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Reversed-phase high-performance liquid chromatography of polar lipids II. Procedure for a phospholipid derivative of methotrexate☆

V. Ioffe*, T. Kalendarev, I. Rubinstein, R. Zabetski, G. Zupkovitz[1]

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

Abstract

Phospholipid derivatives of methotrexate (MTX) having enhanced membrane penetration (DP-71 being the most important) are potential prodrugs for treatment of autoimmune and inflammatory diseases as well as diseases involving abnormal cell proliferation. The previously published reversed-phase HPLC methods for similar compounds, phospholipid derivatives of valproic acid and non-steroidal anti-inflammatory drugs (NSAIDs), could not be used for MTX derivatives due to highly basic character of the MTX core molecule. The new HPLC procedure using gradient elution was developed as a compromise between the pharmacopoeial method for MTX and the previous "generic" procedure for phospholipid derivatives of NSAIDs. The newly developed method is sensitive, selective, reproducible, and stability indicating. Identification of major related compounds was carried out. The bioanalytical applications of this method, as well as of the derived isocratic procedure, are discussed and illustrated by examples of pharmacokinetic studies.

Keywords: Pharmaceutical analysis; Gradient elution; Lipids; Phospholipids; Methotrexate

1. Introduction

Phospholipid derivatives of methotrexate (MTX, Fig. 1) are potential prodrugs for treatment of autoimmune disorders, such as psoriasis, diseases involving abnormal cell proliferation, such as tumors, and inflammatory diseases, such as rheumatoid arthritis [1]. These compounds were designed in order to lipophilize the MTX molecule thus enhancing its membrane penetration, which is the further development of the core *D-RAP* (regulated activation of prodrugs) technology of prodrug design, applied to anti-epileptic [2] and non-steroidal anti-inflammatory drugs (NSAIDs) [3]. These molecules may specifically release the active drug at the target site following cleavage by phospholipase A2 (PLA2) associated with excitatory or inflammatory processes.

In our previous publications [4,5], we described the development of isocratic RP HPLC methods for the phospholipid derivatives of valproic acid and several NSAIDs. These methods could not be applied directly to the similarly designed derivatives of MTX due to strongly basic character of the MTX core and, therefore, the whole molecule.

In this paper, we report the development of RP-HPLC methods for analysis of phospholipid derivatives of methotrexate using as an example DP-71 (Fig. 2), the most promising compound in this series, which showed a remarkable anti-proliferative effect on drug-resistant cells [1]. High quality chromatographic separation is required for discriminative analytical methods attended for release and stability testing, as well as for bioanalytical procedures, especially to support metabolic studies. Optimisation of the performance of these methods and their adjustment for bioanalytical applications are discussed.

2. Experimental

2.1. Reagents and materials

MTX drug substance (US Pharmacopoeia) was purchased from Abbott Labs (Abbott Park, IL, USA). DP-71 and its related compounds were synthesized by the Chemical Department, D-Pharm (Rehovot, Israel). Water, acetonitrile and methanol (HPLC-grade) were obtained from Merck (Darmstadt, Germany) or BDH (Poole, UK). Tetrahydrofuran

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* Corresponding author. Fax: +972-8-9300795.
E-mail address: vioffe@dpharm.com (V. Ioffe).
URL: http://www.dpharm.com.
[1] Present address: Institute of Medical Biochemistry, Division of Molecular Biology, Dr. Bohrgasse 9/2, A-1030 Vienna, Austria.

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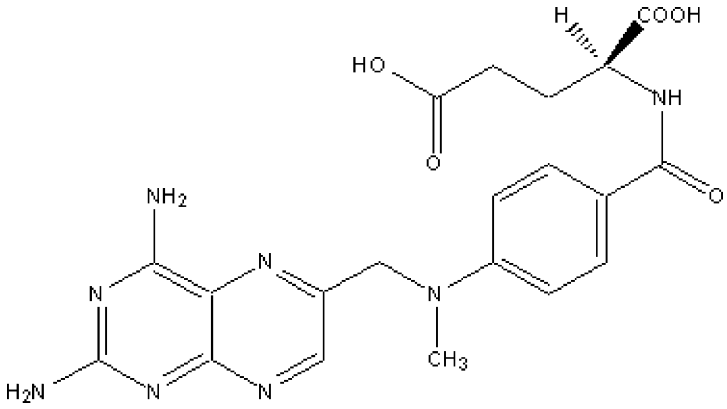

Fig. 1. Methotrexate (MTX) structure.

(THF) (HPLC-grade) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid >99%, citric acid, and sodium hydrogenphosphate decahydrate, analytical grade, were provided by Sigma (St. Louis, MO, USA). *N*,*N*-Dimethylformamide (DMF) was purchased from Bio-Lab (Jerusalem, Israel). Ammonium Acetate HiPerSolv for HPLC was purchased from BDH.

2.2. Chromatographic instrumentation and equipment

The following equipment was used:

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 equipped with 10 mm flow cell, and L-7000 interface.

An Alliance HT 2790 separation module automatic HPLC system with a UV-Vis photodiode array detector (PDA) 996 having a 10 mm flow cell (Waters, Milford, MA, USA), equipped with 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 equipped with 50 mm flow cell, and SN-4000 interface.

HPLC columns: Inertsil C_4, 5 μm, 4.6 mm × 150 mm (GL Sciences, Japan), Luna C_5, 5 μm, 150 mm × 4.6 mm

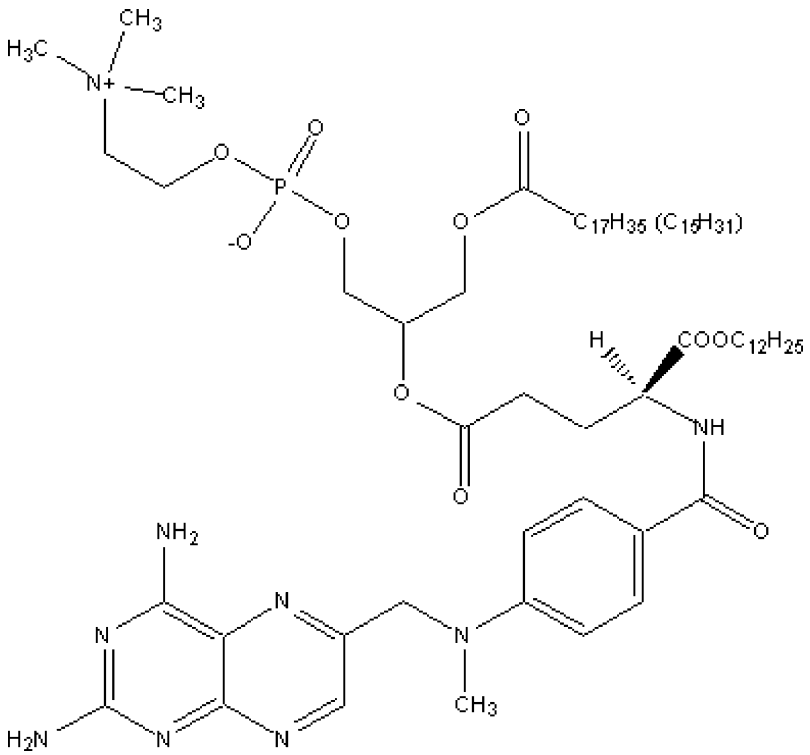

Fig. 2. DP-71 structure.

(Phenomenex, USA), Supelcosil LC-ABZ, 5 μm, 250 mm × 4.6 mm (Supelco, USA), Symmetry Shield RP18 Waters, 5 μm, 150 mm × 3.9 mm (Waters, USA).

2.3. Preparation of buffer solutions for mobile phase

Phosphate–citrate buffer solution: 630 ml of 0.2 M solution of disodium hydrogenphosphate is mixed with 370 ml of 0.1 M solution of citric acid and adjusted to pH 6.0 with one of the components.

Acetate–citrate buffer solution: 630 ml of 0.2 M solution of ammonium acetate is mixed with 370 ml of 0.1 M solution of citric acid and adjusted to pH 5.5 or 6.0 (as specified) with one of the components.

2.4. Chromatographic conditions

The variety of mobile phase compositions is presented in Table 1. The gradient profile for methods applying gradient elution is provided in Table 2. The column temperature was 25 ± 3 °C. For drug substances, the working concentration was 0.5 mg/ml and the injection volume 20 μl. For bioanalytical applications, the injection volume was 50 μl. UV detection was at 302 nm. MS detection was performed using the electrospray ionization (ESI) ion source, after splitting the flow between the UV and MS detectors. The flow rate before the MS detector was 0.3 ml/min. The nebulizer gas was nitrogen, 350 l/h. The probe temperature was 100 °C. The capillary voltage was 3.3 kV. The cone voltage was +20 V for positive ions mode and −30 V for negative ions mode.

Table 2
Gradient profile

Time (min)	Buffer solution (%)	Acetonitrile (%)
0	90	10
5.0	90	10
7.5	55	45
15.0	55	45
17.5	10	90
25.0	10	90
27.5	90	10
30.0	90	10

2.5. Preparation of samples for bioanalytical studies

2.5.1. Rat serum and plasma

The samples from serum and plasma were prepared according to three procedures.

Scheme 1: To a 200 μl of serum/plasma sample 100 μl of buffer solution (50 mM of citric acid and 100 mM of disodium hydrogenphosphate, adjusted to pH 5.0) was added and mixed. Then 30 μl of methanol and 600 μl of chloroform were added and vortexed for 20 min. The mixture was centrifuged in Eppendorf tubes for 10 min at 10 000 × *g*. The chloroform (lower) layer was collected and evaporated to dryness. To the residue 200 μl of methanol was added, mixed thoroughly and filtered through a methanol-resistant 0.2 μm membrane filter. The filtrate was injected onto the HPLC system. The recovery of the procedure is 85%.

Scheme 2: To 200 μl of serum/plasma sample 400 μl of methanol was added. The mixture was vortexed for 20 min

Table 1
HPLC conditions

Set of conditions	Sample	HPLC column	Mobile phase composition	Flow rate (ml/min)
A	DP-71	Supelcosil LC-ABZ, 5 μm, 250 mm × 4.6 mm	Methanol–acetonitrile–water (85:15:5, v/v) [1,5]	1.0
B	MTX		Phosphate–citrate buffer solution: acetonitrile, 90:10, (v/v) [7]	1.2
C	DP-71		Phosphate–citrate buffer solution: acetonitrile, 50:50 (v/v)	1.2
D	DP-71 (and related compounds)	Symmetry Shield RP18, 5 μm, 150 mm × 3.9 mm	Acetate–citrate buffer solution (pH 6.0): acetonitrile, 60:40–20:80 (v/v)	0.8–1.0
E			Gradient: acetate–citrate buffer solution (pH 5.5)—acetonitrile (Table 2)	1.0
F	Methotrexate (MTX) γ -Dodecyl ester of MTX α-Dodecyl ester of MTX DP-71 (both homologues) Bis-dodecyl ester of MTX	Inertsil C_4, 5 μm, 150 mm × 4.6 mm	Gradient: acetate–citrate buffer solution (pH 5.5)—acetonitrile (Table 2)	1.3
G	DP-71 in rat serum, plasma and cerebral spinal fluid	Inertsil C_4, 5 μm, 150 mm × 4.6 mm	Acetate–citrate buffer solution (pH 5.5): acetonitrile, 30:70 (v/v)	1.0
H	DP-71 in rat brain	Luna C_5, 5 μm, 150 mm × 4.6 mm	Acetate–citrate buffer solution (pH 6.0): acetonitrile, 30:70 (v/v)	1.0
I			Gradient: acetate–citrate buffer solution (pH 6.0)—acetonitrile (Table 2)	1.3

and centrifuged in Eppendorf tubes for 10 min at 10 000 × *g*. The supernatant was collected and injected onto the HPLC system. The recovery of the procedure is 35–40%.

Scheme 3: To 200 μl of serum/plasma sample 400 μl of a mixture of acetate–citrate buffer solution (pH 5.5) with acetonitrile (30:70, v/v) was added. The resulting mixture was vortexed for 20 min and centrifuged in Eppendorf tubes for 10 min at 10 000 × *g*. Supernatant was collected and injected onto the HPLC system. The recovery of the procedure is 85%.

Based on the recovery results, as well as taking into account interference of matrix peaks with the peaks of interest, Scheme 3 appeared to be the preferable one.

2.5.2. Rat cerebrospinal fluid

Cerebrospinal fluid was used for injection onto the HPLC system without any pretreatment.

2.5.3. Rat brain

0.5 g of brain was homogenized in 2.5 ml of phosphate–citrate buffer solution, 3.0 ml of methanol was added, and the mixture was vortexed for 1–2 min, then 6.0 ml of chloroform was added. The resulting mixture was vortexed for 20 min and centrifuged in Eppendorf tubes for 10 min at 10 000 × *g*. The chloroform (lower) layer was collected and evaporated to dryness. To the residue 500 μl of methanol was added, mixed thoroughly and centrifuged under the same conditions. Supernatant was collected and injected onto the HPLC system.

3. Results and discussion

3.1. Starting points of the development of HPLC method for DP-71

Due to a strong chromophore of the MTX core molecule (Fig. 3A), all the phospholipid derivatives of MTX also have a pronounced absorption in UV-Vis spectrum (Fig. 3B). Therefore, the detection for the HPLC method could be carried out at a comparatively high wavelength (302 nm), which guarantees minimum interruption, caused by baseline noise or biological matrix effect. The high extinction coefficient at this wavelength (25 119 L M^{-1} cm^{-1} [6]) ensures sufficient

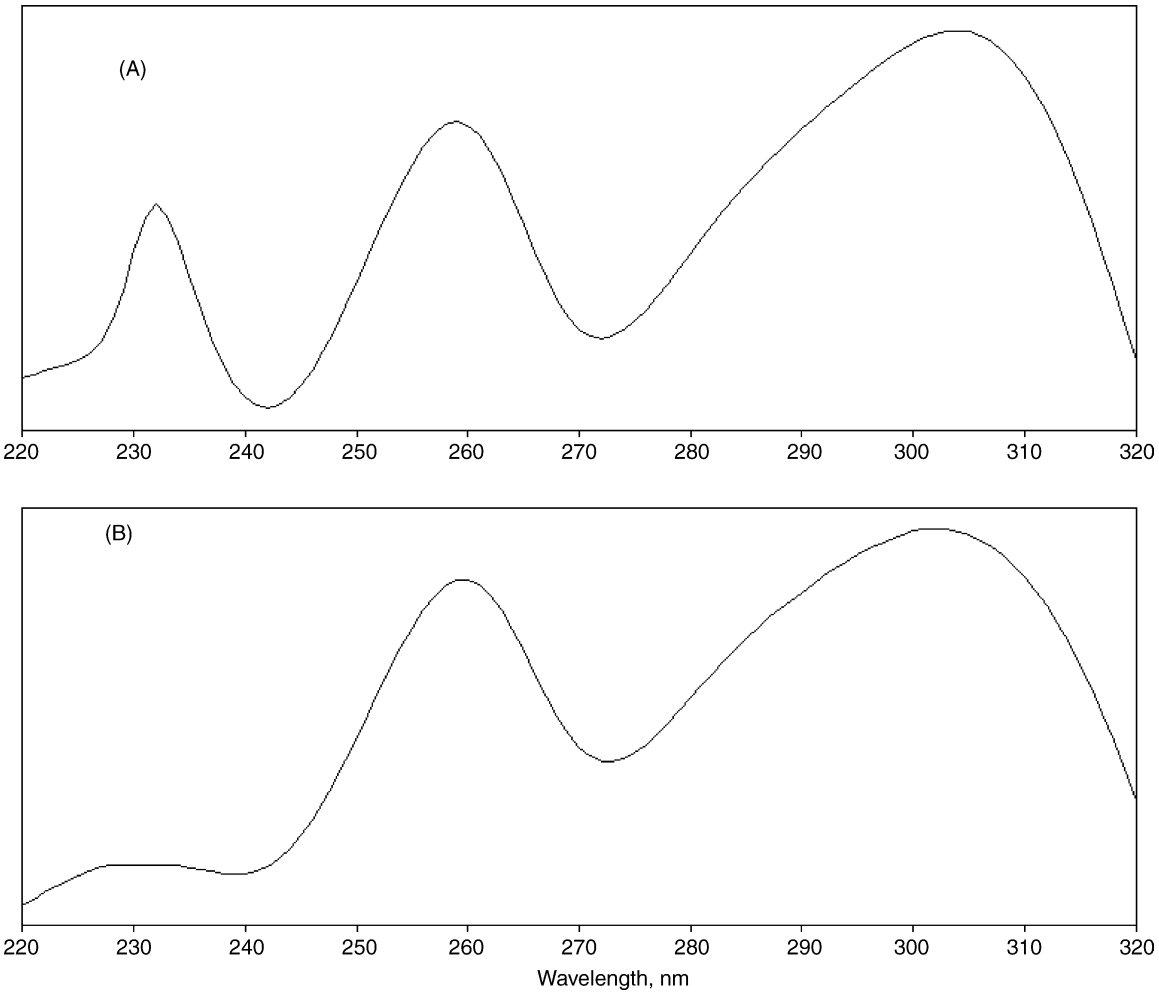

Fig. 3. UV spectra of the HPLC peaks of MTX (A) and DP-71 (B) from diode-array detector.

sensitivity of a UV detection, which allows bioanalytical applications, especially when the detector is equipped with a 50-mm flow cell.

The analytical HPLC method used for phospholipid derivatives of valproic acid and NSAIDs [4,5] is suitable, after minor adjustments, to a variety of similarly designed molecules. However, running this "generic" method (Table 1, A) did not result in elution of DP-71 from the HPLC column. Being designed in the same manner, DP-71 differs from the above molecules due to the presence of seven amino groups in the MTX core. This determines the strongly basic character of the whole molecule, which requires specific approach to the design of the HPLC procedure.

For MTX itself and its related compounds (synthetic impurities and degradation products) there is a well-established pharmacopoeial HPLC method [7] (Table 1, B). This analytical procedure, which comprises in its mobile phase a phosphate buffer at pH 6.0, takes into account the pronounced basic character of MTX. But due to the lipophilic nature of the "DP-template"—the lysolecithin moiety—this method could not be applied for analysis of the various DP-MTX derivatives.

Therefore, the main approach to method development for DP-71 was an attempt to achieve a compromise between the pharmacopoeial procedure for MTX [7] and the "generic" method designed for various phospholipid derivatives [5].

3.2. Reaching a compromise—development of isocratic method

Due to a composition of natural phospholipids, starting material for the synthesis, DP-71 consists of a mixture of homologues (1-palmitoyl- and 1-stearoyl- with respect to the glycerol moiety) in the ratio of 10:90–0:80.

The tolerable compromise method has been designed. This isocratic procedure, based on the use of the pharmacopoeial buffer solution as an aqueous part, together with elevated quantities of the organic solvent (Table 1, C), allows to achieve more than a baseline separation between the two peaks of C16 and C18 homologues of DP-71 (Fig. 4). As a method for determination of only DP-71, this procedure looks quite satisfactory and could also be a good starting point for development of bioanalytical procedure for pharmacokinetic studies. Method performance can be improved by using the symmetry shield column and optimizing the ratio of the aqueous and organic parts in the mobile phase, and the procedure is adjusted to be run on LC–MS instrument by substituting the non-volatile sodium phosphate buffer with volatile ammonium acetate, and (Table 1, D). However the isocratic method still does not allow determining of all the possible impurities and degradation products of DP-71. Related compounds, which are more polar than DP-71, are eluted together with or close to the solvent front and not separated, more lipophilic ones do not elute at all.

3.3. Development of gradient method—the discriminative stability indicating procedure

Preparation of DP-71 is a multistage synthesis starting from MTX and including its esterification with 1-dodecanol followed by reaction with 2-unsubstituted lysolecithin. As a result of the synthetic procedure, the possible impurities could be: MTX (Fig. 1), a starting material, which could also be a degradation product of DP-71; α-dodecyl MTX (Fig. 5),

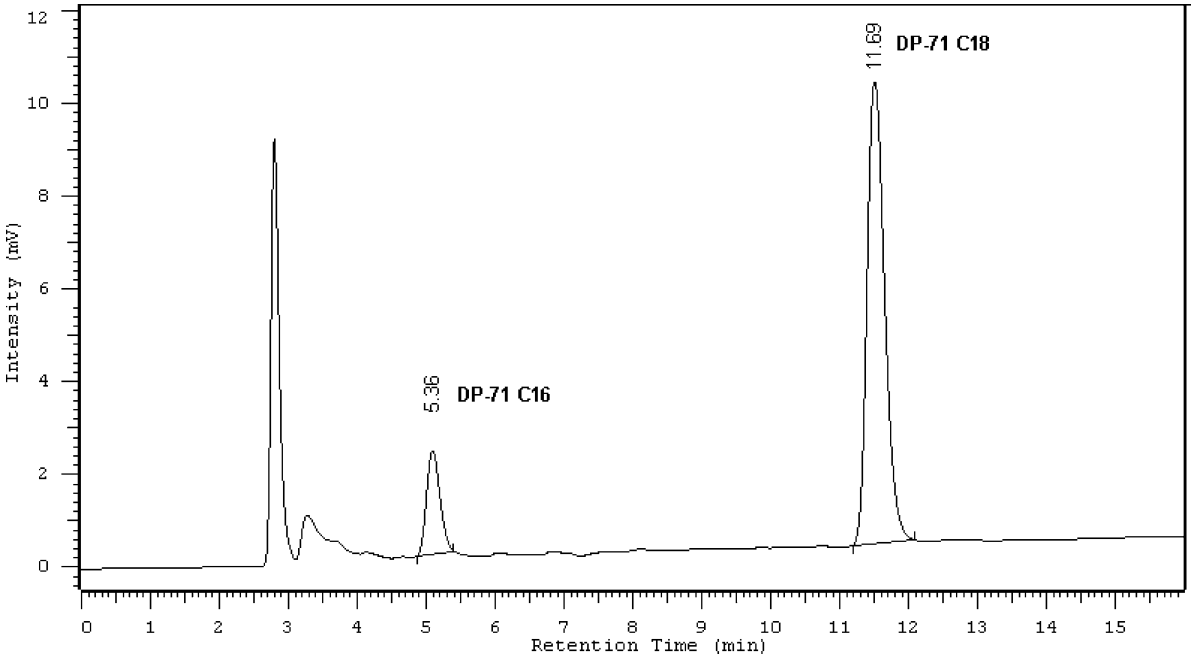

Fig. 4. Separation of homologues of DP-71 running HPLC procedure C.

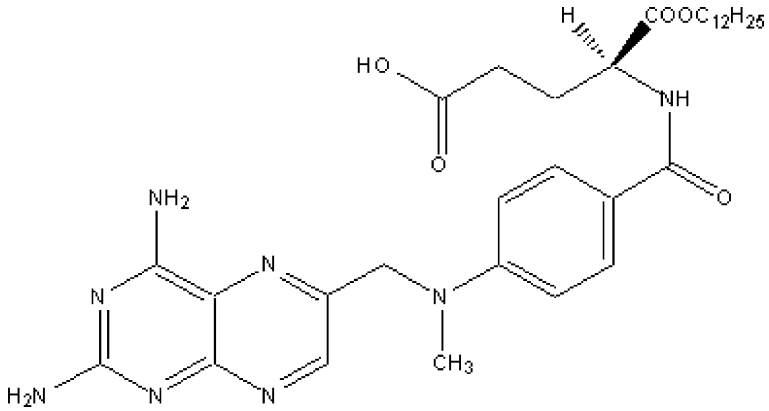

Fig. 5. α-Dodecyl MTX structure.

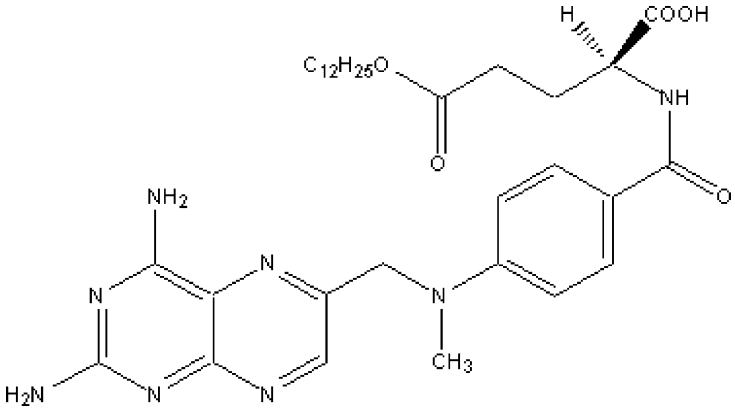

Fig. 6. γ-Dodecyl MTX structure.

an intermediate product, which could also be a degradation product of DP-71; its isomer γ-dodecyl MTX (Fig. 6), and bis-dodecyl MTX (Fig. 7), both being the by-products of preparation of α-dodecyl MTX. Using isocratic HPLC methods, it was impossible to elute all these compounds, starting with the most polar—MTX itself—and through the most lipophilic—bis-dodecyl MTX—within a single chromatographic run. Applying the gradient from higher content of aqueous component to higher content of organic solvent in the mobile phase (Table 1, E; Table 2) resulted in a chromatographic procedure, which allows to achieve at least baseline separation between the homologues of DP-71 and two very similar related compounds—α- and γ-dodecyl MTX.

However, the chromatographic performance of this procedure was still low. The peaks obtained were of unsatisfactory shape, and the column efficiency (theoretical plates count) was also low. Most probably, this could be attributed to the fact, that the C_{18} column is too lipophilic,

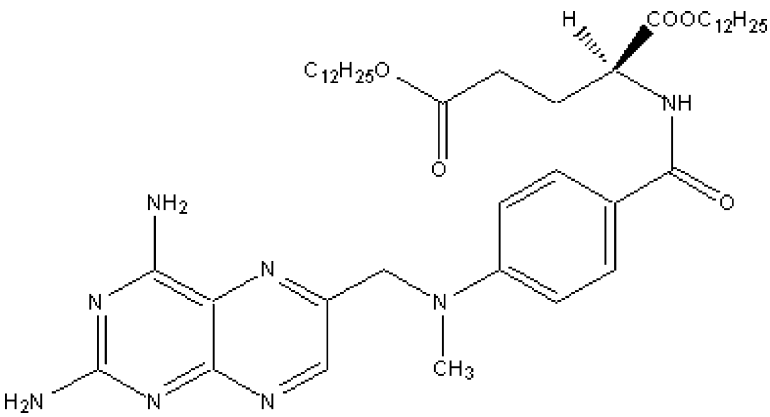

Fig. 7. Bis-dodecyl MTX structure.

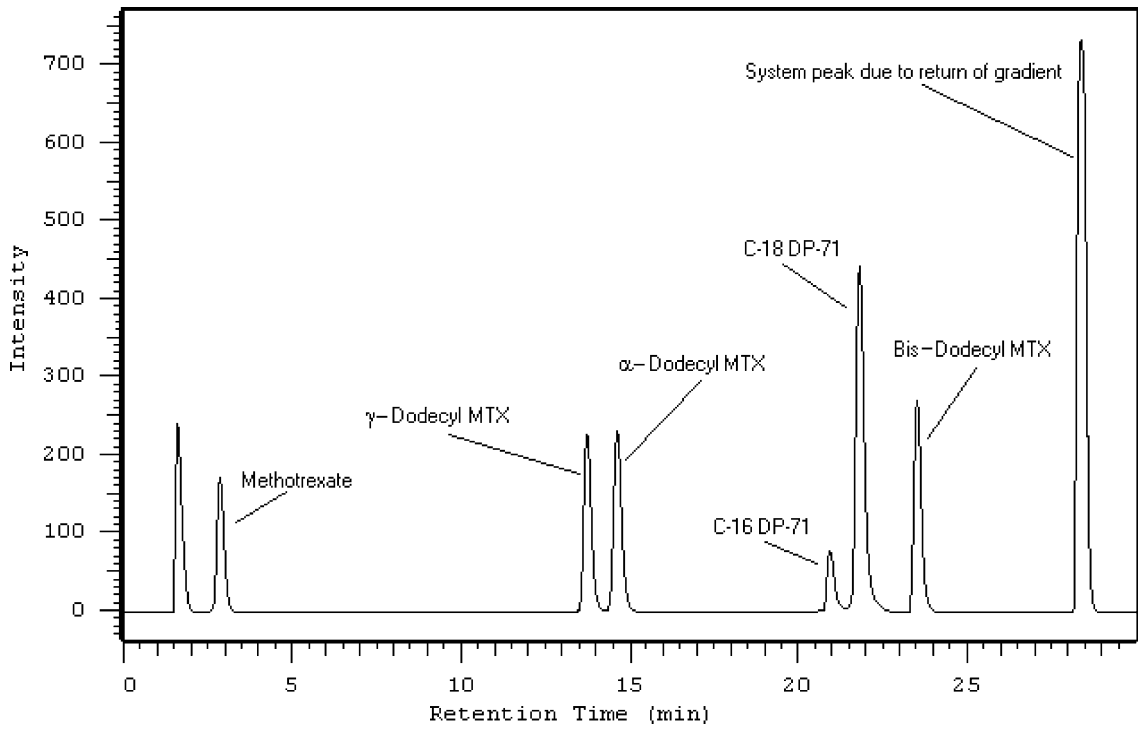

Fig. 8. HPLC procedure F: separation of homologues of DP-71 and related compounds.

and therefore has a strong tendency to retain compounds with long aliphatic chains and comparatively high molecular mass (1200 DP-71). Moreover, bis-dodecyl MTX, due to its very high lipophilicity, could not be eluted from the C_{18} column. Even a C_8 stationary phase is helpless. Therefore, it was decided to use a reverse d-phase chromatographic column with a much shorter organic chain and lower hydrophobicity—the C_4 brand of Inertsil, which is characterized by very even coverage of the silica surface with organic modifier and very low silanol activity. The designed procedure (Table 1, F; Table 2) resulted in a chromatographic method, which allows obtaining peaks of both homologues of DP-71 and all the known related compounds within a single chromatographic run (Fig. 8). The method is characterized by high performance, with very narrow and symmetric peaks of all the compounds having different polarity and lipophilicity. At least baseline separation was reached for both critical pairs of closely eluted peaks (both homologues of DP-71 and two isomeric dodecyl-derivatives).

Clear separation between the peaks of main compounds and possible impurities and degradation products, as well as high sensitivity allow to apply the developed method as a stability indicating analytical procedure.

The use of volatile buffer permits running the developed method with MS and MS–MS detectors thus facilitating the upgrade of this procedure to highly sensitive bioanalytical method. LC–MS technique used together with this separation method allowed verifying the identification of all the separated peaks.

3.4. Achieving bioanalytical HPLC applications for DP71

3.4.1. Determination of DP-71 in rat serum and plasma for pharmacokinetic studies

The gradient elution in this case was not required since DP-71 is the only target compound to be determined. The chosen chromatographic conditions for the work with biofluids were derived from the corresponding gradient procedure (Table 1, G).

Sample preparation procedure was also optimised, as follows.

The extraction procedure (Section 2.5.1, Scheme 1) was found to be insufficient: the retention time of one of the matrix peaks was the same as the retention time of the C18-homologue of DP-71, and therefore, the baseline subtraction was required, which reduced the method accuracy (Fig. 9).

Excluding chloroform from the sample preparation (Section 2.5.1, Schemes 2 and 3) resulted in no overlapping between the system peak and any of the peaks of DP-71.

However, extraction with methanol (Section 2.5.1, Scheme 2) had low recovery (only 35–40%). The recovery was improved (85%) applying the extraction with mobile phase (Section 2.5.1, Scheme 3).

3.4.2. Determination of DP-71 in rat cerebrospinal fluid

The rat cerebrospinal fluid (CSF) was injected onto the column without any pretreatment. Testing under the same conditions, which were found to be optimal for serum (Table 1, G), provided a good separation between C16 and C18 homologues, no overlapping with matrix peaks, and

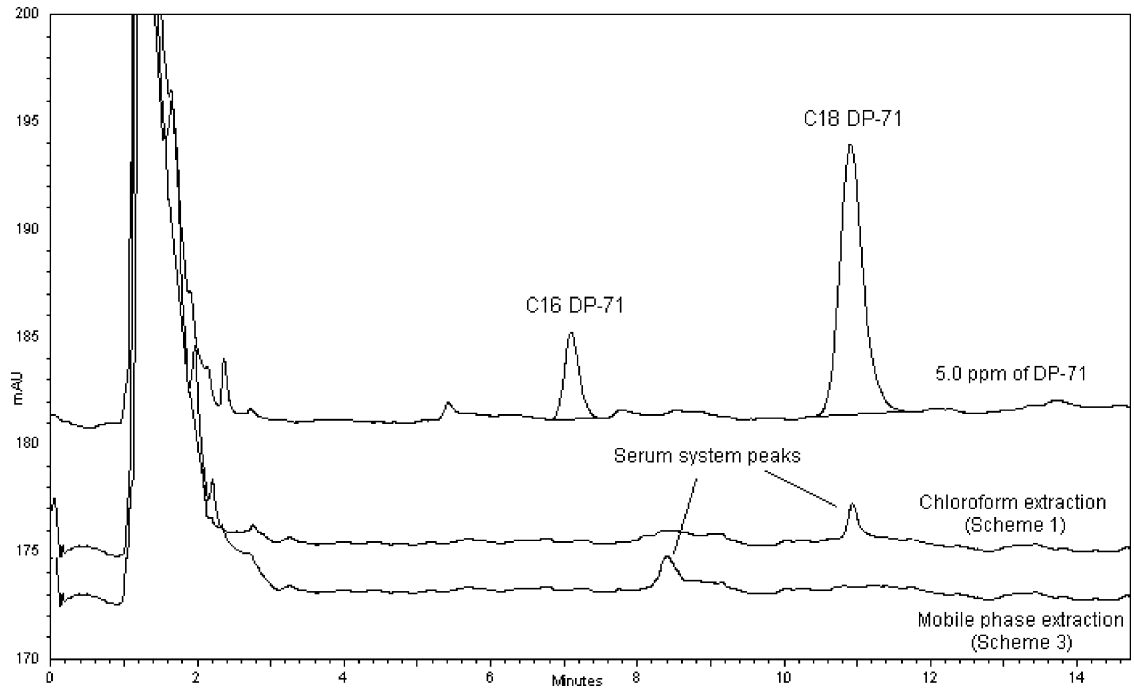

Fig. 9. HPLC procedure G: testing DP-71 in serum samples.

sufficient sensitivity using UV detection at a concentration level of about 1 ppm (Fig. 10).

3.4.3. Determination of DP-71 in rat brain

The initial testing conditions for DP-71 in brain samples were similar to those for the rat serum and plasma (Table 1, G). This procedure did not provide separation between the DP-71 and matrix peaks, even varying pH from 5.5 to 6.0, which could be due to high content of lipids in brain.

Replacement of the column with slightly more lipophilic (Phenomenex Luna C_5 instead of Inertsil C_4, Table 1, H) allowed to achieve separation of the homologue peaks with no interference from the biological matrix (Fig. 11A).

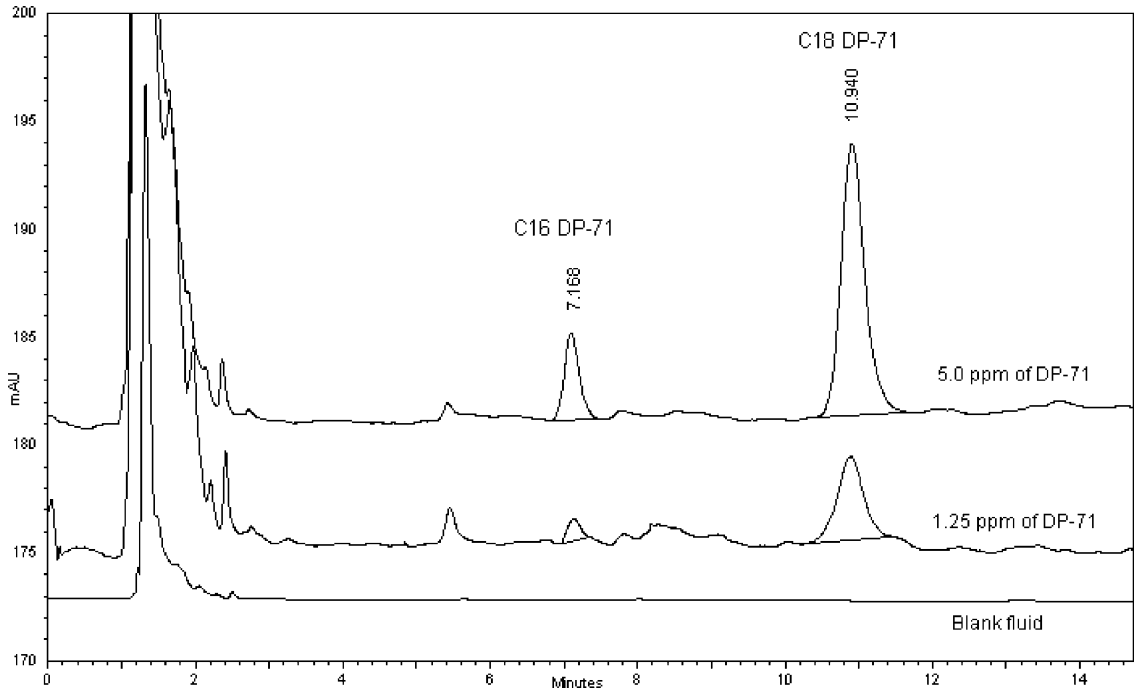

Fig. 10. HPLC procedure G: testing DP-71 in CSF samples.

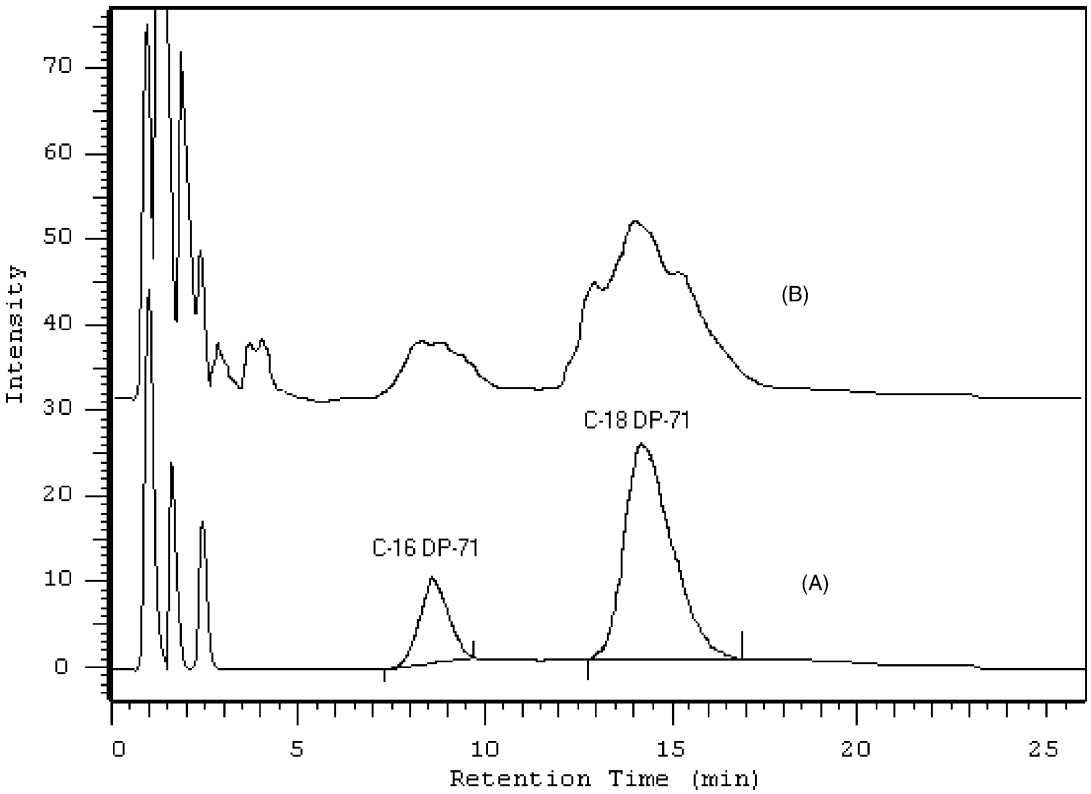

Fig. 11. HPLC procedure H: testing DP-71 in brain samples. (A) Chromatogram of the first injection and (B) after 20 injections.

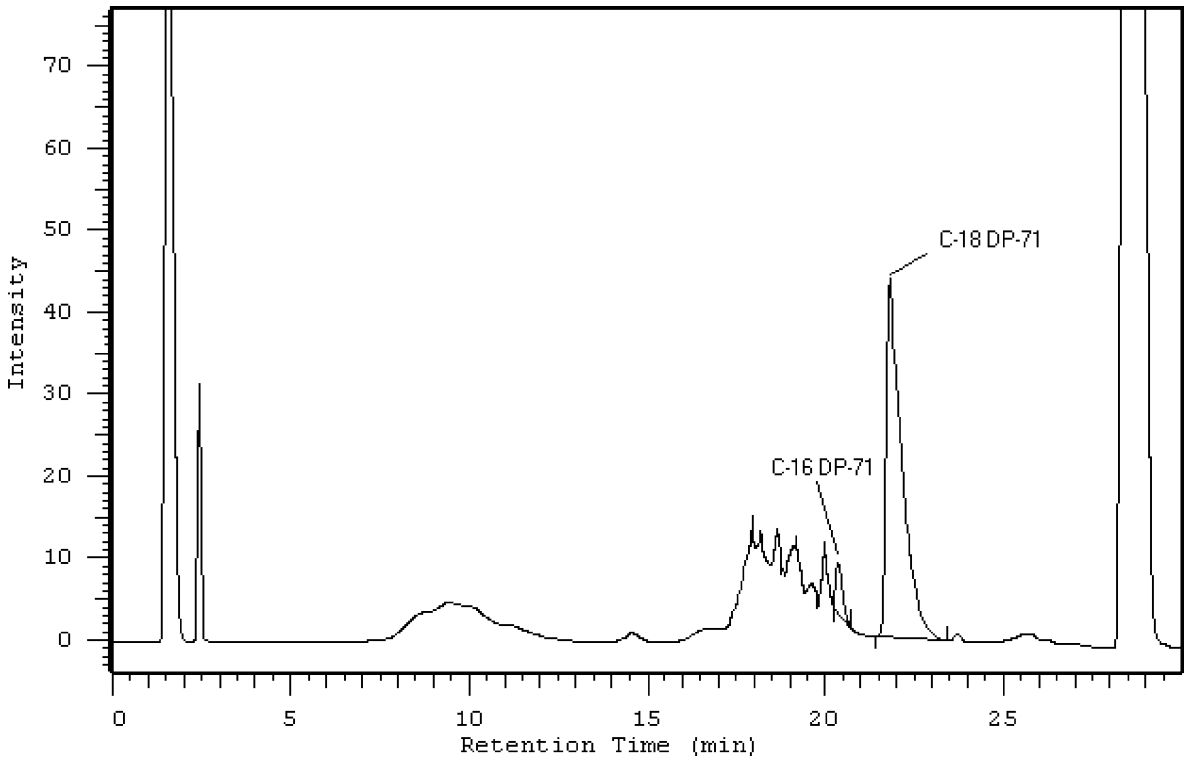

Fig. 12. Gradient HPLC procedure I: testing DP-71 in brain samples.

However, the chromatogram appeared to be irreproducible: the repeated injections resulted in broadened and split peaks (Fig. 11B). This could happen because the isocratic elution does not effectively wash out all the matrix stuff from the column, which was verified by washing the column with strong organic solvent (5% THF in methanol) after 20 injections of brain samples. Applying the gradient elution (Table 1, I) solves this problem (Fig. 12). The peak of the major C18 homologue of DP-71 is baseline separated from all the matrix peaks, and this picture remains reproducible due to the gradient, which allows performing cleanup of the column after every injection. The peak of the minor C16 homologue is in less favorable position, but still its integration is reproducible.

However, the problems of UV detection are easily solved when using LC–MS. It also facilitates identification required for metabolic studies and can substantially improve sensitivity for routine pharmacokinetic assays, especially when using a single ion monitoring mode.

All the developed bioanalytical methods demonstrate acceptable linearity in the range 0.2–20.0 μg of DP-71 (sum of both homologues) in 1 g (ml) of sample. The linear correlation coefficients r^2 are 0.9896, 0.9895 and 0.9828 for serum, CSF and brain matrices, respectively.

4. Conclusions

A gradient method for determination of purity profile and assay of DP-71 drug substance was developed. This method allows to quantify a diversity of related compounds and degradation products, from the most polar MTX through the most lipophilic bis-dodecyl MTX, within a single chromatographic run.

In addition, an isocratic method for DP-71 determination in biological fluids (rat serum, plasma and CSF) was developed. This method can be also used for analysis of DP-71 drug substance when a determination of purity profile is not required.

In sample preparation, the extraction procedure for rat serum and plasma was optimised, which allowed eliminating the overlapping of the main peaks of DP-71 with matrix peaks and increased the accuracy of the method.

The gradient procedure, used for the drug substance, was found to be suitable for testing of DP-71 in rat brain.

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