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HIGH-EFFICIENCY LIQUID CHROMATOGRAPHY IN PHARMACEUTICAL ANALYSIS

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SUMMARY

An account is given of the application of high-efficiency liquid chromatography in pharmaceutical analysis. The review examines samples arising from fermentation and from formulated pharmaceuticals, including creams, ointments, and tablets.

INTRODUCTION

The introduction of commercial liquid chromatographs some four years ago opened up what was virtually a new field to the analytical chemist. Liquid partition chromatography had been used for many years in pharmaceutical analysis, for the separation of closely related impurities, and for the quantitative estimation of many natural products in pharmaceutical formulations[1,2]. The technique is time consuming and extremely tedious to laboratory staff and in many cases only used as a last-resort technique.

New instrumentation changed this old image by giving separations comparable to gas chromatography in time and efficiency. The new column packing materials which are available and the ultrasensitive, highly stable ultraviolet (UV) detectors enable nanogram quantities of material to be chromatographed in minutes.

EXPERIMENTAL

Apparatus

A DuPont 830 liquid chromatograph was used throughout this work. The instrument was fitted with a standard DuPont 254-nm detector and connected in series with this was a Cecil 212 UV monitor. This latter detector enables variable wavelength selection from 220–360 nm, employing a grating monochromator. The columns used are all commercially available DuPont packed columns.

The instrument was also fitted with a gradient elution accessory.

Applications

Fermentation products. Griseofulvin is an antifungal agent produced by

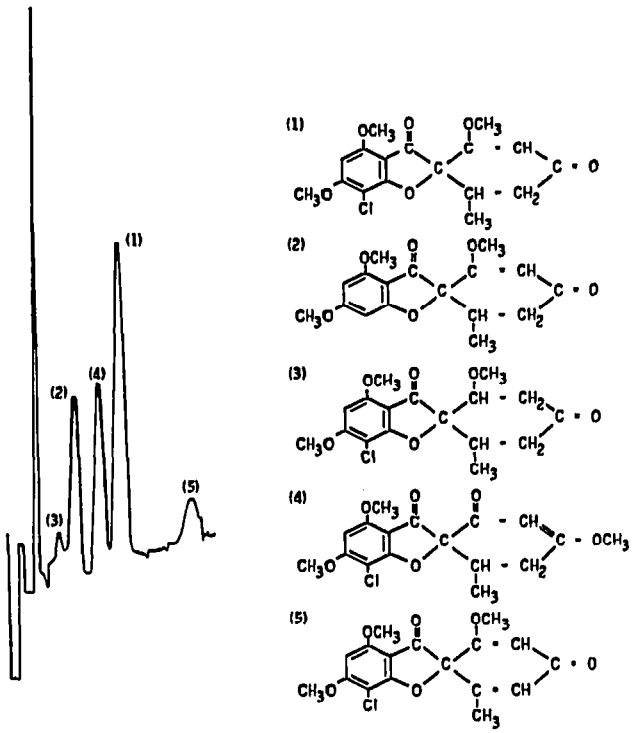

Fig. 1. Chromatogram of the separation of griseofulvin from its related impurities in a fermentation medium. Column, Permaphase ETH; system, 5% chloroform in hexane; temperature, ambient; attenuation, 4×10^{-2}; pressure, 500 p.s.i.

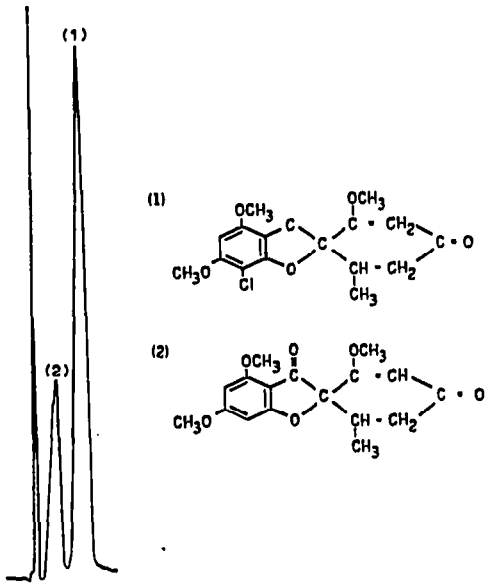

Fig. 2. Chromatogram of the separation of griseofulvin from dechlorogriseofulvin. Column, Permaphase ETH; system, 8% chloroform in hexane; temperature, ambient; attenuation, 8×10^{-2}; pressure, 550 p.s.i.

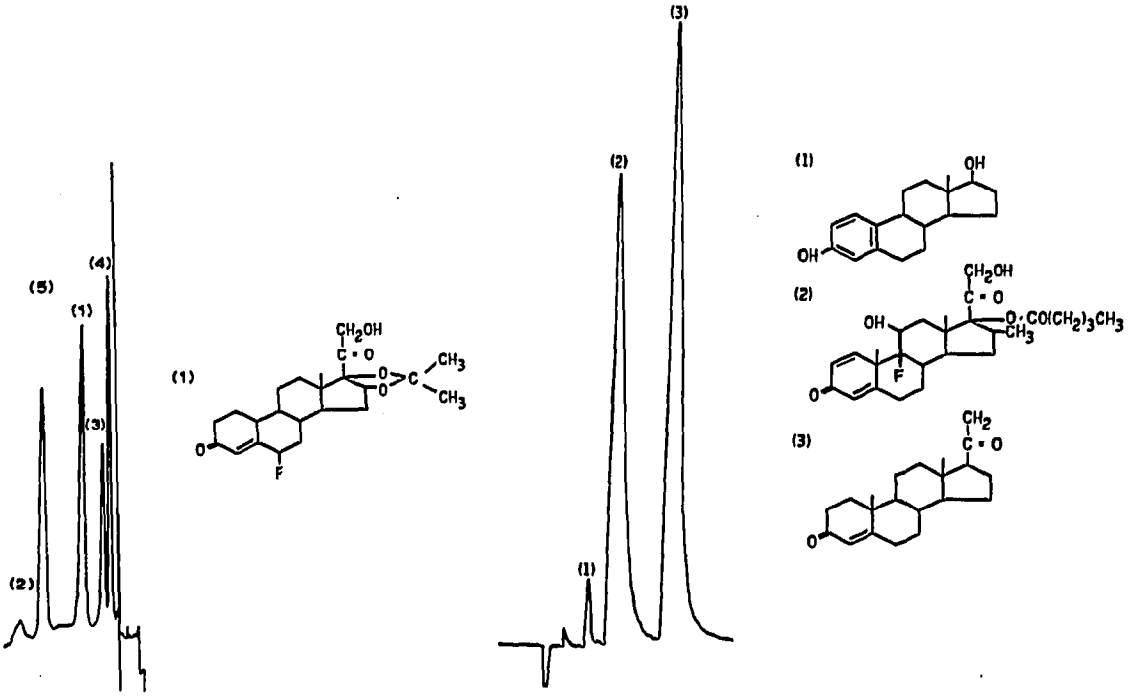

Fig. 3. Chromatogram of a steroid fermentation mixture. Compound 1 is being converted to its 11β-OH derivative, with the 11-keto derivative appearing as impurity. Bromobenzene is used as internal standard. Column, Permaphase ODS; system, 7.5% methanol in water; temperature, 40°; attenuation, 4×10^{-2}. (1) see insert; (2) 21-acetate; (3) 11β-OH; (4) 11-keto; (5) bromobenzene (I.S.).

Fig. 4. Separation of three commonly occurring steroids. (1) Oestradiol; (2) betamethasone-17-valerate; (3) progesterone. Column, Permaphase ODS; system, 20% methanol in water; temperature, 43°.

fermentation. The crude mycelium obtained after filtration of the broth contains many closely structurally related compounds. Attempts to separate these compounds in the past by a single partition chromatograph have failed —a minimum of two column systems was required, one of which had to be monitored at the two wavelengths 265 and 292 nm as separation of the iso- and dechlorogriseofulvins was not achieved.

The relatively simple separation of these compounds by high-efficiency liquid chromatography (HELC) is shown in Fig. 1, when the five compounds are separated in a single run within 20 min.

After isolation the only impurity remaining is dechlorogriseofulvin. A chromatogram of this separation for quantitative estimation is shown in Fig. 2.

The microbiological transformation of steroids to introduce an 11β-OH group is becoming increasingly important and control and assessment of material produced in the fermentation within certain small limits is essential. This can be achieved on a single chromatogram in 15 min, as shown in Fig. 3.

The chromatographic separation of three commonly used steroids is shown in Fig. 4. This is a reversed-phase system using aqueous methanol as eluant phase and

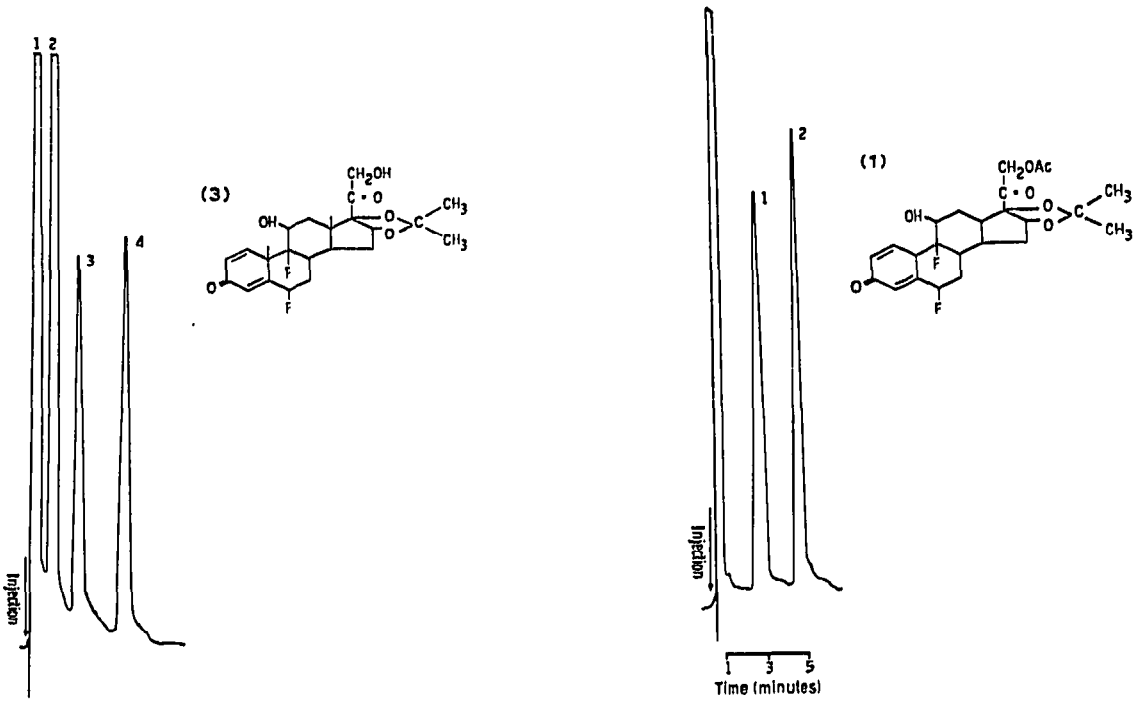

Fig. 5. Chromatogram of a simple extract of a steroid cream. Toluene is used as an internal standard. Column, Permaphase ODS; system, 7.5% methanol in water; temperature, 40°. (1) Methyl *p*-hydroxybenzoate; (2) propyl *p*-hydroxybenzoate; (3) fluocinolone acetonide (see insert); (4) toluene.

Fig. 6. Chromatogram of a simple extract of a steroid ointment. Toluene is used as an internal standard. Column, Permaphase ODS; system, 30% methanol in water; temperature, 40°. (1) Fluocinolone acetonide acetate (see insert); (2) toluene (I.S.).

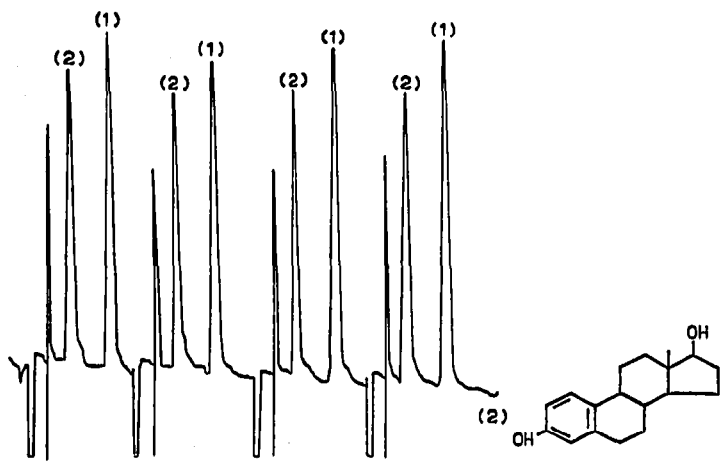

Fig. 7. Chromatogram of a simple extract of oestradiol tablets. Toluene is used as internal standard. The first two chromatograms are duplicate injections of a standard and the next two chromatograms duplicate injections of the sample. Column, Permaphase ODS; system, 20% methanol in water; temperature, 45°; attenuation, 8×10^{-2}; pressure, 1,500 p.s.i. (1) Toluene (I.S.); (2) oestradiol (see insert).

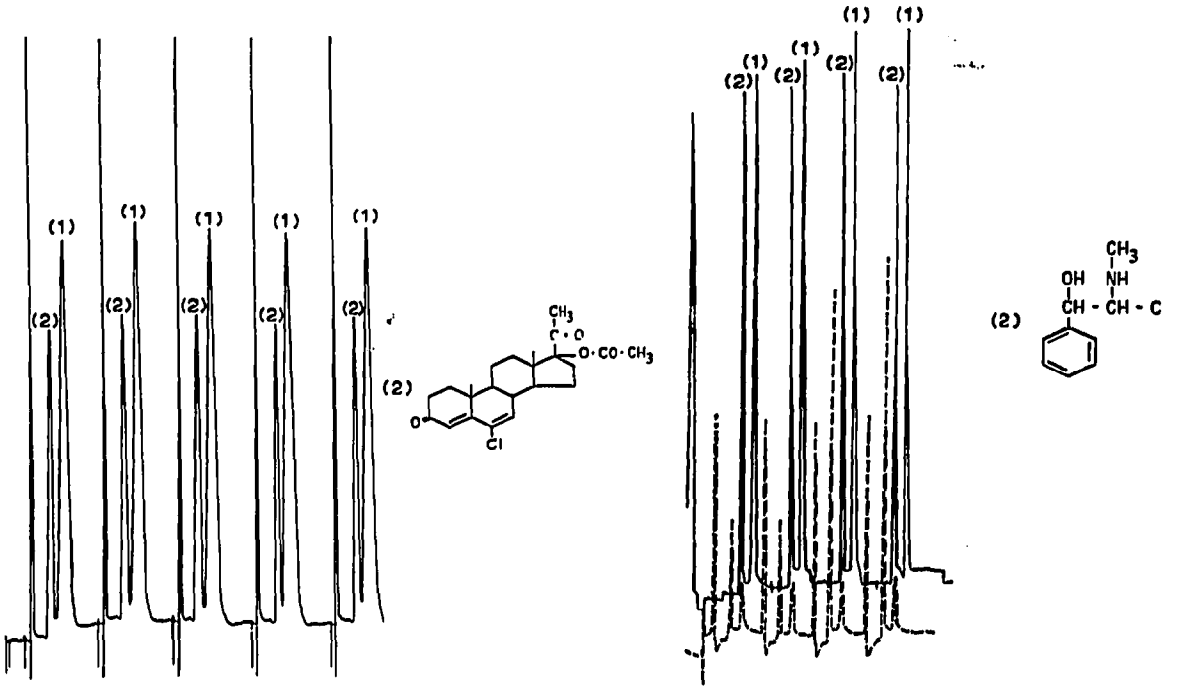

Fig. 8. Chromatogram of a simple extract of chlormadinone tablets. Bromobenzene is used as internal standard. The first three chromatograms are replicate injections of the standards and the next two chromatograms, duplicate injections of the sample. Column, Permaphase ODS; system, 28% methanol in water; temperature, 45°; pressure, 1,000 p.s.i.; attenuation, 2×10^{-2}. (1) Bromobenzene (I.S.); (2) chlormadinone (see insert).

Fig. 9. Chromatogram of a simple extract of ephedrine tablets. Toluene is used as internal standard. The first two chromatograms are duplicate injections of the standard and the next two chromatograms are duplicate injections of the sample. The dotted line is the comparable response of a differential refractometer detector. Column, Permaphase ODS; system, 40% methanol in water; attenuation 16×10^{-2}. (1) Toluene (I.S.); (2) ephedrine (see insert).

a Permaphase octadecylsilane (ODS) column. In reversed-phase operation the most polar compound elutes first, *i.e.* oestradiol, then betamethazone 17-valerate, and progesterone, being the least polar, elutes last.

Creams and ointments[3]. This technique offers a second bonus in addition to speed and efficiency of separations when applied to formulations, in that the pre-column preparation can be much simplified. For instance, in the case of creams and ointments a simple partition of the excipients into isooctane and the active agent into aqueous alcohol is all that is required.

Fig. 5 shows a fluocinolone acetonide cream extracted in this way and Fig. 6 a fluocinolone acetonide acetate formulation.

Tablets. In the case of tablets the pre-column preparation is again much simplified when compared to conventional analytical techniques. For all the tablets studied to date, it is sufficient to grind the tablets in methanol, filter the resulting solution and make to volume.

Examples of three tablets extracted in this way are shown in Fig. 7 (oestradiol

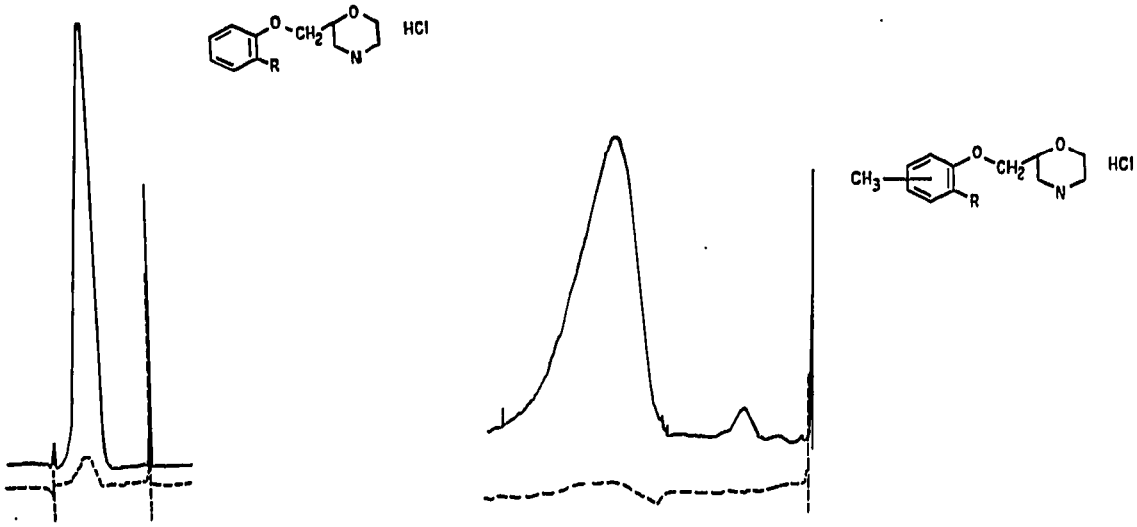

Fig. 10. Chromatogram showing the comparative responses of a single wavelength UV detector monitoring at 254 nm and of a CE-212 grating monochromator UV detector monitoring at 220 nm. Both detectors were set at the same sensitivity, 0.040 absorbance units full scale. Column, SCX; system, 0.2 *M* boric acid adjusted to pH 9 with 0.1 *M* NaOH and 0.2 *M* Na_2SO_4; temperature, 40°. ———, Cecil monitor, 220 nm; — — —, DuPont monitor, 254 nm.

Fig. 11. Chromatogram showing the comparative responses of a single wavelength UV detector monitoring at 254 nm and of a CE-212 grating monochromator UV detector monitoring at 220 nm. Both detectors set at the same sensitivity. Note the impurity peaks clearly visible in the detector monitoring at 220 nm. Column, SCX; system, 0.2 *M* boric acid adjusted to pH 9.6 with 0.1 *M* NaOH and 0.2 *M* Na_2SO_4. ———, Cecil monitor, 220 nm; — — —, DuPont monitor, 254 nm.

tablets), Fig. 8 (chlormadinone tablets), and Fig. 9 (ephedrine tablets).

Many compounds of pharmaceutical interest have very little or no UV absorbance at 254 nm, hence one of the original limitations of this technique was one of detection. With the introduction of a spectrometric detector using a grating monochromator it became possible to select a wavelength of maximum UV absorbance of the compound from 220–360 nm, hence the sensitivity of detection is greatly enhanced. Figs. 10, 11 and 12 demonstrate the type of sensitivity increase one could expect on using this kind of detector, compared to the 254-nm single-wavelength detector.

CONCLUSIONS

The contribution that this technique has made to pharmaceutical analysis has been tremendous. Nanogram quantities of material from complex mixtures or formulations can be separated in a matter of minutes. The simplicity of extraction and speed of chromatography enable large numbers of individual tablet assays to be carried out simultaneously. Quantitation is carried out using internal standards and peak height measurements.

The Permaphase column packings have been shown to be extremely durable, *e.g.* 2½ years in daily use for an ODS column with no indication of deterioration of column efficiency.

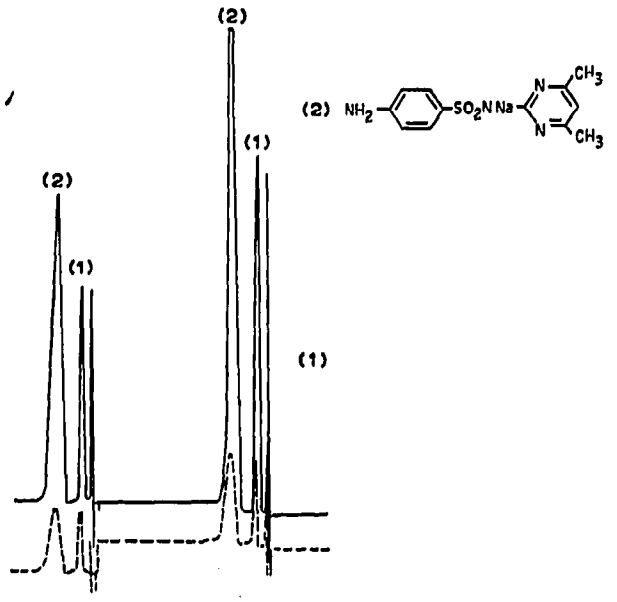

Fig. 12. Chromatogram showing the comparative responses of a single wavelength UV detector monitoring at 254 nm and of a CE-212 grating monochromator UV detector monitoring at 270 nm on a sample of a tablet extract from sulphamezathine tablets; acetylsalicylic acid is used as internal standard. Column, SAX; system, 0.01 *M* boric acid adjusted to pH 9.2 with 0.1 *M* NaOH and 0.01 *M* NaOH; temperature, 40°. (1) Acetylsalicylic acid (I.S.); (2) sulphamethazine (see insert). ———, Cecil monitor, 270 nm; — — —, DuPont monitor, 254 nm.

The introduction of a grating monochromator UV monitor makes the technique more universally applicable.

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