This item was submitted to [Loughborough's Research Repository](#) by the author.
Items in Figshare are protected by copyright, with all rights reserved, unless otherwise indicated.

High temperature liquid chromatography of steroids on a bonded hybrid column

PLEASE CITE THE PUBLISHED VERSION

PUBLISHER

© Springer Verlag

VERSION

AM (Accepted Manuscript)

LICENCE

CC BY-NC-ND 4.0

REPOSITORY RECORD

Al-Khateeb, Lateefa A., and Roger M. Smith. 2019. "High Temperature Liquid Chromatography of Steroids on a Bonded Hybrid Column". figshare. <https://hdl.handle.net/2134/4091>.

This item was submitted to Loughborough's Institutional Repository (<https://dspace.lboro.ac.uk/>) by the author and is made available under the following Creative Commons Licence conditions.

For the full text of this licence, please go to:
<http://creativecommons.org/licenses/by-nc-nd/2.5/>

Lateefa A Al-Khateeb and Roger M. Smith¹

High temperature liquid chromatography of steroids on a bonded hybrid column

Abstract A hybrid stationary phase, XTerra MS C18, has been evaluated for the high temperature reversed phase liquid chromatography of selected hydrophobic steroids. The effects on the retention and efficiency at temperatures up to 130 °C and eluent compositions from methanol-water mixtures to superheated water were studied. The thermodynamic data of the separations were determined. It was shown that increasing the temperature enabled the percentage of methanol to be reduced. High mobile phase flow rates could be used but for these non-polar analytes the retention times with superheated water as the eluent were still high.

Keywords: High temperature liquid chromatography. Superheated water. Steroids. Hybrid stationary phase. XTerra MS C18 stationary phase.

¹ L. A. Al-Khateeb R. M. Smith (✉)Department of Chemistry, Loughborough University, Loughborough Leics, LE11 3TU, UK
e-mail: r.m.smith@lboro.ac.uk
Tel.: +44-1509-222563

Introduction

Temperature changes, in particular elevated temperatures, have become a valuable variable which should be considered during method development in liquid chromatography, because they can lead to a reduction in analysis time, can offer an alternative selectivity and are claimed to increase column efficiency.[1-4]. The reduction in mobile phase viscosity at elevated temperatures, enhances diffusion rates and the mass transfer within the column and reduces column back-pressure so enabling faster eluent flows to be used without loss of efficiency. Alternatively smaller particles sizes can be employed without an excessive back pressure. Another advantage of elevated temperature is that the proportion of organic modifier in the mobile phase can be

significantly reduced and in some cases superheated water alone can be used as the eluent [5-8] providing an environmental friendly separation method.

Previous studies have demonstrated the applicability of high temperature separations to a range of pharmaceuticals [9], with examples such as analgesics [10], vitamins[11] and thalidomide [12] and compounds of environmental interest, such as the triazine herbicides [13]. However, as a method it has been constrained by the poor thermal stability of conventional octadecyl bonded silica based phases but this problem can be overcome by using polymeric materials, such as polystyrene divinylbenzene or alternative thermally stable phases, such as coated zirconia [14] or porous graphitic carbon [13]. More recently the bonded hybrid phases have also shown good stability [15,16]. A repeated concern has been for the thermal stability of the analytes, however, only a few cases of degradation during chromatography have been reported. Thompson and Carr [17] examined a range of pharmaceuticals and suggested that for most analytes the problem would be minimal if the compound had reasonable room temperature stability as the time of exposure to the high temperatures are short. However, Godin and co-workers [18] found that some well retained analytes, such as ferulic acid, suffered degradation at 170 °C in superheated water eluent.

The steroids are an important class of therapeutic compounds and need methods for their assay and clinical monitoring. Typically, relatively high proportions of organic modifier (70-80 %) are employed at room temperature on conventional C18 reversed phase columns. The high temperature liquid phase separation of steroids have been studied previously by Fields and co-workers [19] using a PBD coated zirconia column with superheated water at temperatures up to 160 °C. They demonstrated the need for a preheater and that the column should not be overloaded with sample solvent. The high temperature separations of a group of sterols were examined by Riddle and Guiochon [20] on porous graphitic carbon with methanol/chloroform eluents and polymeric phases at temperatures up to 200 °C. They also examined an ODS column with 70:30 methanol-water at 150 °C and a carbon coated zirconia based column again with methanol-water. However, the peak shapes were generally poor, particularly for cholesterol.

The aim of the present work was to examine the application of hybrid bonded phases to the high temperature RP-HPLC separation of a selected group of relatively hydrophobic steroids using both methanol-water mixtures and superheated water as the eluent. The intention was to demonstrate the applicability of high temperature to reduce or eliminate the organic modifiers in the mobile phase and to show that the thermal stability of the analytes and the stationary phase was not a restriction.

Experimental

Chemicals and reagents

Standard steroids: estriol, 1, 4-androstadiene-3,11,17-trione, 19-nortestosterone, testosterone and progesterone were from Sigma-Aldrich (Poole, UK). Uracil was used as a column void marker. Methanol and acetonitrile were HPLC grade from Fisher (Loughborough) and water was deionised from an ELGA (High Wycombe, UK) system.

High Temperature Liquid Chromatograph

The separations were carried out on a XTerra MS C18 (3.5 μm 150 x 4.6 mm) or (3.5 μm 50 x 4.6 mm) column (Waters (Milford, USA) using a Hewlett Packard 1100 series liquid chromatograph equipped with an autoinjector and diode array detector. A Hewlett Packard 5890 GC (Palo Alto, CA USA) was used as the column oven. A length of stainless steel tubing (90 cm x 0.18 mm I.D.) was used in the oven as a pre-heater. The post-column tubing (100 cm x 0.125 mm I.D.), which also acted as a post-column back pressure restrictor, was placed in a cooling bath after the oven before the detector. The compounds were detected using a UV photodiode array detector at 230 nm and the data was analysed using a Hewlett Packard ChemStation.

Results and discussion

Influence of temperature on retention

Estriol, 1, 4-androstadiene-3,11,17-trione, nortestosterone, testosterone and progesterone were selected as typical moderately hydrophobic steroids and were examined on a methyl-silica hybrid XTerra MS C18 column, which has a tridentate bonded C18 bonded phase. Initially at 40% methanol and 40°C, the retention times of the least polar analytes were excessive and even at 50°C progesterone was only eluted after 154 min. On increasing the proportion of methanol to 50 % at 40 °C this retention was reduced to 37 min. A series of studies were then carried out over the composition range from 20- 50% methanol and a temperature range from 50 - 120 °C (Table 1). In some of the conditions, progesterone was omitted as its retention time was unreasonably long. As expected, the retention factors of all the analytes decreased with increasing temperature and a comparison at 50 °C and 100 °C with 40% methanol as the eluent (Figure 1) shows that over this range there was effectively no change in selectivity or order of elution. The efficiency for the more highly retained analytes changed only slightly constant as the temperature increased (Figure 2). However, the efficiency of the more rapidly eluted analytes, estriol and 1,4-androstadiene-3,11,17-trione, decreased, which might be because the contribution from the extra-column dead volume was more significant. On comparing separations with similar retention factors it can be seen that a 10 °C change in temperature had a similar effect to an increase in the proportion of methanol of about 2.5 %. For example the retention factor of testosterone, which was 29.43 at 50 °C in 40% methanol decreased to 8.78 on raising the temperature by 40 ° to 90 °C. Alternatively keeping the temperature at 50° and increasing the percentage of methanol to 50% reduced the retention to 8.02 suggesting that a 40 ° change in temperature was similar to a 10% change in methanol.

As the temperature increases the viscosity of the mobile phase decreases, which permits higher flow rates to be used. However, on increasing the flow rates from 0.5 ml/min to 2.5 ml/min at 100 °C the more rapidly eluted retained analytes showed a significant loss of efficiency (Figure 3) although the peaks were still well resolved.

The relationships between the retention factors and reciprocal temperature from 50 -90 °C were effectively linear ($r^2 > 0.99$) for each compound at the different eluent

compositions (see Figure 4 for 40% methanol) and from these van't Hoff plots the thermodynamic data for the separations could be calculated (Table 2). The enthalpy became more negative with the retention factors of the analytes and became more negative in each case as the temperature increased. Over a wider range of temperatures a non-linear relationship had observed with the phenyl bonded XTerra column[16].

It was of interest to see if the proportion of methanol could be reduced further in combination with a higher temperature. However, at 20% methanol and 120 °C the retention of testosterone was still significant with a retention time of about 57 min at 1 ml/min. The effect of increasing the flow rate from 1 to 3 ml/min was therefore examined (Figure 5). As before this caused some loss of efficiency in the earlier peaks but the testosterone could now be separated in less than 25 min. and the peak shapes were still symmetrical.

Superheated water separations

As noted earlier, one advantage of elevated temperature is that superheated water can be often be used as the mobile phase to provide an organic solvent free eluent, however, non-polar analytes can be strongly retained. If methanol was omitted from the mobile phase in the present study so that 100% superheated water alone was used as the eluent with a flow rate of 3 ml/min nortestosterone was eluted in around 56 min at 130 °C (Figure 6), however, testosterone was not eluted within a reasonable time. Importantly at this temperature the peaks shapes are still symmetrical and there is no distortion or extra peaks that might be resulting from degradation of either column or analytes. It had not been expected that thermal degradation of the analytes would be a problem as some of these steroids can be separated in the gas phase at 200-300 °C. However, from other studies this temperature was considered to be close to the limit of this column material so that further raising the temperature could reduce the column lifetime. If a shorter 50 mm column was used testosterone was eluted using 5 % methanol at 1 ml/min but even at 130 °C the retention time was 237 min indicating that much higher temperatures would be required for elution with on reasonable time scale.

Conclusion

Although relatively non-polar analytes such as steroids are strongly retained in HPLC and require high proportions of organic solvent for elution, they can be eluted with much lower proportion of solvent and sometimes even just with superheated water by raising the temperature. The increased temperature on a bonded hybrid column also enabled the retention times to be reduced by increasing the flow rate without significant loss of peak efficiency and there was no evidence of analytes or column degradation during the relative brief chromatographic run.

Acknowledgments Thanks to King Abdulaziz University and the Government of Saudi Arabia for a studentship and to Waters Ltd for a gift of columns.

References

1. Vanhoenacker G, Sandra P(2006) J. Sep Sci 29: 1822-1835
2. Lundanes E, Greibrokk T (2006) Adv Chromatogr. 44: 45- 77
3. McNeff CV, Yan B, Stoll DR, Henry RA (2007) J Sep Sci 30: 1672 - 1685
4. Wenclawiak BW, Giegold S, Teutenberg T (2008) Anal Lett 41: 1097-1105
5. Smith RM (2008) J Chromatogr A, 1184: 441-455
6. Smith RM (2006) Anal Bioanal Chem 385: 419-421
7. Hartonen K, Riekkola M-L (2008) Trends Anal Chem 27:1-14
8. Yang Y (2007) J Sep Sci 30:1131 - 1140
9. A.M. Edge AM, Shillingford S, Smith C, Payne R, Wilson ID (2006) J Chromatogr A 1132:206-210
10. Smith RM, Chienthavorn O, Wilson ID, Wright B, Taylor SD (1999) Anal Chem 71: 4493 - 4497

11. Chienthavorn O, Smith RM, Saha S, Wilson ID, Wright B, Taylor SD, Lenz EM (2004) *J Pharm Biomed Anal* 36:477 - 482
12. Giegold S, Holzhauser M, Kiffmeyer T, Tuerk J, Teutenberg T, Rosenhagen M, Hennies D, Hoppe-Tichy T, Wenclawiak B (2008) *J. Pharm. Biomed. Anal* 46:625 - 630
13. Tajuddin R, Smith RM (2005) *J Chromatogr A* 1084: 194 - 200
14. Dunlap C, McNeff C, Stoll D, Carr P (2001) *Anal Chem* 73: 598A - 607A
15. Liu Y, Grinberg N, Thompson KC, Wenslow RM, Neue UD, Morrison D, Walter TH, O'Gara JE, Wyndham KD (2005) *Anal Chim Acta* 554: 144 - 151
16. Al-Khateeb LA, Smith RM (2008) *J Chromatogr A* 1201: 61-64
17. Thompson JD, Carr PW (2002) *Anal Chem* 74: 1017 - 1023
18. Godin JP, Hopfgartner G, Fay L (2008) *Anal Chem* 80:7144 - 7152
19. Fields SM, Ye CQ, Zhang DD, Branch BR, Zhang XJ, Okafo N (2001) *J Chromatogr A* 913:197-204

Figures

- Figure 1:** Comparison of separations at 50 and 100 °C with 40:60 methanol-water as eluent on XTerra MC C18 column. Compounds: 1. uracil, 2. estriol, 3. 1, 4-androstadiene-3, 11, 17-trione, 4. 19-nortestosterone, 5. testosterone, 6. progesterone on XTerra MS C18 column.
- Figure 2:** Effect of temperature effect on plate height for XTerra MS C18 with phase over the temperature range of 50-100 °C. Mobile phase 40:60 methanol-water at 1 ml/min.
- Figure 3:** Effect of flow rate on efficiency of analyte efficiency at 100 °C. Mobile phase 40:60 methanol-water..
- Figure 4:** Relationship between retention factor and reciprocal temperatures for steroids from 50 - 90 °C. Mobile phase 40:60 methanol-water at 1 ml/min.
- Figure 5:** Separation of steroids at 120 °C with increasing flow rates with 20 % MeOH on XTerra MS C18 column. Compounds: 1. estriol, 2. androstadiene-3,11,17-trione, 3. 19-nortestosterone, 4. testosterone
- Figure 6** Separation of selected steroids with superheated water at 130 °C, on XTerra MS C18 column. Mobile phase flow rate 3 ml/min. Compounds: 1. uracil, 2. estriol, 3. 1, 4-androstadiene-3,11,17-trione, 4. 19-nortestosterone

Table 1: Retention factors of steroids as a function of temperature using different proportions of methanol-water as the mobile phase

Mobile phase	Analyte				
Column temperature	Estriol	1,4-Androstadiene 3,11,17-trione	19-Nortestosterone	Testosterone	Progesterone
MeOH-water 50:50					
50 °C	1.38	1.68	5.87	8.02	20.54
60°C	1.19	1.42	4.55	6.14	15.25
70°C	0.97	1.18	3.69	4.92	11.89
80°C	0.79	1.01	2.84	3.74	8.76
90°C	0.71	0.85	2.29	2.98	6.78
MeOH-water 40:60					
50°C	3.44	5.15	20.16	29.43	91.19
60°C	2.58	4.09	14.94	21.47	63.85
70 °C	1.96	3.25	11.09	15.69	44.78
80 °C	1.51	2.61	8.32	11.57	31.73
90 °C	1.19	2.14	6.41	8.78	23.22
100 °C	0.95	1.74	4.94	6.67	16.94
MeOH-water 30:70					
50 °C	9.49	16.58	69.08	107.97	-
60 °C	7.04	12.59	49.30	74.68	-
70 °C	5.13	9.57	35.37	53.22	-
80°C	3.78	7.37	25.53	37.77	-
90 °C	2.95	5.78	18.89	27.43	-
MeOH-water 20:80					
90°C	8.64	19.78	67.01	103.72	-
100 °C	6.25	16.90	46.99	71.01	-
110 °C	4.65	10.74	33.31	49.54	-
120 °C	3.57	8.04	23.77	34.66	-

Table 2: Enthalpy and entropy for the retention of steroids at different proportions of methanol in the mobile phase based on relationship between retention factor and reciprocal temperature over the range indicated

		% of MeOH and temperature range			
		50 %	40 %	30 %	20 %
Analyte		50-90 °C	50-100 °C	50-90 °C	90-120 °C
Estriol	ΔH° (kJ/mol)	-16.92	-25.99	-28.86	-35.05
	ΔS° (J/mol/K)	-49.56	-70.11	-70.57	-78.59
	Correlation coefficient (r^2)	0.9918	0.9993	0.9993	0.9979
1,4-Androstadiene- 3,11,17-trione	ΔH° (kJ/mol)	-16.58	-21.83	-25.85	-35.73
	ΔS° (J/mol/K)	-46.88	-53.83	-56.56	-73.49
	Correlation coefficient (r^2)	0.9998	0.9995	0.9994	0.9996
19-Nortestosterone	ΔH° (kJ/mol)	-22.96	-28.37	-31.79	-41.03
	ΔS° (J/mol/K)	-56.28	-62.68	-63.05	-77.91
	Correlation coefficient (r^2)	0.9995	0.9995	0.9996	0.9998
Testosterone	ΔH° (kJ/mol)	-24.20	-29.94	-33.47	-43.35
	ΔS° (J/mol/K)	-57.52	-64.39	-64.56	-80.69
	Correlation coefficient (r^2)	0.9997	0.9996	0.9997	0.9998
Progesterone	ΔH° (kJ/mol)	-27.13	-33.92	-	-
	ΔS° (J/mol/K)	-58.77	-67.27	-	-
	Correlation coefficient (r^2)	0.9998	0.9995	-	-

Figure 1: Comparison of separations at 50 and 100 °C with 40:60 methanol-water as eluent on Xterra MC C18 column. Compounds: 1. uracil, 2. estriol, 3. 1, 4-androstadiene-3, 11, 17-trione, 4. 19-nortestosterone, 5. testosterone, 6. progesterone on XTerra MS C18 column.

Figure 3

Figure 2: Effect of temperature effect on plate height for XTerra MS C18 with phase over the temperature range of 50-100 °C. Mobile phase 40:60 methanol-water at 1 ml/min.

Figure 3: Effect of flow rate on efficiency of analyte efficiency at 100 °C. Mobile phase 40:60 methanol-water..

Figure 4: Relationship between retention factor and reciprocal temperatures for steroids from 50 - 90 °C. Mobile phase 40:60 methanol-water at 1 ml/min.

Figure 5: Separation of steroids at 120 °C with increasing flow rates with 20 % MeOH on XTerra MS C18 column. Compounds: 1. estriol, 2. androstadiene-3,11,17-trione, 3. 19-nortestosterone, 4. testosterone

Figure 6 Separation of selected steroids with superheated water at 130 °C, on XTerra MS C18 column. Mobile phase flow rate 3 ml/min. Compounds: 1. uracil, 2. estriol, 3. 1, 4-androstadiene-3,11,17-trione, 4. 19-nortestosterone