Journal of Chromatography, 316 (1984) 461–472
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 439

ANALYSIS OF STEROIDS IN BULK PHARMACEUTICALS BY LIQUID CHROMATOGRAPHY WITH LIGHT-SCATTERING DETECTION

PAUL A. ASMUS* and JOHN B. LANDIS
Control Analytical Research & Development, The Upjohn Company, Kalamazoo, MI 49001 (U.S.A.)

SUMMARY

A detector for liquid chromatography based on light-scattering was evaluated to determine its application to pharmaceutical analysis. It performed adequately as a mass detector in liquid–solid chromatography of steroids, since response factors were equal to within 20%. The detection limit was approximately 0.5 μg. In reversed-phase chromatography response factors varied from 0.13 to 1.0 because of partial vaporization of some steroids. Stable baselines were obtained in gradient elution chromatography, but response varied with solvent composition. This detector was useful for determining impurities in bulk pharmaceuticals.

INTRODUCTION

The analysis of pharmaceuticals by high-performance liquid chromatography (HPLC) is now well established. This is partly because the sample is usually analyzed at room temperature and without derivatization. Also, the high separation efficiency and specificity gives the analyst confidence that the major component is separated from impurities. In addition, with gradient elution all the components of the sample can be eluted from the column in a reasonable time. Liquid chromatography in its present form, however, does not directly provide the mass concentration of the separated components. The limiting factor preventing this goal is the detector.

The measurement of impurities by liquid chromatography requires detector calibration with known masses of each impurity to obtain response factors. An instrument which could directly measure the mass concentration of all the components in a sample would be very useful in the pharmaceutical industry, particularly for the determination of purity. Such a device could be called a universal mass detector. Universal detection, *i.e.* the use of a universal detector and complete elution chromatography, is close to that ideal except response factors are not necessarily equal for equal masses of material analyzed. The "universal" detectors developed —the dielectric constant[1], density[2], plasma chromatograph[3], density balance[4], vapor pressure[5] and heat of adsorption detectors[6]— are not useful with gradient elution and, therefore, cannot be used for universal detection. Also, the commonly used UV absorption and refractive index detectors are unresponsive to certain classes of compounds, or are not usable with certain solute–solvent combinations and, therefore, cannot be called universal.

0021-9673/84/$03.00

The transport detectors developed in the early 1960's[7–10] were based on the fact that most liquid chromatographic solvents (mobile phases) are substantially more volatile than the analyte being measured. Typically, the column effluent was deposited on a mechanical carrier, such as a metal chain, wire or disc, and the solvent evaporated in an oven. The solute remaining on the carrier was then examined by a suitable detection procedure, *e.g.*, flame ionization. This detection system is nearly universal, providing the solutes are not volatile and are organic compounds. The transport detectors, however, are very noisy due to the mechanical linkages and, consequently, are not very sensitive. They have detection limits approximately equal to the refractive index detector.

Another detector, initially called an evaporative analyzer[11], accomplishes the same separation of solute from solvent as the transport detectors, but without a mechanical carrier. This detector works by measuring light scattered from the solid solute particles remaining after nebulization and evaporation of the mobile phase. It has been used to analyze carbohydrates[12,13] and polymers[14], and was called "mass detector". It should, more properly, be called a light-scattering detector, as suggested by Stolyhwo *et al.*[15], because this is the physical process used to measure response. It was, however, found to act adequately as a mass detector under certain conditions. It can be considered a universal detector if it is assumed that all desired solutes are non-volatile. This detector has been used with gradient elution over a limited range of solvent strength[12,13].

The name "light-scattering detector" was previously used for a slightly different detector by Jorgenson *et al.*[16]. They constructed a selective detector based on measurement of light scattered from solid particles formed by precipitation from solution. One obvious difference between the two is the phase in which the light-scattering particles are suspended. The two detectors could be differentiated by calling them "precipitation light-scattering" and "aerosol light-scattering detectors".

A commercially available detector based on aerosol light-scattering is produced by Applied Chromatography Systems (Luton, U.K.) and is the detector evaluated in this work. The evaluation consisted of investigating the response-affecting variables of the light-scattering detector and the associated chromatographic systems in order to answer the questions: Under what conditions can mass detection of steroids be accomplished? Can gradient elution be used to achieve universal detection? What are the detection limits and linear ranges for steroids in both adsorption and reversed-phase chromatography?

THEORY

A brief review of the theory of this detector, developed by Charlesworth[14], will aid in understanding the results obtained in this work. The intensity of light scattered from solid suspended particles depends on their particle size. Therefore, with the detector, response is dependent on the solute particle size produced. This, in turn, depends on the size of droplets generated by the nebulizer and the concentration of solute in the droplets. The droplet size produced in concentric nebulizers, like the one in this instrument, depends on the properties of the liquid, *i.e.*, its surface tension, density and viscosity, and the relative velocity and flow-rates of the gas and liquid streams. The droplet size, D_0, can be calculated with the equation developed by Nukiyama and Tanasawa[17].

$$D_0 = \frac{585\sqrt{\sigma}}{u\sqrt{\rho}} + 597\left(\frac{\mu}{\sqrt{\sigma\rho}}\right)^{0.45}\left(\frac{1000\,Q_l}{Q_a}\right)^{1.5} \quad (1)$$

where σ is the liquid surface tension, ρ is the liquid density, μ is the liquid viscosity, u is the velocity ratio between the airstream and liquid stream, Q_l is the volumetric flow-rate of the liquid and Q_a is the volumetric flow-rate of the gas.

The concentration, C_m, of a solute at peak maximum can be calculated from the sample size m and the peak width at the base ω, expressed in volume units. For a Gaussian profile C_m is given by[15]:

$$C_m = \frac{4m}{\omega\sqrt{2\pi}} \quad (2)$$

For example, a 100-μg injection with a peak width of 1.0 ml will give a peak maximum concentration of 160 μg/ml. The nebulizer droplets formed with a typical mobile phase will be on the order of 50 μm in diameter. As the droplet travels through the evaporator, it shrinks until all the solvent is evaporated. To calculate the size of the resulting solute particle, the droplet diameter is multipled by $(C_m/\rho_s)^{1/3}$, where ρ_s is the density of the solute. For $\rho_s = 1$ g/cm^3 the solute particle in this example will be 2.7 μm in diameter.

Charlesworth determined that for particles about 3 μm in diameter light-scattering is due primarily to refraction and, to a small extent, to reflection[14]. Also, he found that the fraction of incident light reaching the detector is rather insensitive to the refractive index of the material. These two factors explained the similarity in response factors for different compounds. Furthermore, the sensitivity was optimal for this particle size. Sensitivity within 10% of the maximum was attained for particles varying from 0.8 to 4.0 μm in diameter. For smaller solute particles, less intense light-scattering was due to Mie scattering, and for larger particles the surface area to volume ratio decreased the response.

EXPERIMENTAL

The liquid chromatograph used consisted of a Model 5060 pump (Varian, Palo Alto, CA, U.S.A.), a Model 7302 filter (Rheodyne, Cotati, CA, U.S.A.), and a Varian 8055 autosampler with a Model AH-CV6-HPax air-actuated injection valve (Valco Instruments, Houston, TX, U.S.A.) with either a 200-μl or 500-μl sample loop. The column was either a Waters μPorasil or a Waters μBondapak C_{18} (300 × 3.9 mm I.D.). The column eluate was monitored with a Varian UV-5 detector, operated at 200 or 254 nm and then passed into the Model 750/14 mass detector (Applied Chromatography Systems). The detector exhaust was then directed into a fume hood for disposal. Nebulizer gas was unfiltered house nitrogen, except for the response *versus* nebulizer gas pressure experiment, where filtered tank nitrogen was used. For response factor and response *versus* solvent composition measurements, a 2-ml stainless-steel tube was substituted for the chromatographic column, so that no solute retention occurred, and peak height could be taken as detector response. Mobile phases were prepared from Burdick & Jackson solvents (Muskegon, MI, U.S.A.) and doubly distilled water. Steroid standards were obtained from The Upjohn Company,

except prednisolone and testosterone acetate, which were purchased from Sigma (St. Louis, MO, U.S.A.).

The adjustable parameters of the Model 750/14 detector are the nebulizer gas pressure (p.s.i.) and the evaporator temperature (arbitrary units). The evaporator temperature control system consists of a comparison between the control panel "Evaporator Set" and a measuring thermocouple in the base of the evaporator. The resulting difference signal controls the heater power. The "Evaporator Set" values have not been correlated to the actual temperature of the evaporator column and, therefore, are arbitrary units. The evaporator Set values are, however, proportional to temperature and will, for the sake of simplicity, be called ESV in this paper (for evaporator set value).

RESULTS AND DISCUSSION

Table I lists the detector response factors for steroids obtained in both reversed-phase and adsorption chromatographic mobile phases. The ESV of the detector was 50 and 25 for the reversed-phase and adsorption systems, respectively. Also, for comparison, the wide range of response factors for UV detection at 254 nm is included. The range in response factors for light-scattering detection was 0.82–1.0 and 0.13–1.0 for the low temperature and high temperature systems, respectively. Even at high temperature, the response factor variation with the light-scattering detector is much less than with UV detection. At the low temperature used for the

TABLE I

DETECTOR RESPONSE FACTORS FOR STEROIDS

Conditions adsorption system: solvent, 5% tetrahydrofuran, 2% methanol, 0.1% water in *n*-butyl chloride; flow-rate, 1.5 ml/min; ESV, 25; nebulizer gas pressure, 10 p.s.i.; time constant, 5 sec. Conditions reversed-phase system: solvent, acetonitrile; flow-rate, 1.5 ml/min; ESV, 50; nebulizer gas pressure, 10 p.s.i.; time constant, 5 sec.

Compound	*Light-scattering detection*		*UV Detection at 254 nm*
	Adsorption system	*Reversed-phase system*	
Cortisone acetate	0.82	0.98	0.53
Hydrocortisone acetate	0.83	0.96	0.65
Prednisolone	0.86	0.90	0.91
Prednisolone acetate	0.86	1.00	0.91
Prednisone	0.86	0.94	0.93
Dehydroepiandrosterone	0.89	0.21	0.01
Androsterone	0.89	0.13	0.01
Epiandrosterone	0.89	0.14	0.01
Methylprednisolone	0.90	0.99	1.00
Hydrocortisone	0.90	0.96	0.74
Testosterone	0.91	0.28	0.63
Testosterone acetate	0.91	0.20	0.61
Testosterone cypionate	0.92	0.87	0.57
Estriol	0.96	0.98	0.11
Estradiol	0.98	0.64	0.14
Progesterone	0.99	0.31	0.63
Estrone	1.00	0.56	0.18

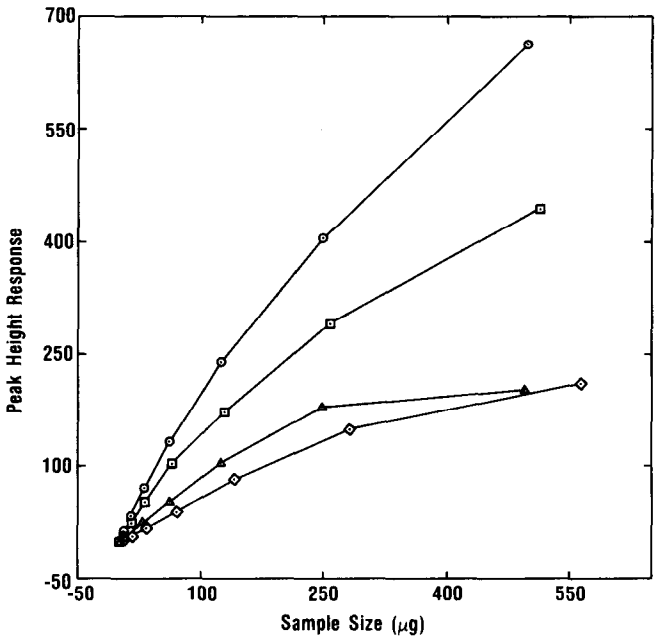


Fig. 1. Standard curves for steroids. Conditions: column, μPorasil; mobile phase, 7% tetrahydrofuran, 2% methanol, 0.1% water in *n*-butyl chloride; flow-rate, 1.5 ml/min.; ESV, 25; nebulizer gas pressure; 10 p.s.i. Key: ⊙ = estrone, ⊡ = androsterone, △ = hydrocortisone acetate, ◇ = prednisone.

evaporation of the liquid–solid mobile phase, all response factors were within 20%. Thus, the instrument is acceptable as a mass detector under these conditions.

The variation in response *versus* the sample size injected on-column for the adsorption system is shown in Fig. 1. All the curves have similar shapes and, as expected, are non-linear. This non-linear behavior has been observed for other compounds in previous studies[12,14,15]. To determine the linear portions of the calibration curves, plots of detector peak height response as a function of sample size (defined as sensitivity) were constructed and are shown in Fig. 2. The linear portion of the standard curve has been defined as the sample size range where the instrument sensitivity is within 10% of its maximum value. For example, the maximum sensitivity for androsterone is 1.65 (arbitrary units), and the linear range is the sample size range where the sensitivity is at least 1.49 (1.65–0.165), *i.e.* 15–90 μg.

Also apparent from Fig. 2 is that the compounds have maximum sensitivity at different sample sizes. This is due to the fact that each compound has a different retention time and, therefore, because of the normal band broadening of a peak with greater retention, a different peak width. From eqn. 2 it is seen that the maximum concentration, C_m, is inversely proportional to the peak width. As displayed in Table II, the range of maximum concentrations (55–83 μg/ml) is much less variable than the sample size for maximum sensitivity (30–130 μg/ml). The average value of the peak maximum concentration is 70 μg/ml, and all values lie within 20% of this mean. At this peak maximum concentration and a droplet size of 60 μm, the resulting solid solute particle is 2.5 μm in diameter, which is a value very close to that previously observed for maximum sensitivity[14].

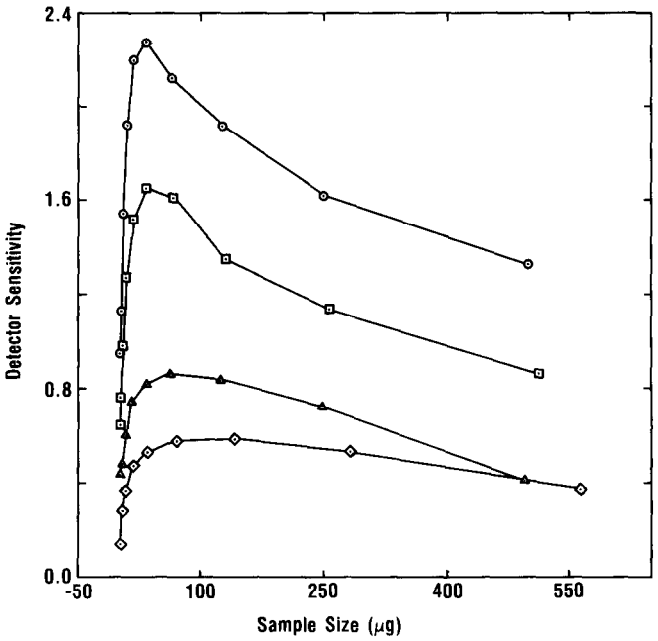


Fig. 2. Plot of sensitivity against sample size. Conditions and key as in Fig. 1.

An isocratic adsorption chromatogram of 10 μg of each steroid standard is shown in Fig. 3. Detection limits estimated from the chromatogram are approximately 0.5 μg for estrone and 1.5 μg for prednisone. This rather modest sensitivity is on the order of that obtainable with refractive index detection. However, other advantages of this detection method overcome, to some extent, this low sensitivity. Improvements in detection limits are probable, since the response-affecting variables were not optimized.

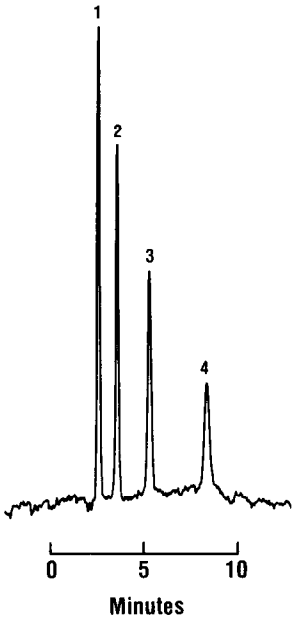
Because of the importance of reversed-phase chromatography in modern liquid chromatography, the behavior of this detector was examined under these conditions. A chromatogram of some standard steroids eluted from an octadecylsilica column with 40% acetonitrile is shown in Fig. 4. The detection limits are significantly higher with this system than with the adsorption system: approximately 1.5 μg for predni-

TABLE II

DETECTOR LINEAR RANGES AND MAXIMUM SENSITIVITIES

Conditions as in Fig. 2.

Compound	*Linear range (μg)*	*Maximum sensitivity*	
		Sample size (μg)	*Peak maximum concentration (μg/ml)*
Estrone	8–85	30	55
Androsterone	15–90	42	65
Hydrocortisone acetate	20–200	85	83
Prednisone	35–300	130	75
			Average 70



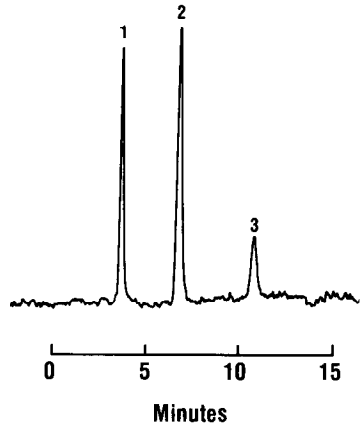


Fig. 3. Adsorption chromatogram of steroid standards. Conditions: column and mobile phase as in Fig. 1; ESV, 20; nebulizer gas pressure, 20 p.s.i.; sensitivity, 2; attenuation, 16; time constant, 5 sec; sample size, 10 μg each compound. Peaks: 1 = estrone, 2 = androsterone, 3 = hydrocortisone acetate, 4 = prednisone.

Fig. 4. Reversed-phase chromatogram of steroid standards. Conditions: column, μBondapak C_{18}; mobile phase, 40% acetonitrile; flow-rate, 1.5 ml/min; ESV, 60; nebulizer gas pressure, 20 p.s.i.; sensitivity, 2; attenuation, 32; time constant, 5 sec. Peaks: 1 = prednisone (28 μg), 2 = hydrocortisone acetate (49 μg), 3 = estrone (52 μg).

sone and 13 μg for estrone. One factor that reduces sensitivity in the reversed-phase system is the higher noise level. This is probably caused by difficulty in evaporating the mobile phase, which has a high water concentration. Pneumatic nebulizers are known to produce a wide range of droplet sizes[17] and some larger droplets probably survive the evaporator column and cause a large and variable signal. In order to minimize the noise, the ESV was 60 for this chromatogram.

In addition to affecting noise, the evaporator temperature influences the response of the steroids tested. Fig. 5 is a plot of detector response against evaporator temperature over the ESV range of 30–90. The responses of prednisone and hydrocortisone acetate increase slightly up to an ESV of 50 and then quickly decrease. The responses of estrone, testosterone and androsterone decrease over the entire range. This decrease in response at higher temperatures suggests that partial vaporization of the solute particle occurs as previously observed with other slightly volatile compounds[14].

Data from a signal-to-noise ratio *versus* evaporator temperature experiment for several steroids with a mobile phase of 40% acetonitrile is shown in Table III. It is seen that for the less volatile solutes, hydrocortisone acetate and prednisone, detectability reaches a maximum at an ESV of about 60, while for the more volatile solute, estrone, maximum sensitivity is obtained at 50. The signal-to-noise ratio in-

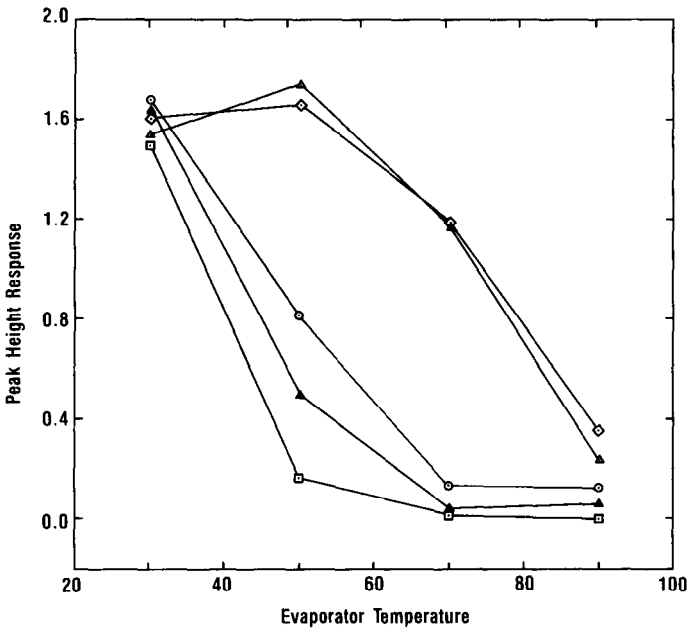


Fig. 5. Detector response variation with temperature. Conditions: solvent, acetonitrile; flow-rate 1.5 ml/min; nebulizer gas pressure, 10 p.s.i.; time constant, 5 sec. Key: ⊙ = estrone (96 μg), ⊡ = androsterone (97 μg), △ = hydrocortisone acetate (91 μg), ◇ = prednisone (102 μg), ▲ = testosterone (96 μg). Peak height response is normalized per μg.

creases because the mobile phase is more successfully evaporated as the temperature increases, but reaches a maximum when the vaporization of the solute becomes significant and the signal decreases.

As stated previously, the ability to perform gradient elution is a requisite for universal detection. Fig. 6 is a chromatogram of steroid standards separated by gradient elution with 30–60% acetonitrile. The baseline is smooth and the peaks are symmetrical. This figure also shows the same chromatogram detected by UV absorption at 200 nm. There is less baseline drift with the light-scattering detector, and the response factors are more nearly equal even though the ESV is 60 and estrone, androsterone, and epiandrosterone are significantly volatile.

A complicating factor in the use of light-scattering gradient elution chromatography is that the detector response varies as the solvent composition varies. Fig. 7 is the result of an experiment where a 400-μg sample of hydrocortisone acetate was repeatedly injected into the detector (no chromatographic column) as the solvent composition was varied linearly from water to acetonitrile. The response increased by a factor of approximately 4 as the chromatographic solvent changed from water to acetonitrile. This can be accounted for by the fact that the droplet size generated by the nebulizer is a function of the properties of the liquid being atomized, *i.e.*, its surface tension, density and viscosity (eqn. 1). When the appropriate values of σ, μ, and ρ for water and acetonitrile are substituted in that equation, it is found that the size of the droplets produced by atomizing acetonitrile is only 60% of that produced from water. The solute particles formed from these droplets will then be smaller

TABLE III

SIGNAL-TO-NOISE (S/N) RATIOS *VERSUS* EVAPORATOR TEMPERATURE AND NEBULIZER GAS PRESSURE

Compound	*S/N vs. temperature**				*S/N vs. pressure***			
	Evaporator set value	*S*	*N*	*S/N*	*Nebulizer pressure (p.s.i.)*	*S*	*N*	*S/N*
Prednisone	40	137	7.8	18	10	122	6.9	18
	50	178	2.9	61	14	115	2.5	47
	60	186	2.4	77	20	93	1.3	73
	70	166	2.2	75	25	78	1.1	73
	80	107	3.0	36	30	72	0.9	78
Hydrocortisone acetate	40	87	7.8	11	10	60	6.9	8.7
	50	115	2.9	40	14	60	6.9	20
	60	127	2.4	53	20	34	1.3	27
	70	115	2.2	52	25	26	1.1	24
	80	67	3.0	22	30	22	0.9	24
Estrone	40	59	7.8	7.6				
	50	42	2.9	15				
	60	21	2.4	8.6				
	70	12	2.2	5.3				
	80	6.3	3.0	2.1				

* Conditions: solvent, 40% acetonitrile; flow-rate, 1.5 ml/min; nebulizer gas pressure, 10 p.s.i.; time constant, 5 sec.

** Conditions: solvent, 40% acetonitrile; flow-rate, 1.5 ml/min; ESV, 60; time constant, 5 sec.

by the same factor. Since the volumetric flow-rate of both solvents was the same, the surface area to volume ratio of the solute particles formed from the acetonitrile solution droplets was larger by a factor directly proportional to the ratio of the droplet radii, or 1.67. Since response is linearly dependent on particle surface area for particles larger than about 3 μm[14], the response should be $(1.67)^2 = 2.8$ times larger for the acetonitrile solution than for the aqueous solution. It should be noted at this point that the difficulties with response variation due to solvent changes would be solved if the nebulizer produced the same sized droplets for all solvents.

Another factor which influences the size of the droplets formed in the nebulization process is the nebulizer gas pressure, which affects the flow-rate of the gas (eqn. 1). The detector response *versus* nebulizer gas pressure for hydrocortisone acetate and prednisone is seen in Table III to decrease continuously as the pressure is increased from 10–30 p.s.i. Although the size of the solute particles decrease with increased gas flow and the total surface area of the solute increases, the decreases in light-scattering intensity due to predominance of Mie scattering overrides the surface area effect, and the response decreases. The signal-to-noise ratio, however, increases as the pressure is increased and reaches a maximum at about 20 p.s.i. More effective evaporation of the small mobile phase droplets is the likely explanation for decreased noise with increased nebulizer gas pressure.

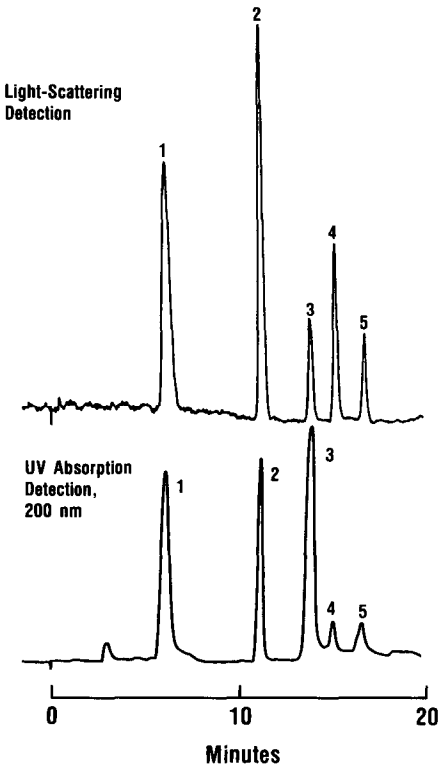


Fig. 6. Gradient elution chromatograms of steroid standards with light-scattering detection and UV absorption detection at 200 nm. Conditions: column μBondapak, C_{18}; gradient, 30–60% acetonitrile in 15 min; flow-rate, 1.5 ml/min; ESV, 60; nebulizer gas pressure, 10 p.s.i.; sensitivity, 2; attenuation, 64; time constant, 5 sec. Peaks: 1 = prednisone (51 μg), 2 = hydrocortisone acetate (51 μg), 3 = estrone (57 μg), 4 = epiandrosterone (115 μg), 5 = androsterone (90 μg).

Applications

The major areas of application for this detection principle to pharmaceutical analysis are envisioned to be (1) impurity assays, (2) reference standard characterization and (3) elucidation of degradation mechanisms. In all of these areas it is desirable to have a universal detection method where response factors are all approximately equal. Although quantitative high-performance thin-layer chromatography (HPTLC) can be useful for these applications, it is difficult to quantitate both organic and inorganic constituents in one HPTLC analysis.

An example of an impurity assay of prednisolone is shown in Fig. 8. This chromatogram was obtained by injecting 7.5 mg of sample on-column and using a 30–100% acetonitrile gradient. Important differences are noted between the UV absorption and light-scattering chromatograms. Peaks 2, 3 and 10 are very small with the UV detector, but are quite prominent with the light-scattering detector. Conversely, peak 5 is very small in the light-scattering tracing and fairly large with the UV detector. Since 7.5 mg of sample was injected on column, the trace impurities at the 0.1 to 1.0% level represent 7.5 and 75 μg, respectively. This is in the linear range

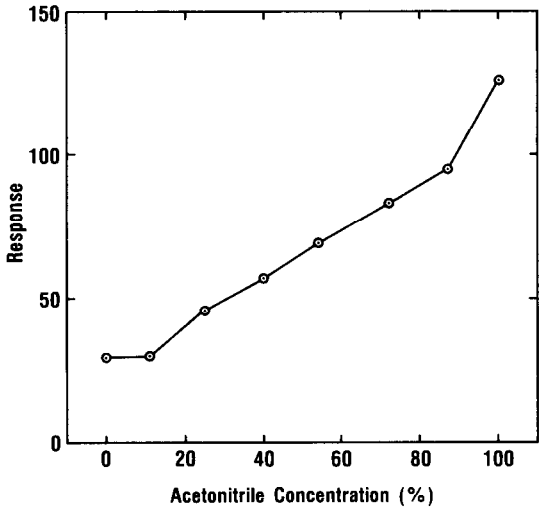


Fig. 7. Detector response *versus* acetonitrile concentration in the solvent. Conditions: sample, hydrocortisone acetate (400 μg); solvent flow-rate, 1.5 ml/min.; ESV, 60; nebulizer gas pressure, 10 p.s.i.; sensitivity, 1; attenuation, 8; time constant, 5 sec.

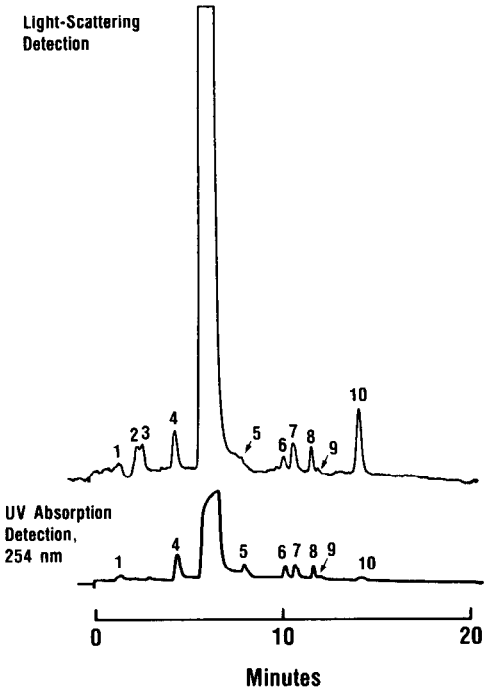


Fig. 8. Chromatogram of prednisolone and impurities with light-scattering detection and UV absorption detection at 254 nm. Conditions: column, μBondapak C_{18}; mobile phase, 30–100% acetonitrile in 15 min; flow-rate 1.5 ml/min; ESV, 70; nebulizer gas pressure, 10 p.s.i.; sensitivity, 2; attenuation, 128; time constant, 5 sec. Numbered peaks are unidentified impurities.

of the detector for the peaks in this chromatogram, since their volumes are all about 1 ml. If sample volatilization and solvent composition effects can be ignored, then the relative peak heights of these impurities are proportional to their mass concentrations. A liquid–solid gradient would certainly solve the volatility problem and perhaps the solvent composition difficulties as well. Further work in this area is continuing.

CONCLUSIONS

(1) The light-scattering detection principle for liquid chromatography is useful as a mass detector for steroid analysis, provided the material being analyzed is relatively non-volatile at the operating temperature of the instrument.

(2) Universal detection can be performed with this detector since a good baseline is obtained with solvent gradients. However, changes in the solvent during gradient elution change the number and size of the droplets generated by the pneumatic nebulizer. This results in a change in the sensitivity of the detector during the gradient.

(3) This detector appears especially useful for impurity screening of new products. When 10 mg of sample is analyzed, trace impurities in the sample ranging in concentration from 0.1 to 1.0% are easily measured, because they are presented to the detector at its optimum sensitivity and linear range. The signal-to-noise ratio is then favorable even when gradient elution is used. Under these conditions, all impurities in the sample can be accurately ranked in terms of mass concentration. This would aid in determining which impurities are most important to be isolated and identified.

ACKNOWLEDGEMENTS

The authors are grateful to the Anspec Co. (Ann Arbor, MI, U.S.A.) for the loan of the mass detector used in this study, and also to R. D. White for helpful discussions.

REFERENCES

1 H. Poppe and J. Kuysten, *J. Chromatogr.*, 132 (1977) 369.
2 J. Francois, M. Jacob, Z. Grubisic-Gallot and H. Benoit, *J. Appl. Polym. Sci.*, 22 (1978) 1159.
3 F. W. Karasek and D. W. Denney, *Anal. Lett.*, 6 (1973) 993.
4 R. Quillet, *J. Chromatogr. Sci.*, 8 (1970) 405.
5 R. E. Poulson and H. B. Jensen, *Anal. Chem.*, 40 (1968) 1206.
6 J. L. Cashaw, R. Segura and A. Zlatkis, *J. Chromatogr. Sci.*, 8 (1970) 363.
7 A. T. James, J. R. Ravenhill and R. P. W. Scott, *Chem. Ind.*, (1964) 746.
8 E. O. A. Haahti and T. Nikkari, *Acta. Chem. Scand.*, 17 (1963) 2565.
9 R. P. W. Scott and J. G. Lawrence, *J. Chromatogr. Sci.*, 8 (1970) 65.
10 O. S. Privett and W. L. Erdahl, *Anal. Biochem.*, 84 (1978) 449.
11 D. L. Ford and W. Kennard, *J. Oil Colour Chem. Assoc.*, 49 (1966) 299.
12 R. Macrae and J. Dick, *J. Chromatogr.*, 210 (1981) 138.
13 R. Macrae, L. C. Trugo and J. Dick, *Chromatographia*, 15 (1982) 476.
14 J. M. Charlesworth, *Anal. Chem.*, 50 (1978) 1414.
15 A. Stolyhwo, H. Colin and G. Guiochon, *J. Chromatogr.*, 265 (1983) 1.
16 J. W. Jorgenson, S. L. Smith and M. Novotný, *J. Chromatogr.*, 142 (1977) 233.
17 H. C. Lewis, D. G. Edwards, N. J. Goglia, R. I. Rice and L. W. Smith, *Ind. Eng. Chem.*, 40 (1948) 67.