Temperature Effects on Packed-Capillary Liquid Chromatography of the X-ray Contrast Agent Iodixanol

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Abstract

The effect of varying the operating temperature from 6 to 90°C on the chromatographic performance of the *exo–exo* and *exo–endo* isomers of the X-ray contrast agent Iodixanol in packed-capillary reversed-phase liquid chromatography shows increasing interconversion rates between the two isomeric conformers with increasing temperature. At 90°C, Iodixanol elutes as one sharp peak due to an increased interconversion rate between the two isomeric conformers. Consequently, increased sensitivity is achieved. Temperature programming from 6 to 40°C is utilized to optimize the resolution and determination of the *exo–exo* and *exo–endo* isomers. Temperature programming provides a significant decrease in the retention times in comparison with the isothermal separations while still preserving baseline separation of the isomers.

Introduction

Nearly all X-ray contrast agents for medical diagnosis are tri-iodinated benzene derivatives with hydrophilic substituents (to achieve high water solubility and low toxicity) (1). Iodixanol (Figure 1) is a third-generation X-ray contrast agent and thus a dimer of a nonionic tri-iodinated aromatic compound. The pharmaceutical formulation of Iodixanol is isotonic and isoosmotic with blood (2). Iodixanol is a complex mixture of stereoisomers and rotational isomers (due to stereocenters and chiral axes arising from hindered rotation) (1). The carbonyl oxygen atoms of the two acetyl groups in the bridge between the two monomers are either in *endo* or *exo* position, specifying their orientation towards or away from the aromatic ring, respectively (1). Thus, Iodixanol has three geometric isomers at equilibrium; *exo–exo*, *exo–endo*, and *endo–endo* forms (3). The population of *exo–exo*, *exo–endo*, and *endo–endo* isomers in Iodixanol at ambient temperature has been measured to be approximately 60, 37, and 3%, respectively (2). However, the interconversion rates between the isomeric forms are highly temperature dependent, with the *exo–exo* isomer being the most stable and *endo–endo* being the least stable specie (2). Thus, changes in the chromatographic performance of Iodixanol are expected when changing the operating temperature.

Packed capillary columns are especially well suited for operation at various temperatures in liquid chromatography (LC), enabling faster thermal equilibrium and thus creating new opportunities for selectivity and retention control utilizing temperature programming (4). Temperature programming utilizing packed capillary columns has successfully been performed as an alternative to traditional solvent gradient elution, which is not simple with miniaturized columns (5–10). In addition, operation at elevated temperatures in LC has been reported to increase the separation efficiency (11–17) and reduce the analysis time (5–17). Packed-capillary columns are also characterized by low mobile phase and stationary phase consumption, high resolution of complex mixtures when using long columns, increased mass sensitivity due to reduced dilution, and excellent suitability for direct connection to mass spectrometers and Fourier transform infrared spectrometers (18).

To the authors' knowledge, all published studies regarding the chromatographic determination of Iodixanol have employed conventional LC at ambient temperature, usually utilizing reversed-phase solvent gradient elution (2,3,19–21). In addition, Nomura et al. (22) utilized size-exclusion chro-

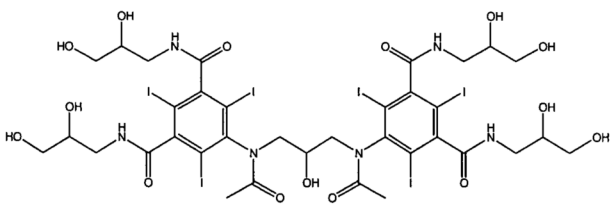

Figure 1. Chemical structure of Iodixanol.

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matography (SEC) for the total determination of Iodixanol in plasma samples, eluting all isomers of Iodixanol as a single peak. Jacobsen et al. (21) used solvent gradient elution reversed-phase LC for the separation and determination of the *exo–exo* and *exo–endo* isomers of Iodixanol, suggesting that the characteristic ratio between the isomer peaks could be exploited for the identification of Iodixanol in plasma and urine samples.

The aim of the present study was to make accessible both the single-peak determination of total Iodixanol and selective separation of the *exo–exo* and *exo–endo* isomers utilizing the same chromatographic system. Packed-capillary LC, at elevated temperatures or with temperature programming from subambient temperatures, offers both opportunities.

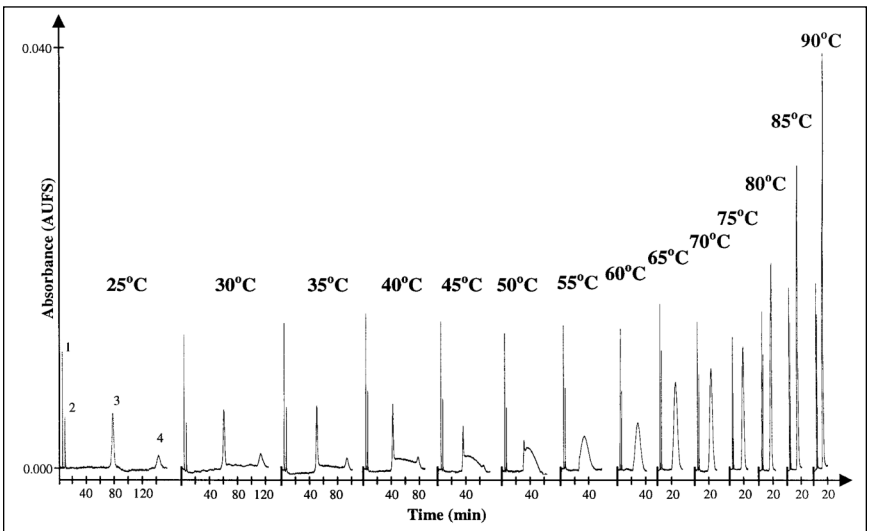

Figure 2. Isothermal separations of uracil (peak 1), 5410 (peak 2), and the *exo–exo* (peak 3) and *exo–endo* (peak 4) isomers of Iodixanol using a mobile phase of 5% acetonitrile in water.

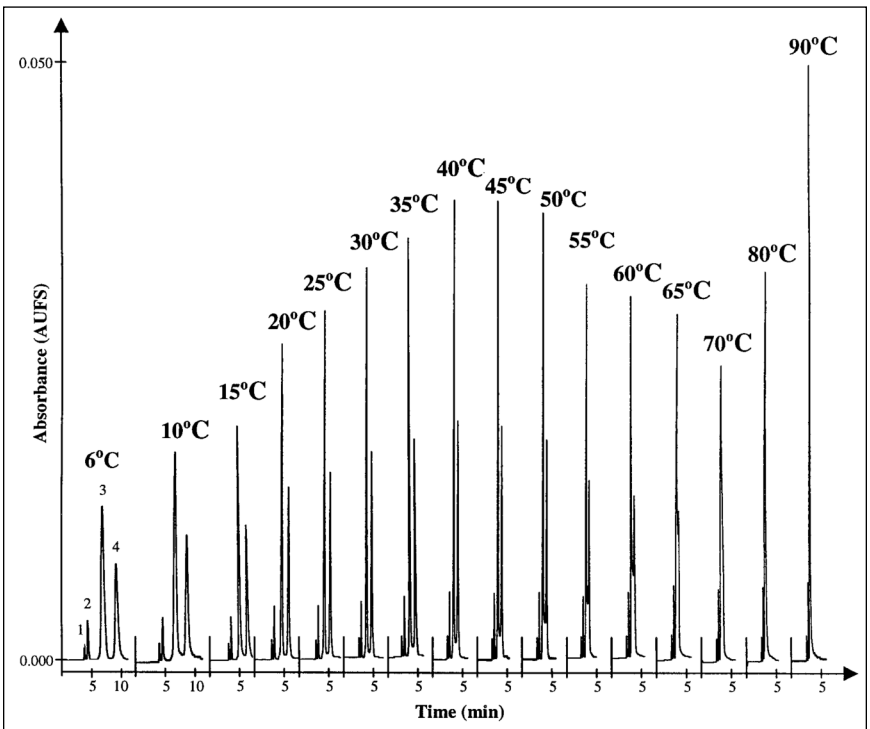

Figure 3. Isothermal separations of uracil (peak 1), 5410 (peak 2), and the *exo–exo* (peak 3) and *exo–endo* (peak 4) isomers of Iodixanol using a mobile phase of 12% acetonitrile in water.

Experimental

Materials and reagents

Acetonitrile (high-performance liquid chromatography grade) was obtained from SDS (Peypin, France). Water was deionized and glass distilled. All fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ). Helium (> 99.998% purity) was purchased from AGA (Oslo, Norway). 3,3',5,5'-tetrakis(2,3-dihydroxypropylcarbamoyl)-2,2',4,4',6,6'-hexaiodo-*N,N'*-(2-hydroxypropane-1,3-diyl)diacetanilide (Iodixanol) and 5-acetylamino-*N,N'*-bis-(2,3-dihydroxy-propyl)-2,4,6-triiodo-isophthalamide (compound 5410) (3) were supplied by Nycomed Imaging (Lindesnes, Norway). Uracil was obtained from Fluka (Buchs, Switzerland).

Column preparation

The packed-capillary columns were prepared according to a method previously described (7), using supercritical CO_2 as the slurry medium. The initial packing pressure was 100 bar, and the packing proceeded with a pressure ramp of 10 bar/min to 550 bar, which was held for 30 min. Valco ZU1C unions (Valco Instruments, Houston, TX) with 2-µm Valco 2SR1 steel screens served as column end fittings, and the columns were connected to the end fittings by Valco FS1.4 polyimide ferrules and steel nuts. The column body was of fused silica (320-µm i.d., 450-µm o.d.) with a polyimide protection layer, and the columns were prepared in lengths of 25 cm. The stationary phase material was 3-µm Hypersil C-18 particles (Hichrom, Reading, U.K.).

Instrumentation

The LC instrument consisted of a Merck Hitachi (Darmstadt, Germany) L-7110 isocratic piston pump. The injections were performed with a Valco C4 manually operated injection valve equipped with a 50-nL internal loop. A Mistral column oven (Spark, Emmen, The Netherlands) was employed. On-column detection at 254 nm was performed using a Linear ultraviolet–visible UV-vis 200 detector with a fused-silica capillary detection cell (100-µm light path) coupled to a Shimadzu (Kyoto, Japan) R5A integrator. A fused-silica restrictor (15 cm × 15-µm i.d.) was connected

to the end of the detection capillary to prevent the mobile phase from boiling when operating at elevated temperatures. The mobile phase was degassed with helium every day for 10–15 min. The flow rate was 5 µL/min throughout the whole study.

Preparation of standard solutions

Solutions of Iodixanol were prepared in water at a concentration of 10 mg/mL. The solutions were spiked with approximately 1 mg/mL of the intermediate product from the synthesis of Iodixanol, 5410, which is likely to be present in in-process samples. In addition, various amounts of uracil (0.03–1 mg/mL) were added as t_0 markers.

Results and Discussion

Temperature effects

Several research groups have studied changes in molecular shape as a function of temperature utilizing LC. The majority of these studies have investigated *cis–trans* isomerism of peptides and proteins and reported the changes in the peak shapes of the isomers with temperature due to interconversion during the chromatographic run (23–27). In the present study, the effect of temperature changes on the peak shapes of the *exo–exo* and *exo–endo* isomers of Iodixanol has been investigated.

In order to achieve a wide chromatographic window, a mobile phase composition of 5% acetonitrile in water was initially utilized for the separation of the different isomers of Iodixanol when investigating the temperature interval from 25 to 90°C. As shown in Figure 2, the *exo–exo* and the *exo–endo* isomers were well resolved at 25°C with retention times of 77 and 143 min, respectively. At 30°C, a plateau was observed between the *exo–exo* and *exo–endo* isomer peaks. The magnitude of this plateau increased with increasing temperature, slowly resulting in a broad, dominating peak, with retention time in between the two isomers. This phenomenon is caused by the reversible interconversion between the two isomeric species during the chromatographic run. Thus, the solutes eluting in the middle of the plateau have spent equal time as *exo–exo* and *exo–endo* isomers during the chromatographic run. The isomerization rate of Iodixanol is temperature dependent, with *exo–exo* as the most stable isomer (2). In accordance, the peak areas of the fraction of the *exo–exo* and *exo–endo* isomers that did not undergo interconversion subsequently decreased with temperature in the present study, and their characteristic peaks were no longer visible on the chromatogram at 55 and 50°C, respectively. At 60°C, only one broad peak was present, indicating that nearly all of the molecules did undergo interconversion at this temperature. When increasing the temperature further, this single peak became narrower, illustrating that the isomerization rate increased with temperature and that an expanding population of the solutes did likewise interconvert. In addition, reduced peak resolution is commonly observed in reversed-phase LC systems at elevated temperatures.

Fossheim et al. (2) calculated the time to reach equilibrium between the *exo–exo* and *exo–endo* isomers of Iodixanol at 10, 25, and 40°C to be 250, 27, and 4 h, respectively. Thus, low temperatures can be utilized for the determination of the individual isomers. It was necessary to increase the portion of acetonitrile in the mobile phase to elute the isomers within reasonable time at subambient temperatures. Separations of the isomers of Iodixanol at temperatures ranging from 6 to 90°C are shown in Figure 3 utilizing a mobile phase composition of 12% acetonitrile in water. At 6°C, the *exo–exo* and *exo–endo* isomers were baseline separated with a total analysis time of less than 10 min. When increasing the temperature further, the resolution between the isomers decreased, and complete baseline separation was not achieved. Due to the short retention times and the rather poor peak resolution, a narrow chromatographic window was observed. Consequently, visual determination from the chromatograms of the interconversion between the *exo–exo* and *exo–endo* isomers was difficult. Based on the results of Fossheim et al. (2), it is reasonable to assume that a smaller population of the isomers did undergo interconversion during the chromatographic run when the mobile phase of 12% acetonitrile in water was utilized as compared with using the mobile phase of 5% acetonitrile in water. This is due to the fact that the isomers spent a significantly shorter time on the chromatographic column at all temperatures when using the stronger mobile phase. Analogously, Jacobson et al. (23) observed similar behavior using different volumetric flow rates for the separation of *cis–trans* Proline. Nevertheless, at 45°C, the peak heights of both the *exo–exo* and *exo–endo* isomer of Iodixanol decreased, displaying the obvious effects of interconversion. At 70°C, only one single-isomer peak was present, and when increasing the temperature stepwise to 90°C, the peak height increased correspondingly.

The *endo–endo* isomer of Iodixanol usually has a slightly longer retention than the *exo–endo* isomer in reversed-phase LC systems (3) because of the increased surface area of the *endo–endo* isomer. However, the *endo–endo* isomer was not visible in the chromatograms in this study due to the fact that the population of this isomer is only 3% at 25°C.

Determination of total Iodixanol

As shown in Figures 2 and 3, the sharp single peak of Iodixanol occurring at 90°C, regardless of the mobile phase composition, can be employed for sensitive total determination of Iodixanol, with the mobile phase composition of 5% acetonitrile in water offering the highest selectivity. Nomura et al. (22) utilized SEC for the total determination of Iodixanol in human plasma and eluted the different isomers as a single peak after 10 min, reporting a limit of detection (LOD) of 0.5 µg/mL plasma, which is equivalent to a mass LOD (mLOD) of 2.5 ng (22). In the present study, the mLOD for the single peak of Iodixanol at 90°C was determined to be 0.5 ng (signal-to-noise ratio = 3), even though a flow cell with a light path of only 100 µm was employed. Thus, by exploiting the enhanced mass sensitivity of packed-capillary columns, elevated-temperature packed-capillary LC has a high potential for the total determination of low concentrations of Iodixanol in limited sample volumes by utilizing focusing techniques upon injection of the total sample volume.

Temperature-programmed isomer separation

For the separation of the individual isomers of Iodixanol, temperature programming was employed in an attempt to reduce the retention times and enhance the separation. Utilizing the mobile phase composition of 5% acetonitrile in water, temperature programming from 25 to 70°C resulted in abnormal peak shapes and poor resolution due to the interconversion. Consequently, temperature programming from 6 to 40°C was performed using the mobile phase composition of 12% acetonitrile in water. A temperature ramp of 5°C/min was found to be most appropriate, resulting in the baseline separation of the *exo–exo* and *exo–endo* isomer with a total analysis time of less than 5 min (Figure 4). This is equivalent to a reduction of 44% in comparison with that of the separation at 6°C, which was the highest isothermal temperature achieving baseline separation of the isomers.

It is well known that all chromatographic systems need time for resetting the initial conditions after gradient separations, which is an obvious disadvantage with respect to total analysis time, if several analyses are to be performed in a row. However, further development of miniaturized systems designed for temperature-programmed LC will probably reduce the resetting time to a minimum.

Conclusion

The chromatography of the *exo–exo* and *exo–endo* isomers of the X-ray contrast agent Iodixanol is strongly temperature dependent. This study showed that temperature programming from subambient temperatures could be utilized for the selective separation of *exo–exo* and *exo–endo* isomers in short analysis times. If increased sensitivity is required, total determination of Iodixanol at elevated temperatures is accessible using the same chromatographic system. Further emphasis should be directed towards high-temperature and temperature-programmed packed-capillary LC determinations of low concentrations of Iodixanol in limited biological sample volumes, and thereby exploit the enhanced mass sensitivity of packed-capillary columns.

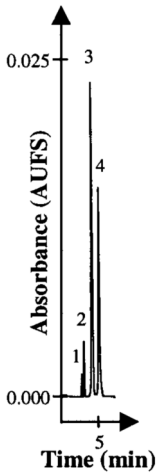

Figure 4. Temperature-programmed separation of uracil (peak 1), 5410 (peak 2), and the *exo–exo* (peak 3) and *exo–endo* (peak 4) isomers of Iodixanol using a mobile phase of 12% acetonitrile in water, starting at 6°C and then increasing 5°C/min to 40°C.

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