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High-performance liquid chromatographic analysis for a non-chromophore-containing phosphatidyl inositol analog, 1-[(1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero)-phospho]-1*D*-3-deoxy-*myo*-inositol, using indirect UV detection

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Abstract

Phosphatidylinositide-3-kinase (PI3 kinase) is an important constituent of growth factor regulation. It is also involved in oncogene signaling pathways. An ether-containing phosphatidyl inositol(PI) analog, OMDPI, 1-[(1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero)-phospho]-1D-3-deoxy-*myo*-inositol, is a potent inhibitor of this pathway and may be clinically useful in the treatment of a variety of neoplasms. OMDPI is currently being investigated as an anti-tumor agent by the National Cancer Institute, NIH. OMDPI, a non-chromophore-containing PI analog, is not directly adaptable to the commonly used UV detection of HPLC. This paper reports the development and validation of an HPLC assay for OMDPI based on indirect UV detection, in which a UV-absorbing ion-pair reagent (the probe), protriptyline, is added to the mobile phase to induce a signal for the compound. The method is sensitive (limit of detection <5 μl of 1 μg/ml or 5 ng), precise (R.S.D.$<2.5\%$), linear ($r^2=0.9995$) and accurate (error$<0.7\%$). It is superior to refractive index detection and evaporative light scattering detection in either sensitivity or linearity and does not require special equipment.

Keywords: Mobile phase composition; Detection, LC; Indirect detection; Ion-pairing reagents; Octadecylmethylglyderophosphodeoxyinositol; Enzyme inhibitors; Protriptyline; Butylparaben; Phospholipids; Phosphatidyl inositol

Introduction

Phosphatidylinositide-3-kinase (PI3 kinase) is an important constituent of growth factor regulation. It is also involved in oncogene signaling and transformation. OMDPI, 1-[(1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero)-phospho]-1D-3-deoxy-*myo*-inositol, an ether-containing analog of phosphatidyl inositol(PI) (Fig. 1), is a potent inhibitor of PI3 kinase ($IC_{50}=2.5$ μ*M*) [1] and may be clinically useful in the treatment of a variety of neoplasms. OMDPI is currently under investigation as an anti-tumor agent.

Naturally occurring phospholipids are composed of multiple molecular species of varying fatty acid chain lengths and degrees of unsaturation. Phospholipids are typically amphiphilic compounds. They can be classed according to their polar head and fatty acid chains. This duality can be utilized both in normal and reversed-phase chromatography. There are numerous reports on the separation for phos-

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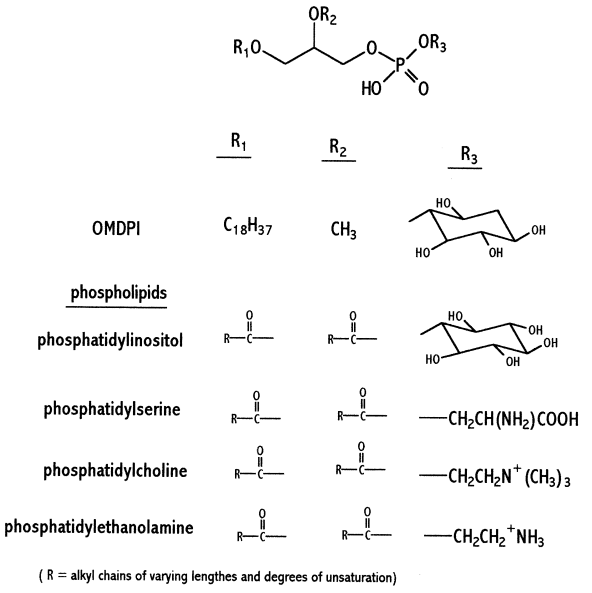

Fig. 1. Structure of OMDPI and phospholipids.

pholipids by HPLC [2]. Generally, separation of phospholipids by class is based on the polar head. Normal-phase chromatography with silica column and non-polar mobile phase has been most commonly used in these cases. The separations of multi-molecular species within the same class are commonly performed with reversed-phase chromatography based on the hydrophobicity and degree of unsaturation of the fatty acid chains. Hax and Geurts van Kessel established a normal-phase chromatography with silica gel column and hexane–isopropanol–water solvent system [3]. Patton et al. reported that major phospholipids were separated and recovered from a silica column by normal-phase HPLC [4]. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine were then further chromatographed on a C_{18} reversed-phase column to isolate individual molecular species. Bonanno et al. described a mixed phase separation for phospholipids from pulmonary surfactant [5]. Using an on-line coupled silica/reversed-phase HPLC system, phospholipids can be separated into classes and molecular species as well. Detection in all of the above examples was by UV at 205/206 nm, utilizing UV absorption of the fatty acid carbonyl group. Detection limits are 0.1 μg or 10 μl injection of 10 μg/ml for UV detection.

Because of the poor UV absorptivity of phospholipids, there were also attempts to use other detection methods. Ma and Kim developed an on-line HPLC–thermospray mass spectrometry method to monitor/identify phospholipid molecular species after a gradient elution from a C_{18} column [6]. MS detection was also useful in identifying individual molecular species within each phospholipid class. The detection limit was greatly improved to 20–50 ng. Since linearity of response was inconsistent, it is not suitable for quantitative analysis. Evaporative light scattering detection (ELSD) is becoming a popular method for detection of non-UV absorbing substances in chromatography. Sas et al. reported a method with a diol–silica column and ternary gradient elution with ELSD for the separation and quantification of major phospholipid and lysophospholipid classes [7]. The typical linearity range reported was 4–10 μg. The detection limit was 1 μg.

OMDPI is related in structure to phospholipids. Its chromatography behavior is expected to be similar to that of phospholipids. Since OMDPI is a non-carbonyl-containing phospholipid analog, it is not directly adaptable to the commonly used approach of UV detection at a short wavelength. Derivatization will add a time-consuming step or a technical complication to the analytical procedure. Refractive index (RI) detection, ELSD or MS are among the alternative detection methods. However, the sensitivity for RI is low and the linearity for ELSD and MS is not well established [7–10].

Indirect detection is another alternative for non-UV-absorbing compounds. The theory of indirect detection has appeared in a review [25] and related papers [24,27–29]. The basic principle is that an additive (probe, P) with detectable properties and affinity for the stationary phase (S) is included in the mobile phase. Once equilibrium is reached, the probe concentration in the mobile phase ([P]) remains constant. An aliquot of analyte (A) injected onto the column perturbs the equilibrium of [P] and gives rise to detection signals.

There were extensive mechanistic studies of the response patterns for ionic and neutral molecules in indirect detection. The types of compounds used in these studies were aliphatic alcohols, alkylamines or alkylammoniums, alkyl sulfonates or sulfates, and carboxylic acids [11–16]. Indirect detection has also

been used for amino acids [17], dipeptides [18], carbohydrates and polyols [19] and choline [20,21]. Chiral separation was also achieved for some poor UV-absorbing carboxylic acids and sulfonic acids with quinine or quinidine as the chiral UV probe [22]. The application of indirect detection for lipid-type compounds is rare, with the exception of a report on glycerol monostearate [23]. No indirect detection method has been reported for the detection of phospholipids.

Because of the pharmaceutical interest of OMDPI, there is a need for a reversed-phase HPLC method for its assay. In this manuscript, we describe the development and validation of an indirect UV detection method for stability-indicating analysis of OMDPI. It applies protriptyline hydrochloride as an UV-absorbing reagent in the mobile phase. The method is linear, sensitive, stable and easy to perform with conventional HPLC equipment.

2. Experimental

2.1. Reagent

OMDPI was received from the National Cancer Institute (MD,USA). *p*-Hydroxybenzoic acid *n*-butyl ester (butylparaben) and protriptyline (PTL) hydrochloride were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile (ACN) was from Mallinckrodt (Paris, KY, USA). Water was purified through a Millipore Super-Q Pure Water System (Waltham, MA, USA)

2.2. Sample preparation

Test solutions for assay were prepared by dissolving 1-mg portions of OMDPI samples in 0.5 ml of isopropanol and 0.5 ml of water. Sample solutions for assay were prepared by dissolving 1-mg portions of OMDPI in 1.00-ml aliquots of the internal standard (I.S.) solution (50 mg butylparaben in 100 ml water–isopropanol, 1:1). Forced decomposition samples were 1.0 mg/ml solution with 50% isopropanol and 50% of one of the following aqueous media: water, 0.1 *M* HCl or 0.1 *M* NaOH. The solutions were heated in a heating block at 80°C for 1 day.

2.3. HPLC

HPLC was performed with a HP-1100 system (Hewlett-Packard, Wilmington, DE, USA). Data were collected and processed with the HP 3D ChemStation. Aliquots of 5 μl of OMDPI test solution were injected onto an Alltech Allsphere ODS-1 column (5 μm, 250×4.6 mm I.D., Alltech, Deerfield, IL, USA). Elution was isocratic with a mobile phase of a mixture of acetonitrile–water (55:45), each containing 0.08 m*M* protriptyline hydrochloride (24 mg/l) at 1 ml/min flow-rate. Detection was by UV at 230 nm.

3. Results and discussion

3.1. Choice of the probe

Choosing the proper probe is the first important consideration in the development of an indirect HPLC assay. High absorptivity of the probe is a key factor for detection sensitivity. Strong, specific binding between the analyte and the probe and an environment that reinforces the binding can induce a potent signal. Optimal signal intensity is achieved when the affinities of the probe and the analyte to the stationary phase (S) are similar [25].

OMDPI is a phosphoric acid ester with a readily ionizable proton, which makes it negatively charged within the whole pH range applicable to HPLC. It also has a highly lipophilic alkyl side-chain. UV-absorbing compounds, which have similar affinity to the stationary phase as OMDPI, may be considered for signal induction. A probe for OMDPI could also be an UV-absorbing cation which may employ the ion-pairing mechanism to couple with OMDPI. Researchers have reported that ionic analyte–probe interactions give higher response [16,26].

Our method development started with the search for the probe. Several anionic, cationic and neutral UV-absorbing reagents have been investigated as probe candidates for OMDPI. Their retention on an ODS column with various mobile phase are shown in Table 1. The retention of native OMDPI (detected by MS) is 18 min on an ODS column with a gradient elution of increasing acetonitrile in ammonium acetate buffer (28–73% acetonitrile in 15 min, 0.1 *M*

Table 1
Probe and OMDPI retention on an ODS column[a]

Probe	Mobile phase	k'
1-Naphthol-2-sulfonic acid	Methanol–water (60:40)	0.5
Nicotinamide		1.7
Nitroaniline		2.4
2-Naphthylamine		4.6
Butyl paraben		8.3
Naphthalene		12.2
Phenanthrene		30.0
Anthrancene		38.5
Benzyldimethyl-ammonium bromide	Isopropanol–water (40:60)	1.5
2-Naphthalene sulfonic acid	Methanol–0.05 *M* KH_2PO_4 (pH 4.3) (30:70)	3.7
Protriptyline hydrochloride	Acetonitrile (50–90%)–water	Not eluted ($k'>52$)
	Acetonitrile–50 m*M* NH_4OAc (pH 5.4) (90:10)	14.3
	(80:20)	13.1
OMDPI	Gradient, 28–73% ACN in 15 min, 0.1 *M* NH_4OAc buffer	9.6

[a] Column: Alltech Allsphere ODS-1, 5 μm, 250×4.6 mm I.D.

ammonium acetate). Most anionic and cationic probe candidates do not have as strong retention as OMDPI (Table 1). Neutral compounds may have sufficient retention, but did not give an induced signal for OMDPI, probably due to limited interaction with OMDPI. The chromatographic behavior of protriptyline (PTL) hydrochloride is somewhat special. The fact that it was retained 26 min ($k'=14.3$) with 90% ACN (with buffer) suggested strong hydrophobic interaction; the fact that it needed NH_4OAc to elute and eluted faster (24 min, $k'=13.1$) in a slightly lower ACN content (80 vs. 90%) suggested ionic polar interaction with the residual silanol group on the silica surface. This may best suit the amphiphilicity of OMDPI, which is ionic and yet hydrophobic. When applied to the mobile phase, PTL gave an intense signal for OMDPI. Therefore, PTL was chosen as the probe for further HPLC optimization.

3.2. Optimization of HPLC

Concentration of the probe in the mobile phase can alter the coverage of the probe on the stationary phase, which can affect the analyte's retention time as well as the detection sensitivity [25]. Fig. 2 shows the result of retention, detection response (peak area) and efficiency (shown as plate number N/column) of OMDPI at different concentration of PTL. The optimal probe concentration of 0.08 m*M* PTL was chosen for the assay development.

Concentration of the organic modifier is another factor that can alter the ion-pair formation as well as probe coverage on the stationary phase. Fig. 3 shows typical chromatograms. Fig. 4 shows the retention and response of OMDPI as the percentage of the organic modifier varied. No peak for OMDPI appeared when ACN is less than 50%. This may be due to the highly aqueous environment unfavorable for ion pairing. The peak area is basically unaffected

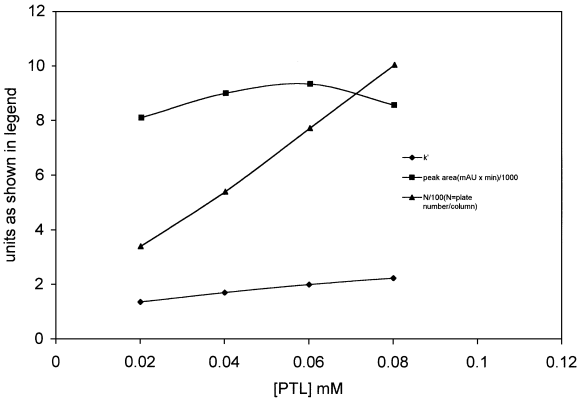

Fig. 2. Effect of PTL concentration on retention, peak area and column efficiency (N). Column: Alltech Allsphere ODS-1 5 μm, 250×4.6 mm I.D.; mobile phase: ACN–water (55:45), with [PTL] 0.02–0.08 m*M*; sample: OMDPI 1.5 mg/ml in isopropanol–water (1:1); injection: 5 μl; detection: 230 nm.

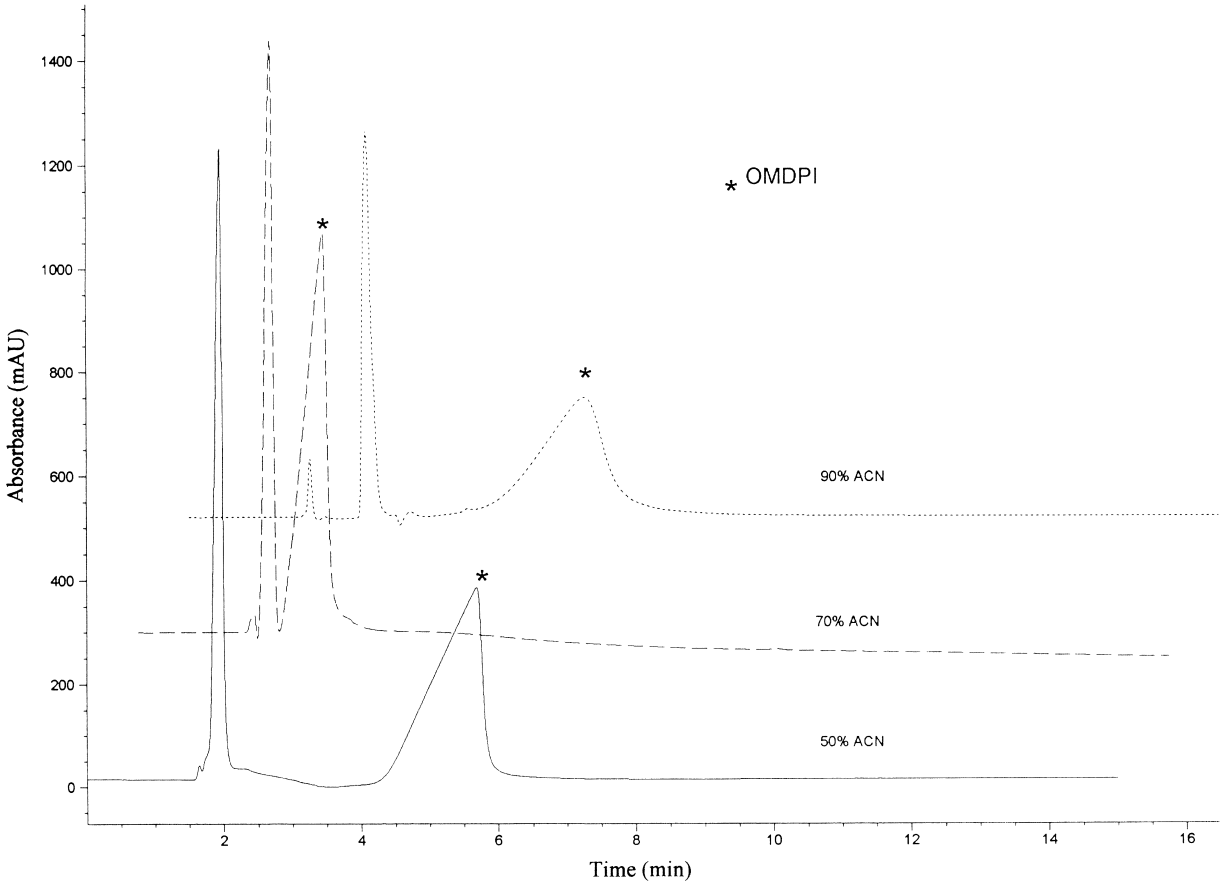

Fig. 3. Chromatograms of OMDPI with various percentages of ACN in the mobile phase Column: Alltech Allsphere ODS-1 5 μ, 250×4.6 mm I.D.; mobile phase: 50–90% ACN in water with [PTL] 0.04 m*M*; sample: OMDPI 1.5 mg/ml in isopropanol–water (1:1); injection: 5 μl; detection: 230 nm.

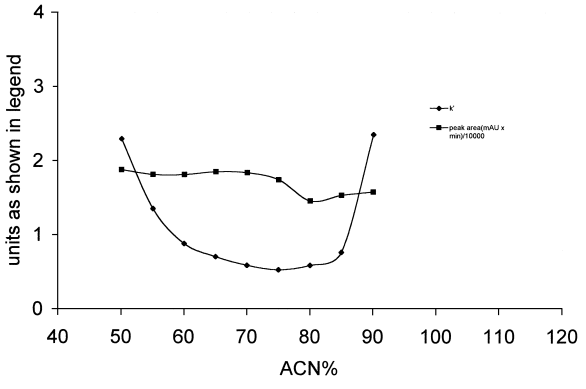

Fig. 4. Effect of ACN content in mobile phase on retention and peak area (see Fig. 3 for HPLC conditions).

between 50 and 90% ACN. The retention varies greatly with ACN content. At constant PTL concentration, OMDPI is most retained at 50 and 90% ACN. It appears that the ion pair exhibits reversed-phase characteristics (t_R decreases when ACN content increases) between 50 and 80% ACN and normal-phase behavior (t_R increases when ACN content increases) between 80 and 90% ACN. This behavior was described by Horváth et al. using the dual retention mechanism [30]. The analyte is retained both by the C_{18}, which shows the reversed-phase behavior, and by the silanol groups, which show the normal-phase behavior. Peaks eluted earlier than 4 min ($k'<1.3$) are generally sharper and higher but are not separated from the unretained system

peak. Therefore, the ACN concentration allowed for assay development is 50 or 90%. The 50% ACN condition was chosen for further optimization.

3.3. Minimize system peaks

In indirect detection, it is often the case that there are many system peaks in a chromatogram. Each component in the sample matrix (analytes, solvents, salts) can cause perturbation of the probe concentration [P] and give rise to a system peak. These system peaks can be confusing and may diminish the separation ability of the method. A buffered system may minimize the ion concentration perturbation and thus eliminate some system peaks. However, when ammonium acetate at 10, 20 or 40 m*M* were added to the mobile phase, the OMDPI peak was not observed under these conditions; probably due to hindrance of the OMDPI-PTL ion pair formation by the buffer ions. Another way to minimize system peaks is to match the sample solvent composition or elutropic strength with the mobile phase. This minimizes the perturbation of [P] by stripping off P from $P^+\cdots S$ (P^+ bound to stationary phase at equilibrium). Fig. 5 shows that the isopropanol peak is distinct at 195 nm but not detected at 230 nm when the mobile phase contains 55% ACN. This remains true when the mobile phase contains≥50% ACN. However, when the mobile phase contains less than 50% ACN, the isopropanol peak begins to be detectable also at 230 nm. Its intensity at 230 nm increases with decrease of ACN in the mobile phase.

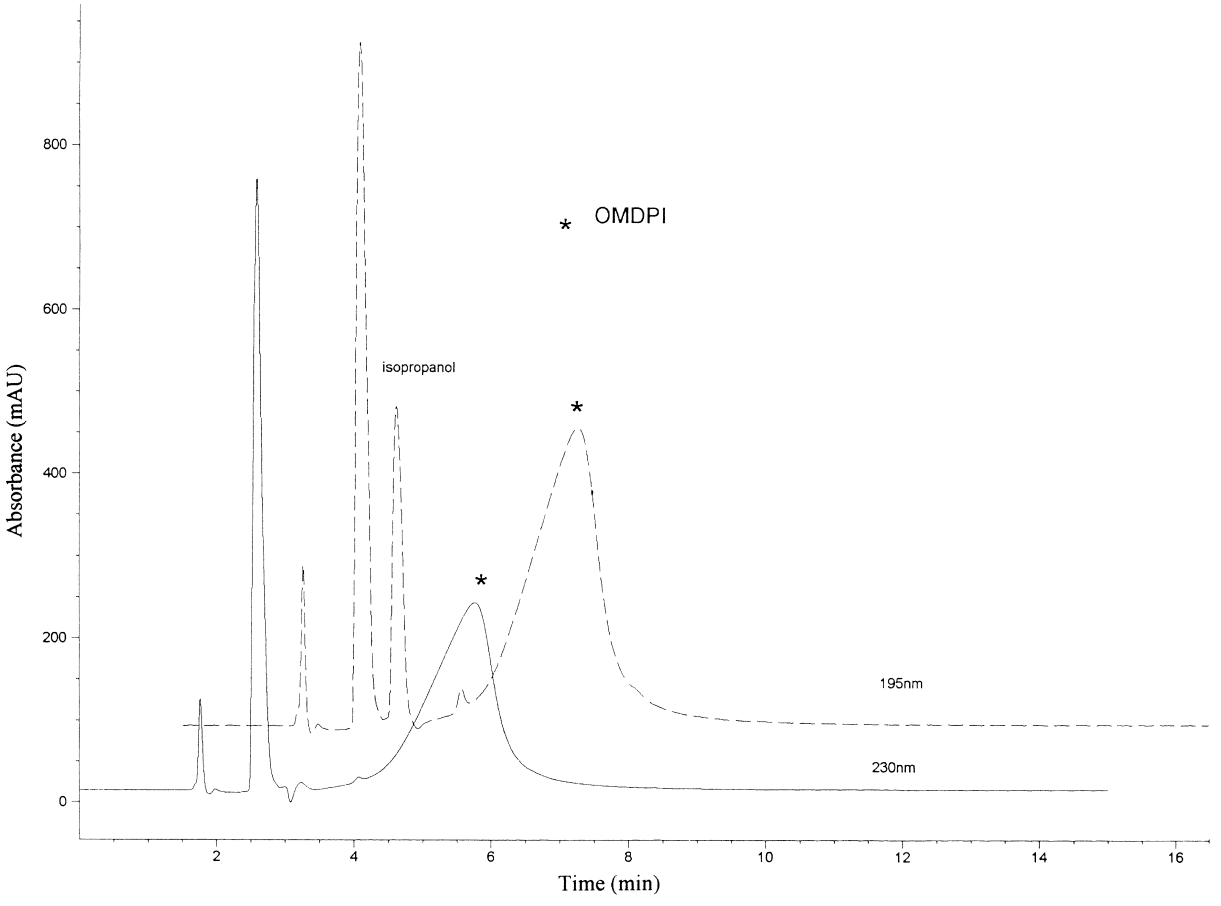

Fig. 5. Chromatograms of OMDPI solution detected at 195 and 230 nm. Column: Alltech Allsphere ODS-1 5 μm, 250×4.6 mm I.D.; mobile phase: 55% ACN in water, with [PTL] 0.04 m*M*; sample: OMDPI 1 mg/ml in isopropanol–water (1:1); injection: 5 μl;.

A probable explanation is that the stronger elutropic isopropanol in the sample solvent stripes the P+ from P+···S. thus, when the elutropic strength of the mobile phase is stronger than that of the sample solvent, system peak(s) due to sample solvent can be minimized. In the current study, the system peak due to isopropanol is minimized when the mobile phase has more than 50% ACN. Although the similar elutropic strength cannot always be achieved (because it depends on the sample solubility and the overall chromatographic requirement for the mobile phase condition), it is worth considering while optimizing the method.

3.4. System conditioning

Protriptyline has strong affinity for the C_{18} stationary phase, probably through both hydrophobic interaction with the C_{18} and ionic interaction with the silanol groups. A large amount of PTL is needed to saturate the stationary phase before PTL concentration in the mobile phase reaches equilibrium. As determined by the absorption isotherm curve breakthrough point and by the baseline rise during conditioning, about 4–5 mg of PTL is needed to saturate the stationary phase. This is equivalent to about 3 h of conditioning under the operating conditions (0.08 m*M* of PTL at 1 ml/min).

3.5. Validation

Based on the optimization results, the final condition for OMDPI's indirect detection are as described in Section 2.2. Fig. 6 is the chromatogram of a test solution for the OMDPI assay. Butyl paraben was chosen as the internal standard, which was well separated from OMDPI and decomposition products (shown in Fig. 7). The significant peak at 1.8 min ($k'=0.06$) was not observed in blank (isopropanol–water, 1:1) injections, and is associated with the sample. When the OMDPI solution was spiked with H_3PO_4 or HCl, the 1.8-min peak was enhanced, along with the appearance of another slightly less retained peak. The 1.8-min peak is probably a 'system peak' caused by the dissociated H^+ of OMDPI which displaced the PTL^+ from the exposed silanol groups in the stationary phase.

The HPLC method presented was validated for OMDPI assay. Results were calculated from the peak area ratio of OMDPI/I.S. At a concentration of 1

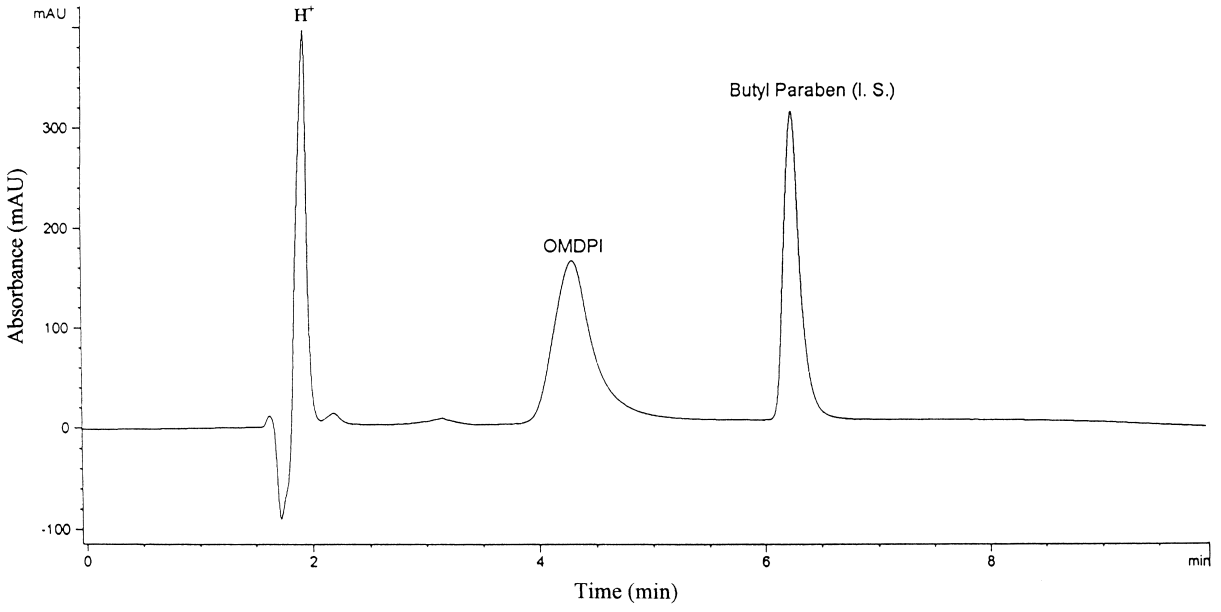

Fig. 6. Chromatogram of an OMDPI assay test solution. Column: Alltech Allsphere ODS-1 5 μm, 250×4.6 mm I.D.; mobile phase: 55% ACN in water, with [PTL] 0.08 m*M*; sample: OMDPI (1 mg/ml) and butylparaben (0.5 mg/ml) in isopropanol–water (1:1); injection: 5 μl; detection: 230 nm.

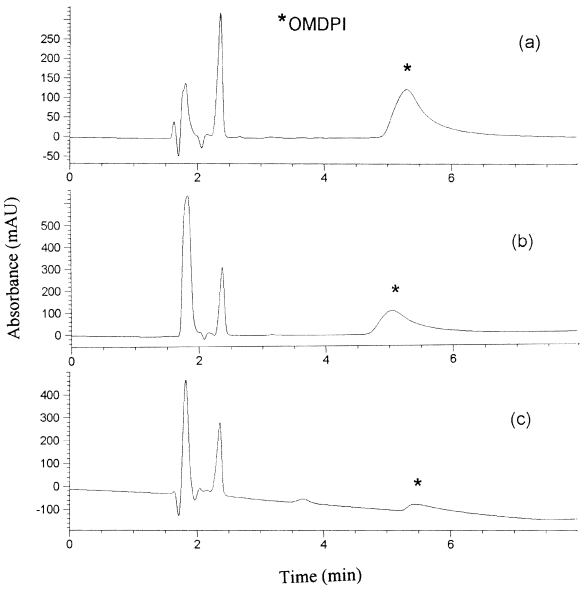

Fig. 7. Chromatograms of forced decomposed OMDPI solutions. in water–isopropanol (1:1), heated at 80°C for 1 day in 0.1 N HCl–isopropanol (1:1), heated at 80°C for 1 day in 0.1 N NaOH–isopropanol (1:1), heated at 80°C for 1 day. Column: Alltech Allsphere ODS-1 5 μm, 250×4.6 mm I.D.; mobile phase: 55% ACN in water, with [PTL] 0.08 m*M*; sample: OMDPI (1 mg/ml) treated as above. Injection: 5 μl; detection: 230 nm.

mg/ml OMDPI, the precision of the HPLC assay is 1.1% R.S.D. ($n=6$) within day and 2.5% R.S.D. ($n=18$) between days. Based on data from five standard solutions ranging from 0.5 to 1.6 mg/ml of OMDPI, the linear regression analysis between response (OMDPI peak area/internal standard peak area, y) and concentration (x) gave $y=1.6121x+0.0341$. The linear correlation coefficient (r^2) is 0.9995. Error of the assay is 0.6%, which was calculated as the average deviation (%) of the back-calculated versus the actual OMDPI concentration. The detection limit was determined by injecting diluted sample solutions until the signal-to-noise ratio became 3. The detection limit is 5 μl injection of 1 μg/ml or 5 ng. The method can separate the compound from its decomposition products and can be used as stability indication assay. Fig. 7 shows the chromatograms of the forced decomposition samples. Hydrolysis products 3-deoxyinositol phosphate (2.4 min, $k'=0.41$), 2-*O*-methyl-1-*O*-octadecylglycerol phosphate (3.7 min, $k'=1.18$) are separated from OMDPI and the internal standard. Identities of OMDPI and the decomposition products were confirmed with LC–MS of the collected fractions from the presented LC system. Another hydrolytic product H_2PO4^- was barely resolved and sometimes co-eluted with the 1.8-min peak.

4. Conclusion

An HPLC analysis method based on ion-pairing indirect detection has been developed and validated for a phospholipid analog, OMDPI. The method is precise (R.S.D.<2.5%), accurate (error 0.6%), linear ($r^2=0.9995$) and specific. The detection limit is 5 ng of OMDPI.

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