Determination of the Alkyl Lysophospholipid Derivative ET-18-OCH₃, a New Antineoplastic Drug, in Plasma

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We describe a sensitive method for measuring the concentration of the new antineoplastic drug ET-18-OCH₃ in plasma. After plasma lipids are extracted, ET-18-OCH₃ is separated from the excess of endogenous lipids by thin-layer chromatography and specific enzymatic hydrolysis of sphingomyelin by the action of sphingomyelinase. Analytical recovery after the complete isolation was 73.5% (CV = 9.8%, n = 15). [³H]-ET-18-OCH₃ is used as internal standard. A densitometric method in which 8-anilino-1-naphthalenesulfonate, Mg salt, is used as fluorescent agent (excitation at 367 nm and emission >390 nm) allows the sensitive determination of ET-18-OCH₃ down to 0.1 mg/L (CV >30%). The day-to-day CV is 25% for concentrations of 0.15 to 0.625 mg/L, 12% for 1.5 to 5.0 mg/L. Preliminary pharmacokinetic data reveal gastrointestinal absorption of ET-18-OCH₃ after multiple oral administration.

Additional Keyphrases: cancer • thin-layer chromatography with densitometry • therapeutic drug monitoring

Alkyl lysophospholipids (ALPs) are synthetic 1-O-alkyl analogs of natural 2-lysophosphatidylcholine (A), an important intermediate in the metabolism of phospholipids (Land's pathway) (1, 2). ALPs represent a new class of antineoplastic drugs, acting by activation of macrophages and by perturbing the phospholipid metabolism of tumor cells (3, 4).

\[
\text{H}_2\text{C}=\text{O}-\text{C}_1\text{H}_{35}\text{H}
\]

A

\[
\text{H}_2\text{C}=\text{O}-\text{C}_1\text{H}_{35}\text{H}
\]

B

The best-investigated ALP, ET-18-OCH₃ (racemic-1-O-octadecyl-2-O-methylglycerol-3-phosphocholine) (B), has already entered phase II trials in human cancer therapy in the F.R.G. (5). Concentrations in plasma and the distribution of total radioactivity in organs in mice after intravenous and oral administration of [³H]-ET-18-OCH₃ have been published (6).

Hitherto, no method has been available for quantifying non-radiolabeled ET-18-OCH₃ in human plasma, because of the excess of endogenous phospholipids present in plasma and the difficulty of detecting ALP in low concentration. Therefore, a prerequisite to more information about the pharmacokinetics of ET-18-OCH₃ in man is development of an assay for ALP in plasma.

Materials and Methods

Samples. Plasma samples were kept at −20 °C until assayed.

Apparatus. We used a chromatogram-spectrophotometer (KM 3; Zeiss, Oberkochen, F.R.G.) in combination with a D-2000 integrator (Merck, Darmstadt, F.R.G.) and a liquid scintillation counter (LB 2760; Berthold, Wildbad, F.R.G.)

Reagents. Sphingomyelinase (EC 3.1.4.12) from Bacillus cereus was from Boehringer, Mannheim, F.R.G.; 8-anilino-1-naphthalenesulfonate, Mg salt, was from Sigma Chemical Co., St. Louis, MO; racemic-1-O-octadecyl-2-O-methylglycerol-3-phosphocholine (ET-18-OCH₃) was from medmark pharma, München, F.R.G.; and racemic-1-O-[³H]-octadecyl-2-O-methylglycerol-3-phosphocholine ([³H]-ET-18-OCH₃) was a gift from Prof. P. G. Munder, Freiburg, F.R.G.

Preparation of ET-18-OCH₃ standards. Dissolve 20 mg of racemic-1-O-octadecyl-2-O-methylglycerol-3-phosphocholine in 20 mL of chloroform and dilute to prepare working standards in concentrations ranging from 10 to 80 mg/L.

Preparation of plasma samples. Add 0.4 μCi of [³H]-ET-18-OCH₃ to 2 mL of plasma and lyophilize. For evaluating the method, we mixed the plasma with ET-18-OCH₃ to achieve final concentrations of 0.15, 0.3, 0.625, 1.5, 2.5, and 5.0 mg/L.

For the pilot pharmacokinetic study, patients received ET-18-OCH₃ dissolved in milk.

Procedures

Extraction and fractionation of plasma phospholipid subclasses. Extract 2 mL of lyophilized plasma two times with 4.5 mL of chloroform/methanol (2/1 by vol). Combine the organic solvents and wash with 1.8 mL of a 7.3 g/L solution of NaCl in water. After centrifugation, discard the aqueous phase and evaporate the chloroform layer at 45 °C under a stream of nitrogen in a water-bath. Redissolve the residue in 500 μL of chloroform and apply this solution in an 8-cm line to a silica gel 60 plate (Merck, Darmstadt, F.R.G.) with a thickness of 0.25 mm. For separating the phospholipids into subclasses use chloroform/methanol/acetic acid/water (55/30/7/2, by vol) as the developing solvent; the nonpolar lipids move with the solvent front, and the [³H]-ET-18-OCH₃ moves coincidently in the sphingomyelin fraction. To detect phospholipids, spray the plate with a 1 g/L solution of diphenylhexatriene in hexane. Mark the end of the lyssolecithin fraction and the beginning of the lecithin fraction.

Develop the plate with hexane to remove the indicator.
from the marked part of the plate. Air-dry the plate and scrape the silica gel in the marked zone into polypropylene tubes.

Extract the silica gel two times with 4-mL portions of chloroform/methanol/water (60/30/5, by vol). Combine the two extracts and add 1.5 mL of a 7.5 g/L solution of NaCl in water to separate the phases. After centrifugation, discard the aqueous phase and evaporate the chloroform layer at 45 °C under a stream of nitrogen in a water-bath.

Elimination of sphingomyelin. Add 1 mL of phosphate buffer (pH 7.4, 50 mmol/L) to the residue of the chloroform extract. Place the mixture for 5 min in an ultrasonic bath. Add 3 U (30 μL) of sphingomyelinase (100 kU/L) and 1 mL of diethyl ether. Incubate at 37 °C for 5 h. After enzymatic degradation of sphingomyelin to phosphorylcholine and ceramide, extract the remaining ET-18-OCH₃ from the phosphate buffer by shaking with 4 mL of chloroform/methanol (2:1 by vol). After centrifugation, remove the chloroform layer and extract the upper phase once more with chloroform/methanol/water (84/16/1 by vol). Evaporate the combined organic solvents under nitrogen in a 50 °C water-bath and redissolve the residue in 50 μL of chloroform/methanol (1/1 by vol). This is the sample solution.

Quantification of ET-18-OCH₃ by reflectance densitometry. Apply 5 μL of the sample solution and 5 μL of ET-18-OCH₃ standards in five different concentrations to an HPTLC-silica gel 60 plate. Use chloroform/methanol/water (65/35/5 by vol) as the mobile phase. After development, dry the plates with a hair dryer. To detect the ET-18-OCH₃ spots, dip the plate into a 300 mg/L solution of 8-anilino-1-naphthalenesulfonate, Mg salt, in acetone. After air-drying, measure the reflected fluorescence intensity of the ET-18-OCH₃ spots with a thin-layer chromatography scanner combined with an integrator. Use an excitation wavelength of 367 nm and measure the emission >390 nm. Calculate from the ET-18-OCH₃ standard curve—which is linear up to 1 μg of ET-18-OCH₃ per spot—the concentration of the sample solution.

Calculation of the ET-18-OCH₃ plasma concentration. Measure the radioactivity of 10 μL of sample solution in a liquid scintillation counter and calculate the analytical recovery. Calculate the ET-18-OCH₃ concentration in plasma after correcting the concentration of ET-18-OCH₃ as measured by densitometry for the analytical recovery.

Results and Discussion

The extraction step. Similarities in chemical structure and polarity between ET-18-OCH₃ and endogenous glycerophospholipids make it very difficult to extract ET-18-OCH₃ from human plasma specifically. Therefore ET-18-OCH₃ has to be separated from natural lipids after the extraction. Separation and quantification in one step is more difficult because of excess of endogenous glycerophospholipids and the lack of chromatophores that would allow sensitive and selective detection of ET-18-OCH₃ in the presence of other phospholipids.

Lipids were extracted from plasma by use of a modification of the method of Polch et al. (7). The analytical recovery was >97%.

Separation of different lipid classes by thin-layer chromatography. The high amount of interfering phospholipids in the extract requires the use of one-dimension thin-layer chromatography for separation. Acid solvent systems are superior to basic mobile phases, because acid solvent systems are able to separate choline-, ethanolamine-, and serine-glycerophospholipids (8). Therefore no lysophosphatidylethanolamine or phosphatidylethanolamine is present in the sphingomyelin fraction. This is important, because ET-18-OCH₃ is located in the sphingomyelin fraction (Figure 1) and sphingomyelin can easily be separated from ET-18-OCH₃ by the specific action of sphingomyelinase. We have used a modification of an acid solvent system described by Skipski et al. (9), which allows reproducible separation of plasma lipids. Although we tested many solvent systems, total separation between ET-18-OCH₃ and plasma glycerophospholipids could not be achieved.

Analytical recovery. A 24% loss of ET-18-OCH₃ is observed during thin-layer chromatography separation and after extraction of the silica gel. The recovery after complete isolation procedure was 73.5% (CV = 9.8%, n = 15). To quantify the loss of ET-18-OCH₃ during extraction we used [³H]-ET-18-OCH₃ as internal standard.

Detection. After isolation, ET-18-OCH₃ was quantified by a densitometric method, with use of 8-anilino-1-naphthalenesulfonate, Mg salt, as fluorescent agent. This reagent is more sensitive than other agents such as rhodamine 6G (10), 2,7-dichlorofluorescein (11), and 2,5-bis-[5-tert-butylbenzoxazolyl(2')]thiophene (12). Traces of sphingomyelin not hydrolyzed by sphingomyelinase do not interfere with the densitometric determination because of the different mobilities (Figure 2).

Quantification. The standard curve for the densitometric determination of standards of ET-18-OCH₃ at concentrations of 0 to 80 mg/L, corresponding to 0 to 400 ng of ET-18-OCH₃ per spot, was linear (Table 1). The mean correlation coefficient (r) was 0.997 (SD 0.002). The limit of detection was 4 mg/L (CV >30%), corresponding to 20 ng of ET-18-OCH₃ per spot. The precision of the method was determined at six drug concentrations. A pooled plasma was split and supplemented to give concentrations from 0.1 to 5.0 mg/L. One sample of each concentration was assayed per day during one month. The interday CV and SD are given in Table 2. The limit of detection was 0.1 mg/L (CV >30%).

Application. For a preliminary determination of the pharmacokinetics of ET-18-OCH₃, three patients were administered an oral dose of 150 mg of the drug every 4 h. Figure 3 illustrates the mean ET-18-OCH₃ concentrations in plasma vs time.

These results demonstrate that, after the oral intake of 150 mg, ET-18-OCH₃ can be quantified in plasma with our

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**Fig. 1. TLC separation of plasma lipids**

LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; NL, neutral lipids
standards. Thus, this would appear to facilitate pharmacokinetic studies with the phospholipid-antimetabolite ET-18-OCH₃ after oral intake of single doses of 150 mg or more.

![Chromatogram of a drug-supplemented plasma sample](image)

**Fig. 2. Chromatogram of a drug-supplemented plasma sample**

*: sphingomyelin not hydrolyzed by sphingomyelinase

### Table 1. Densitometric Determination of ET-18-OCH₃ Standards

<table>
<thead>
<tr>
<th>ET-18-OCH₃ standards, mg/L</th>
<th>ET-18-OCH₃, ng/spot</th>
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<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
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*Arb. units. n = 6.

### Table 2. Precision of the Method

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CV, % Recovery, %

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References