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Superheated water as eluent in high-temperature high-performance liquid chromatographic separations of steroids on a polymer-coated zirconia column

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Abstract

High-temperature liquid chromatography (HTLC), with a superheated water mobile phase, has been shown to be a feasible replacement for medium-polarity acetonitrile–water mixtures as an eluent in reversed-phase HPLC. Instrumental parameters of flow-rate, injection volume and mobile phase preheating were shown to have significant effects on the quality of the chromatographic peaks. The selectivity and retention patterns of testosterone and several related compounds were investigated on a porous zirconia, polybutadiene-coated column at temperatures up to 200°C and compared with that of a porous silica, octadecylsilane-coated column and the zirconia column under traditional reversed-phase conditions of an acetonitrile–water mobile phase at 40°C. The selectivity differences observed for testosterone and related compounds show that the separation mechanisms are complementary and unique selectivity is obtained with the zirconia column under HTLC conditions.

Keywords: Mobile phase composition; Liquid chromatography, high-temperature; Zirconia–polybutadiene stationary phases; Superheated water chromatography; Stationary phases, LC; Testosterone; Steroids

1. Introduction

High-temperature operation in high-performance liquid chromatography (HPLC) provides the opportunity to increase analyte mass transfer rates and thereby decrease peak width. Within the mobile phase, increased temperatures serve to increase diffusion and decrease viscosity, where the ratio of the diffusion coefficient to the viscosity–temperature product is approximately constant over a wide range of pressure and temperature conditions [1]. An additional benefit of lower viscosity is the potential to operate at higher flow-rates and thereby reduce total analysis time [2]. In some cases, temperature can also enhance compound selectivity when changes in mobile phase and column chemistry are not effective.

Elevated temperature (up to about 70°C) has been utilized in HPLC with mixed organic–water mobile phases for many years. In the last few years, the use of water-only mobile phases with significantly higher operating temperatures (up to near the critical temperature) has been demonstrated to be viable and useful for both chromatographic and extraction applications [1–10]. In this report, this operational mode is referred to as high-temperature liquid chromatography (HTLC), within which the mobile phase could

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be either mixed organic–aqueous or only water. In addition to the increased mass transport with a high-temperature water mobile phase, other practical advantages are reduced solvent and waste costs, simpler system preparation, nonflammability, and no organic vapor emission from the eluent waste containers. Since water is the most ultraviolet transparent solvent for HPLC, it can potentially be used down to the optical limits of the detector. The flame ionization detector commonly used for universal detection in gas chromatography has been used with an aqueous mobile phase [4,5,11].

The applicability of HTLC is dependent upon the solvating properties of water. While the dielectric constant decreases from about 80 to about 35 over the range of 25 to 200°C [6], it retains an appreciable density (>0.85 g/ml), cohesive energy and hydrogen bonding potential [8]. As such, it has been described as a moderate polarity solvent [3,6,8] under high-temperature conditions, comparable to traditional organic–water mobile phases. A variety of moderately polar analytes have been separated using a water mobile phase in HTLC [1–11]. The change in solvating power relative to temperature has also been proposed and demonstrated as a means of programming solvent strength [1,3,5,9].

Column durability considerations have been a major reason for only limited development of high-temperature HPLC. In particular, higher temperatures accelerate the dissolution of silica in aqueous solution. Polymeric columns such as styrene–divinylbenzene are much more resistant and stable under such conditions. Porous zirconia columns [10,12] offer an alternative, much more durable substrate compared with silica, while not imparting the high retentive characteristics of the aromatic polymer-based columns. A zirconia column coated with polybutadiene (PBD), operated at 110°C with an acetonitrile–water (40:60) mobile phase, has been used for the separation of a few nonionic, moderately polar analytes [1]. A recent report [10] compared the separation of phenol and four chlorophenols using a zirconia–PBD column with an acetonitrile–water mobile phase at 30°C and 80°C and a water-only mobile phase at 200°C. This column is reported to be stable up to 200°C [13], and was chosen for evaluation in this study.

In this study, the chromatographic characteristics of testosterone were examined at temperatures up to 200°C using a zirconia–PBD column and a water-only mobile phase. The evaluation of instrumental design and operational factors included a study of mobile phase preheating, injection volume, flow-rate, and eluent disposal strategies. A comparison was made between the zirconia–PBD column at high and normal temperatures and a conventional reversed-phase silica column for testosterone and a series of related compounds.

2. Experimental

The HTLC chromatographic system consisted of an HP1100 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) coupled with a Gilson 831 (Gilson, Middleton, WI, USA) column oven placed beside the HP1100 system. A 150 cm or 15 cm long stainless steel tubing of 0.017 cm I.D. (0.007 in. I.D.) was placed in the oven between the injection valve and the column as a preheating coil. Polyether ether ketone (PEEK) tubing of 0.017 cm I.D. placed at the outlet of the detector served as restriction tubing to maintain a constant back pressure in the detector cell. The column used in the HTLC experiments contained a porous zirconia packing and PBD coating (ZirChrom-PBD, 150×4.6 mm I.D., 3 μm particles, 300 Å pore size; ZirChrom Separations, Anoka, MN, USA). Conventional reversed-phase liquid chromatography (RPLC) was performed using an HP1100 system (quaternary pump, variable-wavelength detector; Hewlett-Packard). A Zorbax Eclipse column (XDB-C_{18}, 5 μm, 150×4.6 mm; Hewlett-Packard) was used with the column temperature controlled at 40°C. The mobile phase was water–acetonitrile (65:35, v/v), with a flow-rate of 1 ml/min. An isocratic HPLC system (515 pump, 2487 detector, 717+ autosampler, standard column heater; Waters, Milford, MA, USA) was used with the zirconia–PBD column at lower temperature (40°C) with the same mobile phase as used with the Zorbax column. The detection wavelength was 254 nm for all conditions. Chromatograms were recorded and processed using Turbochrom data analysis software (Perkin-Elmer). Prior to injections, the chromatographic systems were equilibrated until a stable baseline and pressure was observed. Replicate in-

jections of standard solutions were made to verify overall system equilibrium, especially for HTLC operation. Sample solution concentrations were 70 μg/ml testosterone and 2 μg/ml other compounds dissolved in acetonitrile–water (25:75).

Water was obtained from a Milli-Q (Millipore, San Jose, CA, USA) filtration system equipped with a 0.22-μm filter, and acetonitrile was HPLC grade (Burdick and Jackson, Muskegon, MI, USA). Testosterone was a USP reference material (United States Pharmacopeia, Rockville, MD, USA) and the other test compounds were research grade (Steraloids, Wilton, NH, USA).

3. Results and discussion

3.1. Instrumental considerations and performance

In a detailed study of temperature-related instrumental parameters [1], the preinjector heating of the mobile phase was found to significantly reduce the axial temperature gradient across a 4.6 mm I.D. column. A linear extrapolation of this data indicates that there was potentially an axial temperature gradient across our HTLC column of about 7 to 10°C relative to the column oven set point at temperatures of 160 to 200°C. The actual fluid temperature at the end of a preheater is affected by several factors, including the configuration of the tubing and how well the circulating media provides for heat transfer. For example, if the tubing is tightly coiled then the heat transfer process may be less efficient than expected. The tubing in this study was loosely coiled to allow unobstructed air circulation. The expected heat transfer efficiency can be calculated using fundamental equations and the properties of the materials [2]. The temperature of the mobile phase entering the column in our study was calculated using data from an evaluation of heat transfer for various flow-rates in HTLC [2]. That study used an oil bath while our heating media was forced air convection, so the heat transfer coefficient (h) for the media (air) to tubing was assumed to be 1/50 to 1/200 that of oil [14,15]. These calculations indicated that our longer preheater tubing (1.5 m) should be more than adequate to bring the mobile phase up to the intended temperature for temperatures up to 200°C and flow-rates up to 3 ml/min.

The chromatograms in Fig. 1 show the effects of two different preheating coil sizes and mobile phase flow-rates ranging from 0.7 to 1.5 ml/min. The larger coil (~34 μl, 1.5 m) produced good peak shapes over the entire flow-rate range, while peak shape with the smaller coil (~3.4 μl, 0.15 m) deteriorated at 1 ml/min and the peak was split at 1.5 ml/min. By comparison, the preheating coil used by Djordjevic et al. [1] was ~880 μl (450 cm×0.5 mm I.D. stainless steel placed before the injector which was also placed in the oven). In that study, which also used a zirconia–PBD column but with an acetonitrile–water (40:60) mobile phase operated at 110°C and 0.5 ml/min, the retention of 1,4-dichlorobenzene decreased by about 3% with the introduction of the preheater. In this study, with a water-only mobile phase at 160°C, the increased size of the

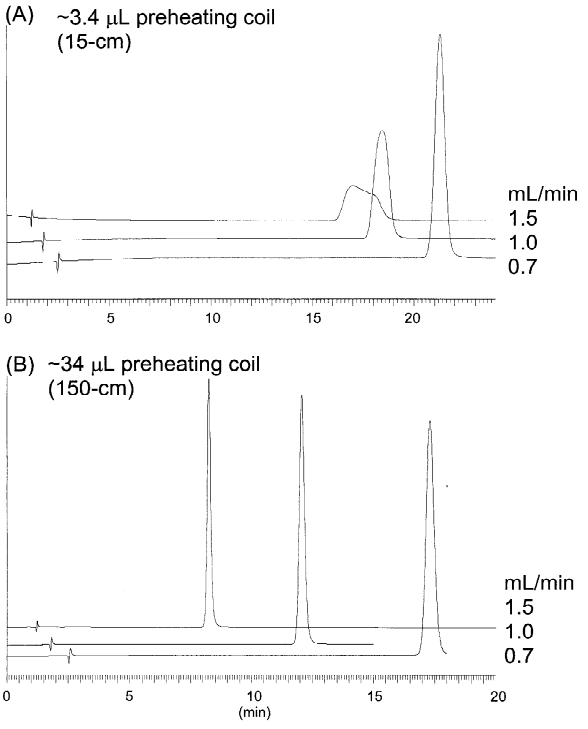

Fig. 1. Effect of preheating coil length and flow-rate on retention and peak shape. Flow-rate indicated on figure, preheating coil volume (A) ~3.4 μl and (B) ~34 μl. Conditions: 160°C, zirconia–PBD column, 7 μl injection of testosterone (A) 65 mAUFS, (B) 100 mAUFS.

preheating coil decreased the retention of testosterone about 20, 50 and 200% at 0.7, 1.0 and 1.5 ml/min, respectively (see Fig. 1).

The sample injection operation in this study was conducted with the autosampler at ambient temperature and the preheating coil placed between the injector and the column. HTLC has also been performed using heated injectors [1,4] and with preheating coils placed between the pump and injector [1,3,8,9]. In this study, the peak shape and repeatability were studied by performing five replicate injections of testosterone for each of five injection volumes (5, 10, 15, 20 and 25 μl). Repeatability for all injection volumes was comparable to that found in conventional HPLC, with low peak area and retention time variability (Table 1 shows data for the 5-μl injection volume). However, as the injection volume increased, the peak shape became distorted and the number of theoretical plates decreased from ~10 000 at 5 μl to <800 at 25 μl (see Fig. 2), and the peak was split at the highest injection volume. Since the temperature of the injected sample solution was similar to that of the mobile phase in the injector, the asymmetric peaks may have been caused by a volume overload of organic solvent that did not allow proper analyte focusing at the head of the column. In comparison, a general guideline [16] is that no more than 25 μl of a sample solution with a stronger solvent be injected into a weaker mobile phase in a 4.6 mm I.D. column. Further study is needed to determine the dependence of peak shape on solvent composition, analyte type, column type, injection volumes and temperature.

The zirconia-based columns have been under development for many years [17–19], and have recently been commercially introduced. It was of interest to make a preliminary determination as to whether retention properties of these test compounds were consistent between columns. Using testosterone as the reference analyte, the relative retention data obtained on two zirconia–PBD columns indicate that variability in column selectivity was very small under the conditions tested for the testosterone-related compounds shown in Fig. 3. The slope of a linear regression analysis of the data was 0.98 with an r^2 of 0.998. The ratios of relative retention times between the columns ranged from 0.98 to 1.03, with a relative standard deviation (RSD) of 1.5%.

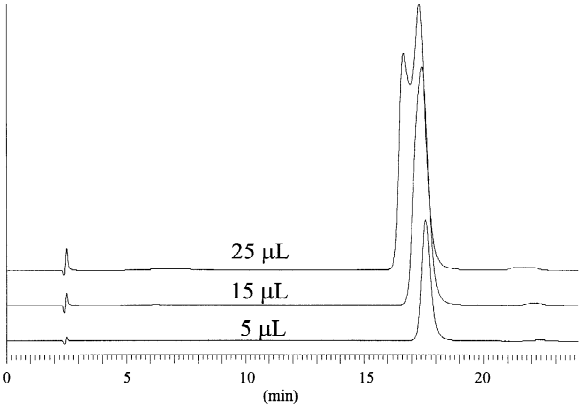

Fig. 2. Effect of injection volume on testosterone peak shape. Conditions: 160°C, zirconia–PBD column, 100% water mobile phase at 1 ml/min.

Often-cited advantages of a water-only mobile phase for HPLC are the reduction in waste costs and its ''environmentally friendliness'', presumably because of the lack of organic modifier (e.g., acetonitrile or methanol) in the mobile phase. However, these presumptions are not true in situations where the sample solution contains some amount of organic

Table 1
Repeatability of chromatographic data in HTLC[a]

	Peak area (RSD, %)	t_R (RSD, %)	Theoretical plates (N) Mean (RSD, %)	Asymmetry factor Mean (RSD, %)
Testosterone (75 μg/ml)	0.2	0.1	7198 (1.0)	1.023 (1.4)
Epitestosterone (2 μg/ml)	1.1	0.1	8428 (1.5)	1.015 (1.3)
Androstenedione (2 μg/ml)	0.6	0.1	6731 (1.0)	0.990 (1.3)

[a] Chromatographic conditions: zirconia–PBD, water mobile phase (1 ml/min), 160°C. Replicate (n=5) injections.

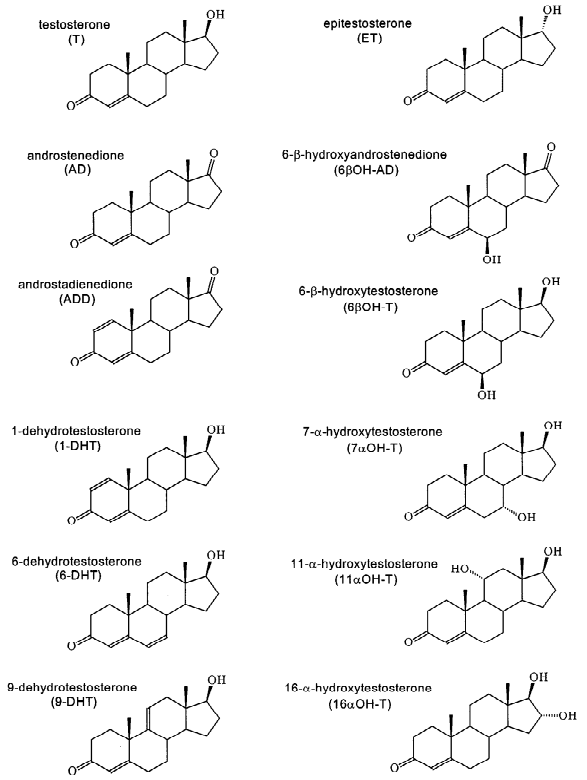

Fig. 3. Structures of testosterone and related compounds.

solvent. Calculations were made to determine the organic solvent concentration in the eluent under a typical operating condition of 1 ml/min and a 10-min analysis time. The calculations assumed that the injections are being made continuously during the operating time of the HPLC system. At 10% organic solvent in the sample, the concentration in the eluent would be 10, 50 and 100 μl/l using 1, 5 and 10 μl injection volumes, respectively. The actual organic concentration will also depend on system equilibration time and any time not used for injections.

3.2. Chromatographic performance

The presence of either axial or radial thermal gradients across an HTLC column can confound the interpretation of chromatographic performance because there is an underlying assumption of isothermal conditions in traditional studies of capacity factor (k') and separation efficiency. Under HTLC conditions, the increased pressure drop across the column and elevated temperature results in a situation where the liquid compressibility and heat of friction may need to be considered in data interpretation [1]. As noted above, there is the possibility of a small thermal gradient across the column used in this study, but calculations indicate that the longer preheater tubing was more than adequate to bring the mobile phase (or injected sample solution) to well within 1°C of the operating temperature.

The effect of flow-rate on retention and efficiency (Fig. 4) for testosterone with the zirconia–PBD column was studied over the range of 0.2 to 3 ml/min. The column void time (t_0) was estimated by finding the earliest baseline deflection for the apparent solvent peak. If higher flows were to induce thermal gradients, the effect should yield a lower actual temperature and subsequent higher k' values. In our study there was no consistent change in k' as flow increased (3% RSD over the entire flow range), which serves to support the calculations above wherein the longer preheater was estimated to provide adequate heat transfer to the mobile phase prior to entering the column.

Some Van Deemter curve profiles in HTLC have been described [9] as showing minima similar to conventional HPLC but that efficiency decreased at higher flow-rates, presumably due to increased thermal gradients. In this study, the plate height vs. flow-rate curve (Fig. 4) indicates that the optimum velocity was as expected, around 1 ml/min, and the minimum reduced plate height corresponded to about 3.4. In comparison, the reduced plate height for a

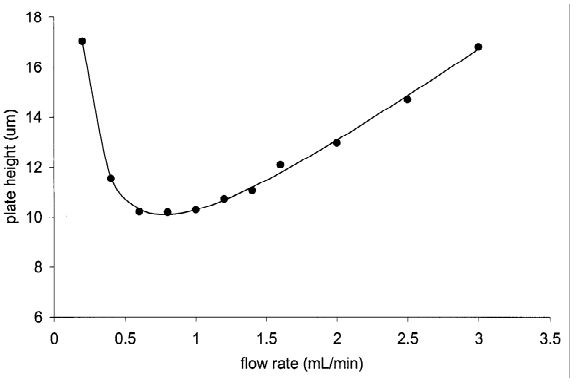

Fig. 4. Plate height vs. flow-rate. Conditions: 160°C, zirconia–PBD column, 100% water mobile phase, 7 μl injection of testosterone.

wide variety of small molecule analytes ranged from 4.0 to 8.0 using the same size zirconia–PBD column at ambient temperature with an organic–buffer mobile phase [10]. In our study, the pressure drop (measured at 165°C) over 0.2 to 3 ml/min changed linearly from 12 to 156 bar.

The dependence of retention on temperature was studied over the temperature range of 170 to 200°C (Fig. 5) for the zirconia–PBD column. The analytes eluted with good peak shape at each temperature tested. A systematic decrease was observed in capacity factor (k') with increasing temperature. The van't Hoff plots in Fig. 5 were linear with correlation coefficients greater than 0.999 for each compound. This linear dependence of retention confirms that the primary retention process in the zirconia–PBD column is the enthalpy of mass transfer between stationary and mobile phases, as would be expected with reversed-phase HPLC separations. This data is consistent with other reported data [8] for various test analytes, where the temperature ranged from 75 to 180°C for a acetonitrile–water (1:99) mobile phase and polystyrene–divinylbenzene column, although some analytes showed convex curves over the wider temperature range. It was suggested [4] that other factors may be affecting retention based on nonlinear dependence of k' vs. $1/T$. But when those data are plotted as ln k' vs. $1/T$, there was a linear relationship for all analytes (except benzamide, which may be affected by very low retention), indicating that the enthalpy effect is the primary factor.

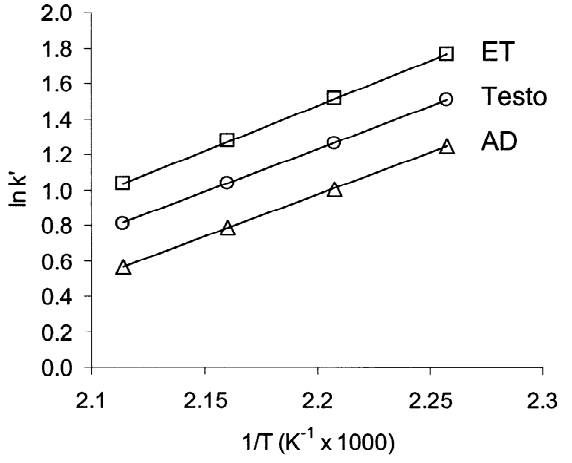

Fig. 5. Dependence of retention on column temperature: van't Hoff plot of ln k' vs. $1/T$ (K^{-1}) with linear regression lines. Conditions: zirconia–PBD column, 7 μl injection of testosterone (Testo), epitestosterone (ET) and androstenedione (AD), temperatures tested were 170, 180, 190 and 200°C.

3.3. Selectivity and resolution of testosterone and related compounds

A series of compounds related to testosterone were used to study the retention on the zirconia–PBD column under both HTLC and RPLC conditions and to compare the selectivity to conventional HPLC with a silica–octadecylsilane column. The compounds included a geometric isomer and compounds that were hydrolyzed (additional hydroxyl groups), oxidized (hydroxyl converted to carbonyl) and/or reduced (additional double bond) at various positions on the testosterone rings (Fig. 3). The traditional RPLC conditions used a Zorbax Eclipse XDB (C_{18}) silica column with an acetonitrile–water (35:65) mobile phase at 40°C. The same mobile phase and temperature were used with the zirconia–PBD column to profile its selectivity under traditional RPLC conditions. The HTLC selectivity was profiled using a water-only mobile phase at 160°C with the zirconia–PBD column. The selectivity differences are shown in Figs. 6–8 as the ratio k'/k'_{testo} for each pairing of the three conditions.

Under the conditions tested here, the rank order of retention was always RPLC silica–C_{18}>HTLC Zir-PBD>RPLC Zir-PBD for the testosterone related compounds. Relative to the RPLC silica–C_{18} conditions, the retention times were, on average, about 30% less for HTLC and about 60% less for RPLC using the zirconia–PBD column. The strongest correlation of selectivity was between the RPLC silica–C_{18} and RPLC Zir-PBD conditions (r^2=0.97, Fig. 6), and the most diversity was found in comparing the RPLC silica–C_{18} and HTLC Zir-PBD conditions (r^2=0.83, Fig. 8). The dehydrotestosterone compound selectivity was affected very little by these changes in operating conditions, while the largest changes were observed for the dione-containing compounds, and smaller changes in selectivity were observed for the hydroxylated compounds.

The relatively strong correlation of selectivity between silica–C_{18} and zirconia–PBD columns

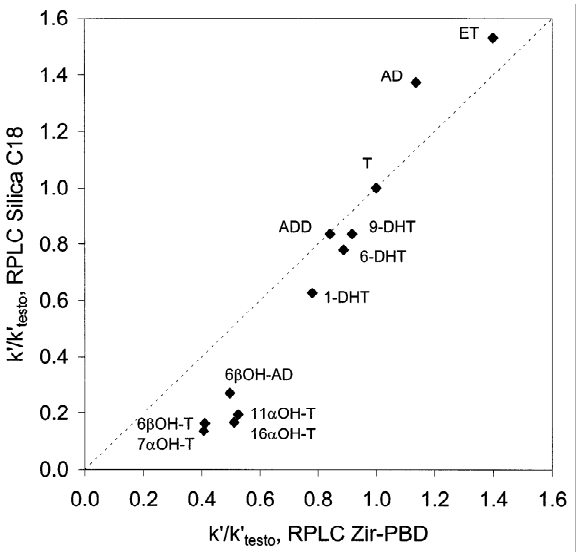

Fig. 6. Comparison of relative selectivity (k'/k' of testosterone) under RPLC conditions (40°C, acetonitrile–water, 35:65) between the silica–C_{18} column (30 μl injection) and zirconia–PBD column (5 μl injection); see Fig. 3 for structures; the line is for reference with a slope of unity.

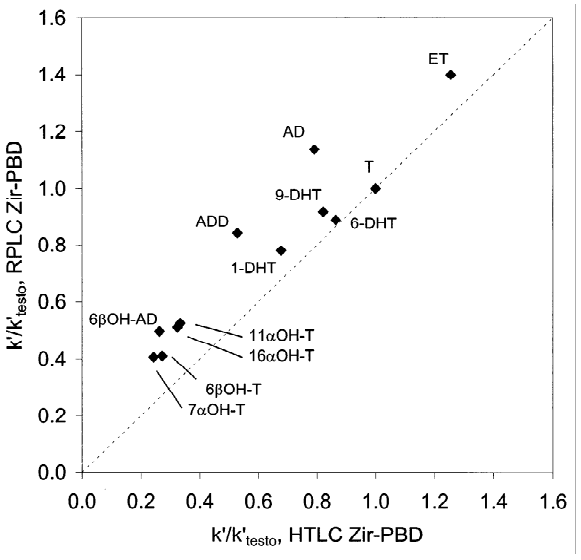

Fig. 7. Comparison of relative selectivity (k'/k' of testosterone) between RPLC conditions (40°C, acetonitrile–water, 35:65) and HTLC conditions (160°C, 100% water) using the zirconia–PBD column (5 μl injection); see Fig. 3 for structures; the line is for reference with a slope of unity.

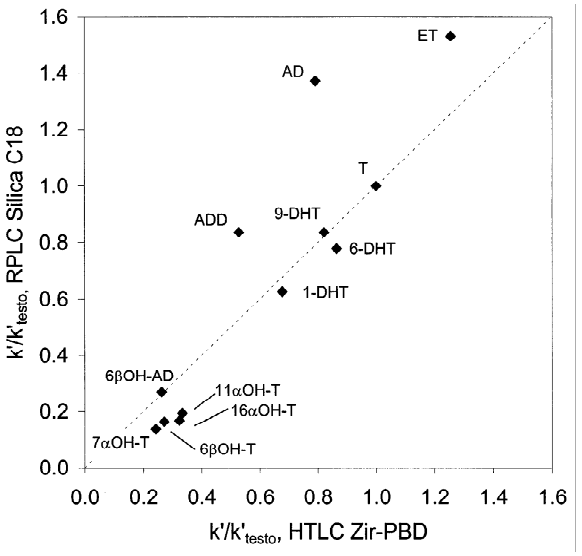

Fig. 8. Comparison of relative selectivity (k'/k' of testosterone) between RPLC (silica–C_{18} column, 40°C, acetonitrile–water, 35:65, 30 μl injection) and HTLC (zirconia–PBD column, 100% water, 5 μl injection); see Fig. 3 for structures; the line is for reference with a slope of unity.

shown in Fig. 6 is similar to that found for cortisone and progesterone related steroids [19] under RPLC conditions (ambient temperature, acetonitrile/water mobile phase) using a Lewis acid site-blocking agent (NH_4F) in the mobile phase. The selectivity differences found between the zirconia–PBD column under RPLC and HTLC conditions (Fig. 8), without the use of blocking agents, demonstrate that HTLC with a water-only mobile phase and the zirconia–PBD column provides selectivity that differs from traditional RPLC using either a silica–C_{18} column or the zirconia–PBD column. The role of various blocking agents and mobile phase combinations, such as acetonitrile and methanol aqueous mixtures, on the selectivity for these testosterone-related compounds is the subject of continuing investigations using the zirconia–PBD column under HTLC conditions.

4. Conclusion

HTLC with a superheated water mobile phase has been shown to be a feasible replacement for

medium-polarity acetonitrile–water mixtures as an eluent in reversed-phase HPLC. Preheating of the mobile phase upstream of the column is essential to maintain good chromatographic integrity. Other considerations that can affect the peak shapes are the volume, and potentially, the composition of the injection solution. The zirconia–PBD column operated under HTLC conditions provides separation efficiencies comparable to conventional RPLC, but also provides a complementary separation mechanism for a variety of testosterone related compounds.

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