Application of Supercritical Fluid Extraction and Chromatography to Assay Fat-Soluble Vitamins in Hydrophobic Ointment

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The determination of retinol palmitate and tocopherol acetate in a hydrophobic ointment by a coupled supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC) system is described. The ointment, triturated with diatomaceous earth powder, was subjected to SFE with carbon dioxide, and the extract containing fat-soluble vitamins was trapped on a trimethylsilyl silica-gel column by reducing the fluid pressure. The trapped components were analyzed on an octadecylsilyl silica-gel column by SFC using carbon dioxide containing ethanol as a mobile phase and UV absorption monitoring at 284 nm. In this method, retinol palmitate and tocopherol acetate in preparations could be determined within ten minutes without any manual products after placing the sample into the system.

Keywords Supercritical fluid extraction, supercritical fluid chromatography, fat-soluble vitamin, retinol palmitate, tocopherol acetate

The determination of fat-soluble vitamins, retinol and tocopherol, in pharmaceutical preparations has been carried out using various techniques, such as spectrophotometry^{1,2}, gas chromatography³ and high-performance liquid chromatography.⁴⁻⁶ However, these methods require manual extraction procedures. Recently, supercritical fluid extraction (SFE) and chromatography (SFC) have been studied regarding the analysis of oily components in both natural products and drugs. Especially, a coupled SFE/SFC system could simplify the analytical procedure.⁷⁻⁹ Many reports concerning SFE and SFC for fatty acids, fats and oils have appeared.¹⁰⁻¹²

This report describes the application of a coupled SFE/SFC system used for the determination of fat-soluble vitamins, such as retinol palmitate and tocopherol acetate, in a hydrophobic ointment.

Experimental

Chemicals

Carbon dioxide with a purity of more than 99.99% was obtained from Kanto Sanso Ind., Co., Ltd., Tokyo. The ethanol used was of analytical grade, and obtained from Wako Pure Chemicals Ind., Ltd., Osaka. Retinol palmitate and tocopherol acetate, used as standard substances, were of raw-material grade based on preparations from Roche Ind., Ltd., Switzerland.

Apparatus

The supercritical fluid extraction and chromatographic system (SUPER-200 SYSTEM 3, JASCO Corp., Tokyo) was equipped with a multi-channel spectrophotometer

(MULTI-340, JASCO Corp., Tokyo) and a UV/VIS spectrophotometer (875-UV, JASCO Corp., Tokyo). Figures 1(a) and 1(b) show diagrams of this SFE/SFC system. The thick line indicates the part that was kept at a higher pressure by using back-pressure regulators.

During the SFE process, the pressure before the trap column was maintained higher; after the trap column the pressure became lower. The density of the carbon dioxide was thus rapidly decreased in the trap column, resulting in the deposition of extracts on the packed bed of the trap column.

Upon completion of extraction, the flow line was switched to the SFC system, and ethanol (as a modifier)

Fig. 1 Systems of SFE & SFC. P1, pump (1)=CO₂; P2, pump (2)=ethanol; EV, extraction vessel; TC, trap column; SC, separation column; D1, spectrophotometer MULTI-340; D2, spectrophotometer 875-UV; ●, back pressure regulator.

was sent from pump (2). Supercritical carbon dioxide containing a modifier was flowed through the trap column on which the extract had been concentrated.

The extract in the trap column was then eluted and introduced into the separation column, where chromatographic separation took place. A multi-channel spectrophotometer was used to monitor real-time three-dimensional chromatograms of the investigation, and a single-channel spectrophotometer was used to accurately calculate the peak areas at 284 nm regarding the determination.

SFE conditions

Extraction was performed at a pressure of 200 kg/cm² and a temperature of 40°C with supercritical carbon dioxide at a flow rate of 4.0 ml/min as liquid carbon dioxide for four minutes. Suprapak SIL-C1 (4.6 mm i.d.×50 mm in length; 5 µm trimethylsilyl (TMS) silica-gel packing material purchased from JASCO Corp., Tokyo) was used as the trap column. As an extraction vessel, a 1 ml vessel for the SFE system was used.

SFC conditions

Finepak SIL-C18 (4.6 mm i.d.×150 mm in length; 5 µm ODS silica-gel packing material purchased from JASCO Corp., Tokyo) was used as the separation column. Supercritical carbon dioxide containing ethanol was used as the mobile phase¹³ at a flow rate of 4.0 ml/min as liquid carbon dioxide and 0.4 ml/min as liquid ethanol at a pressure of 200 kg/cm² and a temperature of 40°C.

Assay procedure

About 1 g of accurately pre-weighed ointment (Table 1) and about 9 g of accurately pre-weighed diatomaceous earth powder were placed in a glass beaker and blended completely. About 20 mg of this mixture (weighed accurately) was placed in an extraction vessel. The components in the sample were extracted and chromatographed under the conditions of the coupled SFE/SFC system. The contents of retinol palmitate and tocopherol acetate were calculated from the peak areas at 284 nm.

Results and Discussion

Extraction time

Extraction was performed at a pressure of 200 kg/cm² and a temperature of 40°C, which are the standard

conditions for SFE in this system; all of the components in this sample could be extracted in less than four minutes (Fig. 2). The time for extraction was thus set at four minutes.

Effect of the pressure, temperature and modifier concentration

The elution parameters, such as pressure, temperature and modifier concentration, were varied in order to determine the optimum condition on an octadecylsilyl (ODS)-silica gel column. At a constant temperature (40°C) and modifier concentration (10% of the flow rate of liquid carbon dioxide), as the pressure was increased the retention was decreased (Fig. 3). This tendency can be explained according to the theory that solubility variations are caused by density variations of the mobile phase. When the pressure was increased at constant temperature, the density of the fluid was increased, and

Fig. 2 Extraction curve of components in hydrophobic ointment on SFE monitoring after the extraction vessel.

Fig. 3 Capacity ratios (k') of retinol palmitate and tocopherol acetate as a function of the pressure on SFC. Temperature, 40°C; modifier concentration, 10%; flow rate of CO₂, 4.0 ml/min.

Table 1 Prescription of preparation

Retinol palmitate	240000 I.U./100 g (141.1 mg)
Tocopherol acetate	100 mg
Ergocalciferol	50000 I.U.
Sulfisomidine	5 g
Allantoin	500 mg
Zinc oxide	5 g

the retention was decreased. Good separation of these components was then obtained at a lower pressure. The pressure at the time of the determination was set at 200 kg/cm² with regard to the time required and the peak shapes. Under these conditions, the resolution (R_s) was 4.2.

At a constant pressure (200 kg/cm²) and modifier concentration (10%), though the temperature was increased, the retention did not vary much. This fact suggests that the density of the supercritical fluid depends on the absolute temperature, and that the variation of the temperature in this experiment was too small to have any effect on the density.

At a constant temperature (40°C) and pressure (200 kg/cm²), as the modifier concentration increased the retention decreased (Fig. 4); good separation of these components was obtained at lower concentration. This tendency can be explained based on the idea that the retention depends on the polarity of the mobile phase with

chromatography. The modifier concentration for the determination was thus set at 10%.

Wavelength

From a three-dimensional chromatogram of the sample, the distortion of base line was observed at a shorter wavelength (Fig. 5). The wavelength at the time of the determination was thus set at 284 nm.

Calibration curves

Calibration curves for retinol palmitate and tocopherol acetate were obtained from 0.5–2.5 µg, respectively. The regression equations were as follows: $Y=197625X-6213$ ($r=0.999$) for retinol palmitate and $Y=21713X-1227$ ($r=0.997$) for tocopherol acetate, respectively, where Y is the peak area and X is the amount of each compound (µg).

Recovery test

Known amounts of retinol palmitate and tocopherol acetate were added to a sample mixture containing a preparation which lacked these two components; the amount of each component was determined using the present coupled SFE/SFC system. The recovery rates for retinol palmitate and tocopherol acetate were 102.0 and 101.5%, respectively. Based on this result, our present method can be satisfactorily used for the quantitative determination of retinol palmitate and tocopherol acetate in this preparation.

Results of determination

The analytical results for the preparation were

Fig. 4 Capacity ratios (k') of retinol palmitate and tocopherol acetate as a function of the modifier concentration on SFC. Temperature, 40°C; pressure, 200 kg/cm²; flow rate of CO₂, 4.0 ml/min.

Fig. 5 Three-dimensional chromatogram of retinol palmitate and tocopherol acetate in sample mixture.

Fig. 6 Chromatogram of retinol palmitate and tocopherol acetate at 284 nm on SFC. Temperature, 40°C; pressure, 200 kg/cm²; modifier concentration, 10%; flow rate of CO₂, 4.0 ml/min.

139.1 mg/100 g of retinol palmitate and 103.2 mg/100 g of tocopherol acetate; the chromatograms are shown in Fig. 6.

From these results it was proved that the present method is applicable to the determination of these two components in this preparation within 10 min without any manual products after placing the sample in the extraction vessel. The accuracy of this method was equivalent to that of the conventional method by solvent extraction and HPLC.

This study suggests that a directly coupled SFE/SFC system should be a valuable method for the quantitative analysis of preparations, including the fat-soluble components.

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