	1.07
6	Ig	DP-b99 (BAPTA diester) decarboxylated	745	767	7.82	1.10
7	Ih	DP-b99 (BAPTA diester) twice decarboxylated	701	723	9.08	1.28
8	Ii	BAPTA triester	945	967	10.48	1.47

RT, retention time.
RRT, relative retention time.

3.3. Further development of HPLC method for DP-109

The previous analytical HPLC method for testing DP-109 [6,7] had the following problems: low repeatability; low precision between standard injections (RSD could exceed 3%) with a tendency of peak area to decrease from run to run; unsatisfactory peak shapes, which did not allow reliable quantitation of minor peaks (related compounds); and rapid aging of columns (back pressure increase and broadening of peaks), which resulted in column change once every 2–3 weeks.

3.3.1. Improving method performance

Low precision of the HPLC method could be attributed to the non-symmetrical peak shape which affects integration (Fig. 6). Insufficient quality of elution of DP-109 from the stationary phase was noticeable by the tendency of the peak area to decrease from run to run (Table 2, Initial).

To improve elution, the quantity of THF in organic solvent B was slightly increased, and acetic acid was added at the same concentration as used in solvent A (test 1). This resulted in visible straightening of the baseline (the gradient ''hill'' was less distinct) and improvement in precision due to more complete elution, where the tendency of the peak area to decrease now disappeared (Table 2).

In the course of experiments aimed at confirming the structure of the impurities of DP-109 using LC–MS, a small amount of ammonium acetate (0.07%, w/v) was added to the aqueous part of the mobile phase in order to enhance ionization in the ESI ion source. It was observed that in presence of ammonium acetate, added either to the aqueous part (test 2) or to both aqueous and organic components of the mobile phase (test 3), the shape of the main peak and impurity peaks substantially improved (Figs. 7 and 8; Table 2). Exactly as in the case of DP-b99, the height and the area of the peak of the first eluting impurity (corresponding BAPTA monoester) in the presence of ammonium acetate were substantially higher than in its absence. Accordingly, the method became more precise (Table 2). It was also proven that the addition of ammonium acetate to

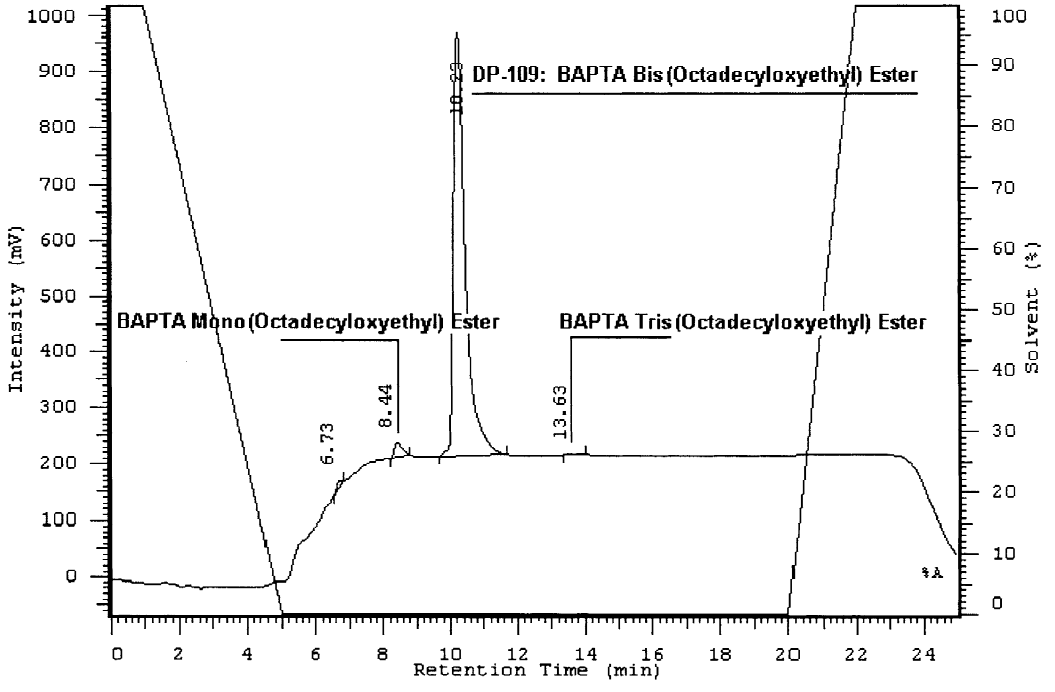


Fig. 6. Chromatogram of DP-109 using the initial phase composition. Both the main peak and the peak of the monoester are broadened.

Table 2
DP-109: repeatability and chromatographic performance

Mobile phase	Initial		Test 1		Test 2		Test 3[a]	
Peak area	11 363 050	14 070 575	11 413 291	14 817 806	13 196 647	11 501 135	6 630 234	7 095 536
	11 126 616	13 769 553	11 450 124	14 762 268	13 086 144	11 484 541	6 647 695	7 131 661
	11 013 590	13 545 273	11 431 874	14 842 696	13 348 756	11 589 290	6 633 350	7 125 321
	10 766 603	13 353 130	11 433 920	14 761 009	13 145 460	11 570 664	6 625 107	7 110 494
	11 246 078	12 961 552	11 377 884	14 864 667	13 194 091	11 675 710	6 656 734	7 134 138
RSD (%)	2.1	3.0	0.4	0.3	0.7	0.7	0.2	0.2
Tailing factor (USP)	1.59	1.29	1.08	1.15				
Peak width on baseline	2 min		1.6 min		1.4 min		1.3 min	
Efficiency (theoretical plates)	2600		5600		8000		7300	

[a] In test 3 the work was performed on another instrument providing different response factors.

the mobile phase does not cause any degradation of DP-109.

3.3.2. Optimization of column wash cycle

Rapid aging of C_4 columns resulted in a necessary HPLC column change once every 2–3 weeks. The deterioration was recognized as back pressure increase and broadening of peaks. It was supposed that the decline of the column could have been caused by insufficient or improper column wash. The routinely used column wash procedure was the same solvent gradient, as in the HPLC method, but without buffer salts, run in triplicate.

The improved wash cycle, which included initial reversed-direction column rinse, was tested in comparison with the usual procedure. The wash cycle comprised 30 min of reversed-direction rinse with methanol, containing 10% THF, then 30 min of

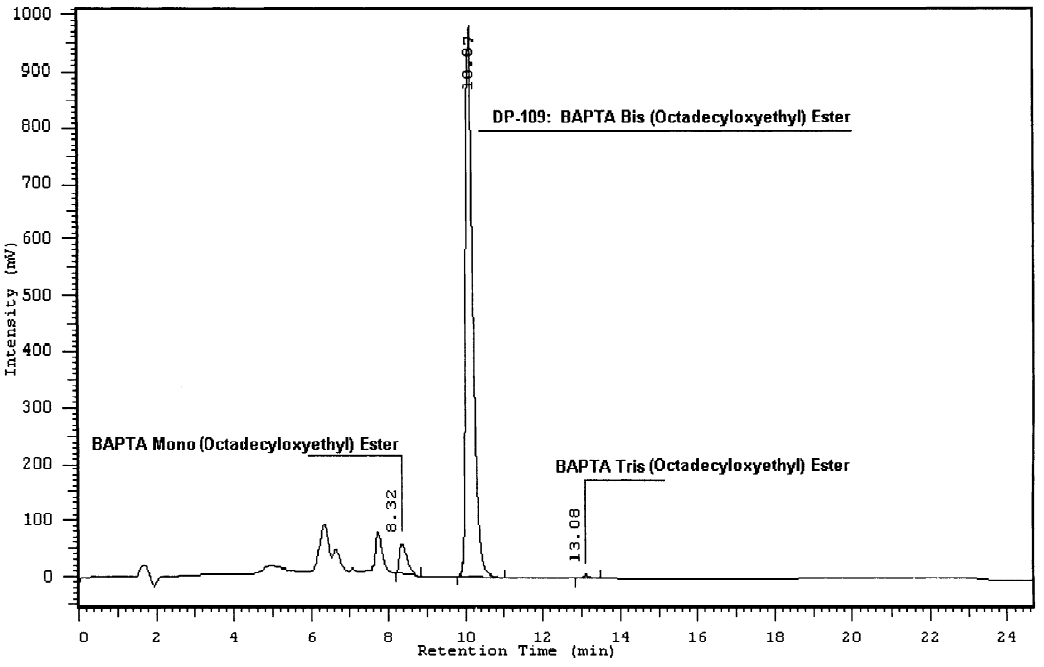


Fig. 7. Chromatogram of DP-109 using the test 2 mobile phase composition. Both the main peak and the peak of the monoester are much narrower. Gradient ''hill'' disappeared.

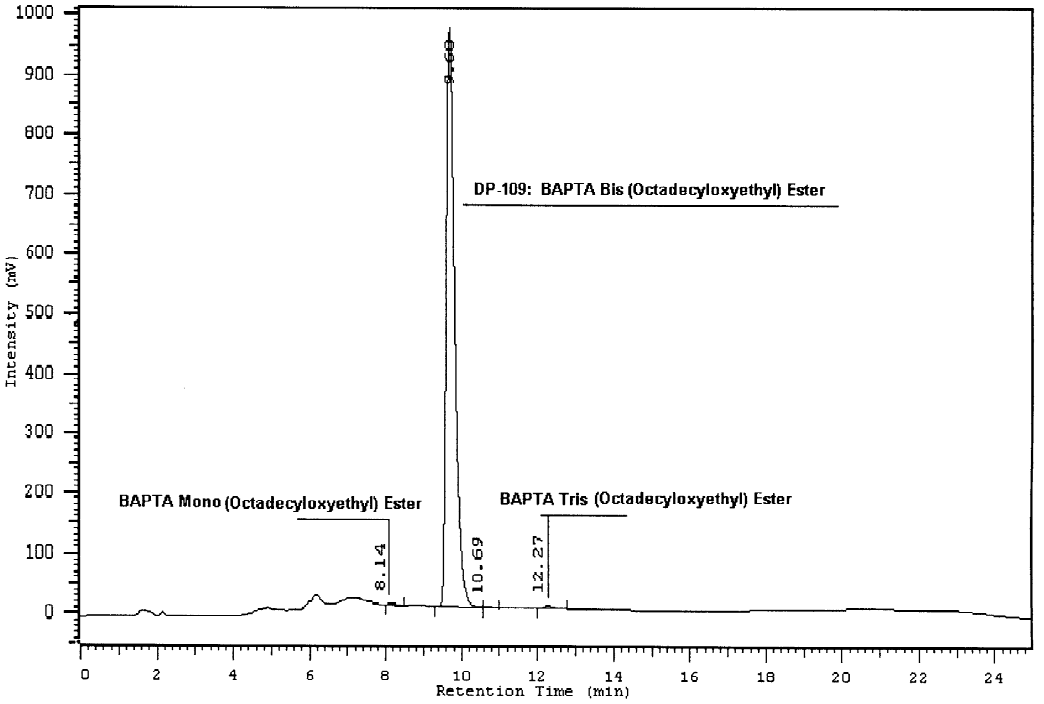


Fig. 8. Chromatogram of DP-109 using the test 3 mobile phase composition.

normal-direction rinse with pure methanol, followed by an additional 30 min using methanol–water (85:15). This procedure solved the problem of column deterioration. The aged columns, which were out of use, were successfully regenerated running this washing scheme, and are currently in use again.

3.4. Bioanalytical applications of HPLC methods for DP-b99 and DP-109 using labeled compounds

Running the HPLC procedure using very sensitive and specific detection methods, such as MS, MS–MS, or various radioactive counters (in the case of work with compounds, labelled with radioactive isotopes), can provide various bioanalytical applications of the method. The technique was successfully used for monitoring pharmacokinetic and metabolic studies.

As an example, applying the chromatographic method, followed by detection on flow scintillation analyser, provides analytical support for in-vitro metabolic studies of tritium-labelled DP-b99 (tritium atoms substitute the protons in the acetic acid moieties). Monitoring the kinetics of metabolism in rat whole blood is illustrated by chromatograms (Fig. 9), thereby presenting evidence of the metabolic transformation of DP-b99 (Ib) into the corresponding monoester (Ic).

The limitations of UV detection, even in the presence of strong chromophore, are attributed to the numerous peaks from the biological matrix, which reduce method sensitivity and specificity (Fig. 10). The work with radioactively labelled compounds, together with the developed HPLC procedure ensures a powerful bioanalytical tool.

Another example of the application of the chromatographic procedure followed by detection on flow scintillation analyser is monitoring the concentrations of DP-109 (Il) and corresponding monoester (Im) in rat serum after intravenous administration (Fig. 11). This provides analytical support for in-vivo pharmacokinetic studies using the tritium-labelled DP-109 (tritium atoms substitute the protons in the acetic acid moieties).

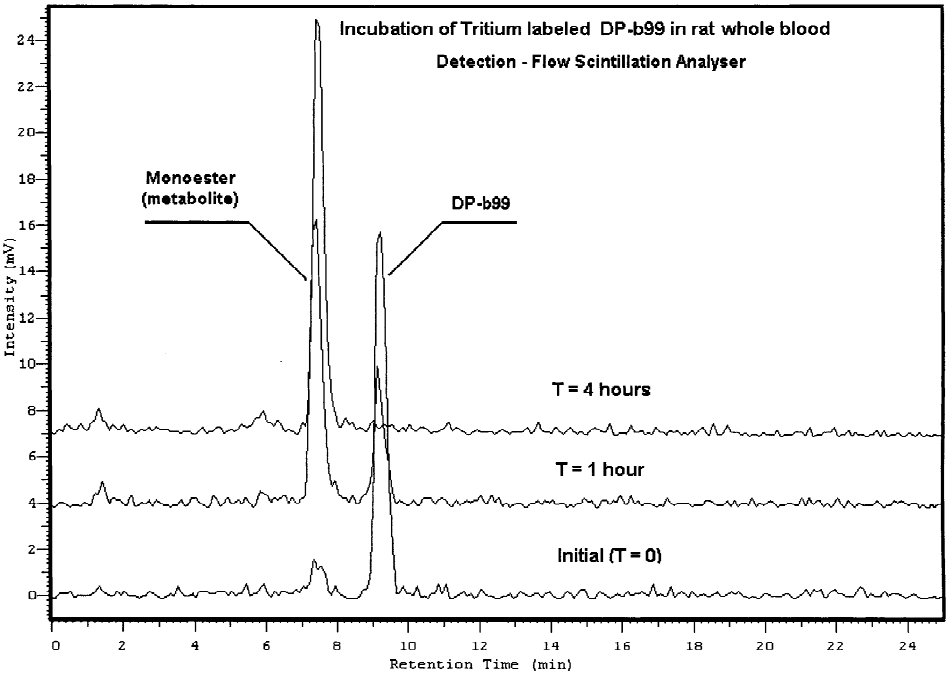


Fig. 9. HPLC monitoring of in vitro metabolic studies of tritium-labeled DP-b99 using flow scintillation detector.

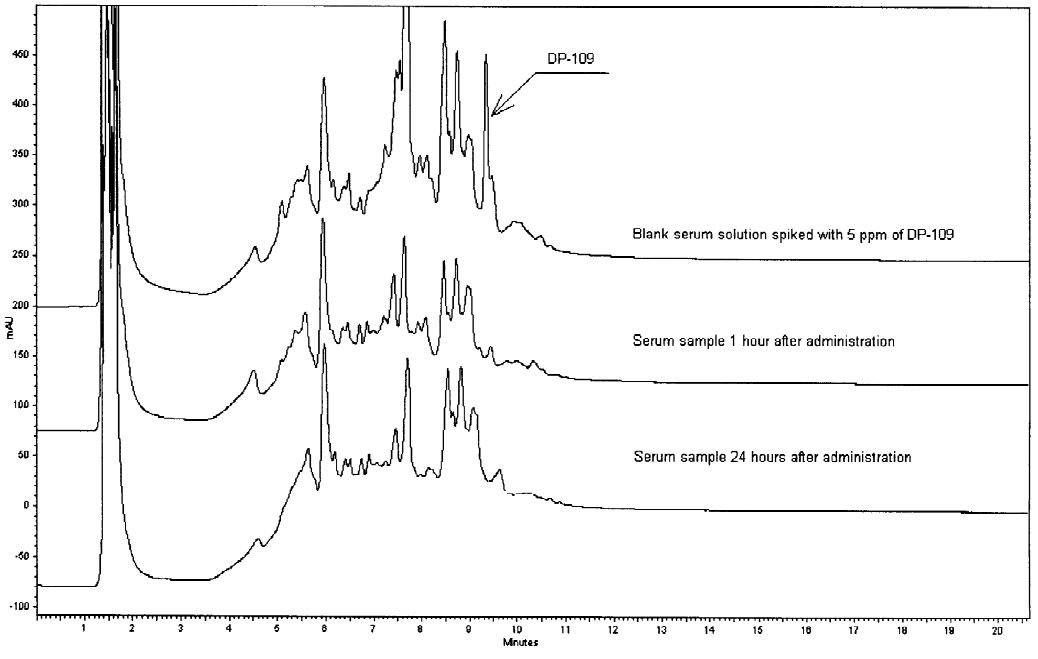


Fig. 10. Monitoring PK studies of DP-109 using UV detection at 250 nm.

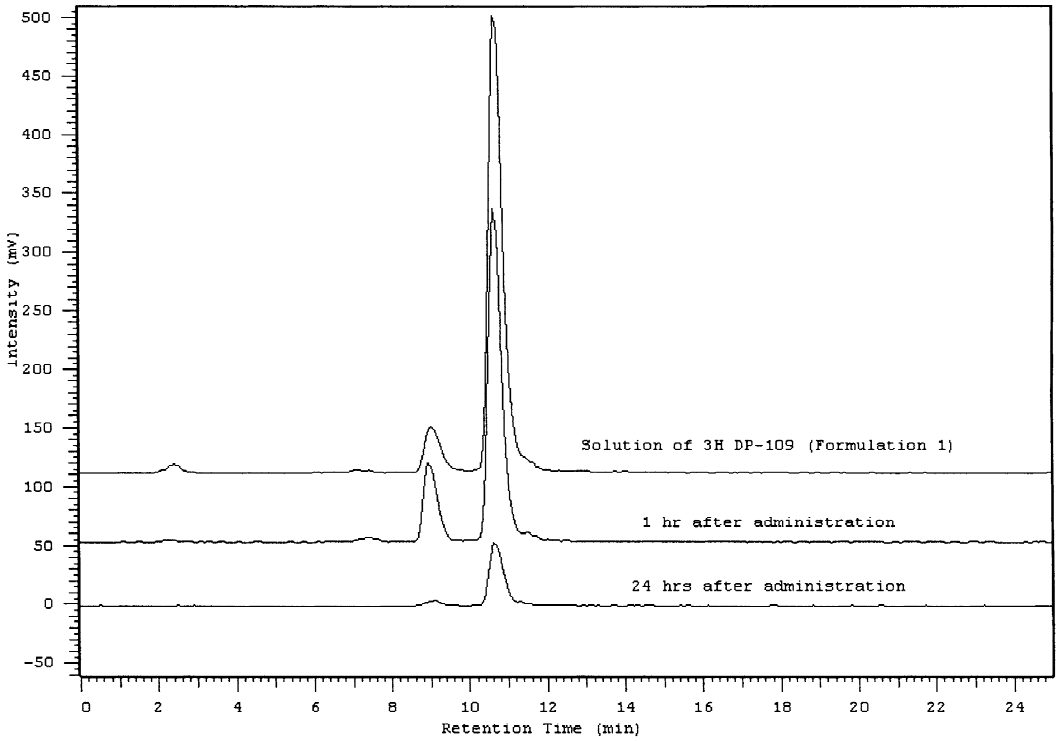


Fig. 11. Monitoring PK studies of tritium-labeled DP-109 using scintillation detector chromatographic performance.

4. Conclusions

(1) The further development of HPLC methods for DP-b99 and DP-109, by modifying the mobile phase composition, has resulted in substantial improvements in chromatographic performance and precision.

(2) This has allowed identification of all potential impurities and degradation products by applying the LC–MS technique.

(3) The method is easily adjustable to be run as a bioanalytical procedure applying mass-spectrometric or flow scintillation detectors.

(4) A novel column washing procedure was developed for the HPLC method for DP-109, which prevented column deterioration and is capable of regenerating aged columns.

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