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Short Communication

An evaluation of the use of supercritical fluid chromatography with light scattering detection for the analysis of steroids

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Introduction

The technique of capillary supercritical fluid chromatography (SFC) has evolved rapidly over the past decade and to a certain extent has been facilitated by the universal nature of flame ionization detection. However, some limitations of the capillary approach have become evident. Lengthy retention times are commonly encountered because mass transfer characteristics are relatively slow compared to gas chromatography. The solvent strength of a supercritical fluid is clearly greater than a gas and this precludes the use of many GC capillary columns leading to a restricted range of column packings. The common use of flame ionization detection severely limits the use of organic modifiers, thus preventing the improved control over solvent strength that these give.

Packed column SFC overcomes some of these constraints. Retention times are greatly reduced over those seen for packed column HPLC, because of the rapid solute diffusivity experienced with supercritical fluids (relative to liquids). The full range of HPLC columns is available giving considerable choice of stationary phase. In addition greater control can be exercised over mobile phase since organic modifiers can be readily added in order to influence retention. As for the capillary case, the use of such modifiers is greatly restricted when flame ionization detection is employed, leaving a real need for a simple universal detector. Ultrasonic detection has been suggested [1] based on the fact that the speed of sound approaches zero at a fluid's critical point. This approach however requires considerable extra work. An NMR interface has been designed for SFC [2], but this is by no means a simple approach. Fourier transform IR spectroscopy [3] and mass spectroscopy [4] also have been used to generate excellent results, but once again these are not simple approaches.

Although light scattering detection (LSD) is becoming well established for HPLC, the combination with SFC has so far received only limited interest [5–7]. Since the mobile phase consists largely of supercritical carbon dioxide with just a small amount of organic solvent, it is obvious that the technique of SFC–LSD should be at an advantage over HPLC–LSD, where it is often necessary for the evaporator to cope with largely aqueous mobile phases. The only interfacing requirement for this detector is a back-pressure restrictor immediately prior to, or integral with, the nebulizer of the LSD. This is needed to maintain supercritical fluid (or at least liquid) conditions as far as the point of vaporization. A crimped section of 1⁄16 inch stainless steel tubing can be used, in exactly the same way as already used for SFC–FID, and SFC–MS studies.

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In order to compare LSD directly with a more traditional approach, a simple series of steroids with UV chromophores was chosen and a UV detector was placed in line just before the LSD. A further advantage of choosing these compounds was that they had already been evaluated in some depth by HPLC with LSD [8], so enabling a direct comparison of results. A few SFC steroid separations have already been reported in the literature [9–13] and these were evaluated. In the event however, an alternative separation was developed, in order to obtain improved peak shape. Two types of light scattering detector were used, one being based on a simple tungsten filament light source, and the other on a laser.

Experimental

A Gilson 303 HPLC pump with a 10-ml head, was used to pump liquid carbon dioxide. The pump head was cooled to prevent cavitation, by circulating an aqueous ethylene glycol mixture at −18°C through an outer metal jacket. Pressure pulsation was reduced by passing the flow through a Gilson 802TI manometric module with titanium diaphragm. Organic modifier was introduced into the flow by means of a Gilson 302 HPLC pump with a 10-ml head, and the liquids were mixed in a Gilson 811 dynamic mixer. Precise control of mobile phase composition was maintained by an IBM PC AT which was interfaced to each pump through a Gilson 621 data module.

Supercritical conditions were established by passing the liquid mixture into a Shimadzu column oven. Samples were introduced into the system via a Rheodyne 7125 valve fitted with a 5μl loop, and mounted through the oven door in order to maintain all of the plumbing at the chosen temperature (50°C).

The column was a Brownlee Spherisorb Phenyl cartridge (10 cm × 4.6 mm i.d.) with a New Guard column using the same packing material, in line.

UV detection was performed by means of a Kratos Spectroflow 757 variable wavelength detector, tuned to 254 nm (attenuation 1.0 AUFS). A high-pressure flow cell was installed enabling the use of pressures of up to 6000 psi. Tungsten filament light scattering (TFLS) detection was carried out using an ACS model 750/14 mass detector (Applied Chromatography Systems), and laser light scattering (LLS) detection was performed with a Varex ELSD II detector. For TFLS detection, the nebulizer gas was filtered compressed air at 20 psi, the evaporator temperature was set at 70°C and an attenuation of 16 was used with a photomultiplier sensitivity of 3 (arbitrary units). The nebulizer tube was crimped in order to provide a suitable back pressure, thus maintaining liquid or supercritical conditions right up to the point of nebulization. For LLS detection, the nebulizer gas was filtered compressed air at 10 psi, the evaporator temperature was set at 70°C, and a range setting of 10 was used. With this system back pressure restriction was provided by crimping the end of the 1/16 inch stainless steel tube linking UV and light scattering detectors.

Integration of the output from UV and LSD detectors was carried out with a Spectra Physics dual channel integrator. For all calibration curves 11 solutions of each steroid were chromatographed in duplicate. Each solution contained an internal standard of medroxyprogesterone acetate at a concentration of 1.6 mg ml^{-1}, with the sample steroid ranging in concentration from 0.08 to 6.4 mg ml^{-1}. Limits of detection were determined individually for each steroid by injection of solutions prepared at a concentration of 0.01 mg ml^{-1}, followed by calculation of the loading required to give a response equal to three times that of the baseline noise.

Results and Discussion

Figure 1 shows a typical chromatogram for a mixture of several steroids. Medroxyprogesterone acetate was found to be well resolved from all of the other steroids under study and was chosen as an internal standard. Calibration curves were generated for the full range of steroids using a TFLS detector. Only methylprednisolone was studied using a LLS detector due to limited availability of this instrument at the time. UV detection was found to give a linear relationship between peak area ratio (sample to internal standard) and column loading. The correlation coefficients given in Table 1 can be improved by discarding results obtained with samples of higher concentration, where some detector overload was experienced. Plots of peak area ratio against column loading for both types of light scattering detector were found to show a slight sigmoidal tendency. Consequently

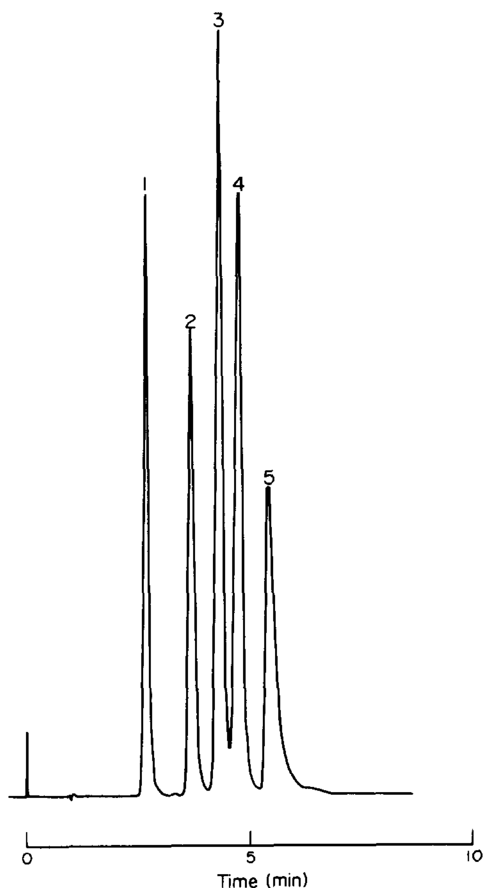

Figure 1
Typical chromatogram for steroids: (1) medroxyprogesterone acetate, 2.0 mg ml^{-1}; (2) cortisone acetate, 2.0 mg ml^{-1}; (3) methylprednisolone acetate, 2.0 mg ml^{-1}; (4) isoflupredone acetate, 2.0 mg ml^{-1}; (5) hydrocortisone, 1.6 mg ml^{-1}.

correlation coefficients obtained by treating the data as if it were linear left some room for improvement. This phenomenon is very common for LSD [14–17]. Regression parameters were generally significantly improved by using a log/log plot (Table 2), although it is important that the potential pitfalls of this approach should be recognized [18].

Limits of detection are shown in Table 3 for a UV detector, TFLS detector, and LLS detector. These were determined for all of the test steroids using each detector, and are quoted as the column loading required to give a peak of height three times the baseline noise. Values measured for the TFLS detector were found to be somewhat dependent upon the condition of the restrictor (and possibly the accuracy of its location within the nebuliser). The values obtained however, show good agreement with results quoted for HPLC/LSD steroid analysis, where using a similar detector, a detection limit of 1.5 μg on column was obtained for prednisone [8], in comparison with the limit of 2.0 μg measured in this study.

The limits of detection measured for the LLS detector were found to show a considerable improvement over those obtained with the TFLS detector. For example, prednisone showed a 40-fold improvement, and several of the steroids gave detection limits of 20 ng on column. This improved sensitivity has been noted in-house when used for HPLC separ-

Table 1
Regression data for steroids using LSD

	Slope	Correlation coefficient	Intercept
(A) UV detector			
Cortisone acetate	0.1026	0.9991	0.0560
Testosterone	0.1520	0.9943	0.1823
Methylprednisolone acetate	0.2202	0.9991	0.0557
Prednisone	0.2103	0.9998	−0.0370
Isoflupredone acetate	0.1806	0.9998	−0.0039
Methylprednisolone	0.2598	0.9989	−0.0824
Hydrocortisone	0.1590	0.9997	0.0188
Prednisolone	0.2437	0.9992	0.0023
(B) TFLS detector			
Cortisone acetate	0.2162	0.9969	−0.2494
Testosterone	0.0685	0.9970	−0.1800
Methylprednisolone acetate	0.2475	0.9954	0.1784
Prednisone	0.1962	0.9973	−0.2270
Isoflupredone acetate	0.2033	0.9977	−0.3871
Methylprednisolone	0.2738	0.9963	−1.4918
Hydrocortisone	0.1918	0.9976	−0.2801
Prednisolone	0.2137	0.9921	−0.3993
(C) LLS detector Methylprednisolone	0.1373	0.9980	−0.1629

In each case data are presented for the plot of peak area ratio (sample to internal standard), against column loading (micrograms).

Table 2
Regression data for log/log plots using LSD

Steroid	Slope	Correlation coefficient	Intercept
(A) TFLS detector			
Cortisone acetate	1.1239	0.9994	−0.8593
Testosterone	1.4206	0.9991	−1.7925
Methylprednisolone acetate	1.3710	0.9982	−1.1611
Prednisone	1.2317	0.9987	−1.0384
Isoflupredone acetate	1.2796	0.9943	−1.1065
Methylprednisolone	1.4710	0.9996	−1.3374
Hydrocortisone	1.2921	0.9973	−1.1351
Prednisolone	1.3011	0.9938	−1.1139
(B) LLS detector Methylprednisolone	1.3845	0.9986	−1.3885

In each case data are presented for the plot of log peak area ratio (sample to internal standard), against log column loading (micrograms).

Table 3
Comparison of limits of detection

Steroid	Limit of detection, μg (on-column) TFLS	LLS	UV
Progesterone	2.1	0.02	0.02
Medroxyprogesterone acetate	1.6	0.02	0.03
Cortisone acetate	1.0	0.03	0.04
Testosterone	2.1	0.04	0.02
Estradiol cypionate	0.9	0.02	—
Hydrocortisone acetate	1.7	0.03	0.03
Methylprednisolone acetate	1.6	0.03	0.02
Prednisone	2.0	0.05	0.03
Isoflupredone acetate	1.5	0.03	0.03
Methylprednisolone	1.3	0.04	0.04
Hydrocortisone	2.1	0.07	0.05
Isoflupredone	2.9	0.05	0.04
Prednisolone	1.9	0.06	0.04
Dehydroisoandrosterone	3.9	0.03	—
Epiandrosterone	4.3	0.03	—
Androsterone	4.2	0.02	—

ations, and is thought to result from improved optics, and minimized stray light. In fact, it can be seen from Table 3 that the values rivalled those achieved with UV detection. It would seem at first glance therefore that light scattering detectors offer little or no advantage over the traditional UV detector. However even in this application, there are steroids such as androsterone that do not have a UV chromophore. With other potential applications the use of packed column SFC has been totally precluded by the lack of a chromophore and the use of LSD could prove invaluable.

Conclusions

In conclusion, the results of this preliminary study show that light scattering detectors can be readily interfaced to SFC. The work presented here was performed with the simplest of interfaces between column and detector. In both of the detectors used, the design of this interface could be significantly improved, and greater sensitivity should result. Even without such improvements, this approach can give a useful extension to the range of detectors available for SFC, and may well allow packed column SFC separations to be developed for the polar, UV transparent compounds that have been ignored to date.

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