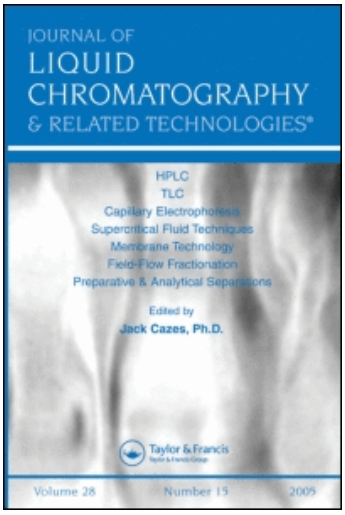

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HPLC, MS AND LC/MS STUDIES OF THE INTERACTION OF GOSSYPOL WITH ALCOHOLS

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ABSTRACT

Gossypol reacts with alcohol solvents to form a complex series of hemiketal and ketal derivatives of its various tautomeric forms, which revert to gossypol or to the readily hydratable anhydrogossypol on attempted isolation and during mass spectrometry.

INTRODUCTION

The phenolic cotton pigment gossypol (1) is orally active as an anti-fertility agent in men and in male animals, interfering with spermatogenesis [1-4]. It has also been demonstrated that gossypol is an active cytotoxic agent against tumour cells in vitro [5] and against the parasite Trypanosoma cruzi which causes

Chagas' Disease in South America [6]. Consequently, there is considerable interest in the development of gossypol and its derivatives as potential clinical drugs.

According to the literature, gossypol has a relatively poor stability and it is most frequently handled as a 1:1 complex with acetic acid [7]. Initial concerns about the purity of the material being studied in vivo led us to establish suitable HPLC methods for the analysis of gossypol and a number of its chemical derivatives, including gossypolone, anhydrogossypol, gossypol hexaacetate and Schiff's bases [8]. In addition, mass spectral (including FD and CI) and linked LC/MS methods for the analysis of these compounds were described [8]. We now report further applications of these techniques to investigate the complex chemistry of the aldehyde group of gossypol, and in particular its reactions with alcohols. The results are of importance in relation to the use of alcohol solutions in chemical and biological studies of gossypol.

MATERIALS AND METHODS

The hplc equipment consisted of a Waters 6000A pump, Rheodyne 7125 injector fitted with a 20 ul loop, a Cecil 2112 variable wavelength detector and a strip-chart recorder. Columns 12.5-25 cm long and 0.45 cm i.d. (analytical) or 0.7 cm i.d (semi-preparative) were filled with 3 um or 5 um Hypersil-ODS by upward slurry packing under pressure. All solvents were either purchased as HPLC grade or were redistilled. Mobile phases were degassed ultrasonically before use.

Mass spectra were recorded on a Varian CH5-D (FD), using benzonitrile-activated emitter wires loaded by the syringe technique. LC/MS (EI source temperature 140°C, vaporiser 200°C) was performed on a Finnigan MAT 4500 mass spectrometer using a moving belt interface and connected to an Incos 2300 data system.

RESULTS AND DISCUSSION

Studies of the stability of solutions of gossypol-acetic acid (5% w/v) in a variety of organic solvents were conducted in order to establish suitable working conditions for chemical and biological studies. Whereas solutions stored in the dark at 4°C were found to be stable for weeks in the case of hydrocarbon (benzene, toluene) and chlorocarbon (chloroform, dichloromethane) solvents, solutions in ethanol appeared to be very unstable, as judjed by reverse phase HPLC analysis. Thus, injections of an ethanol solution after a few hours standing showed the presence of at least three new peaks of shorter retention times than gossypol and the latter compound had disappeared entirely after 2-3 days. Similar results were obtained with methanol. Since ethanol is frequently used as a solvent for gossypol in preparing samples for biological experiments, it was considered to be particularly important to elucidate the nature of the changes occuring in alcoholic solutions.

The possibility that an air-induced oxidative degradation was occuring was examined. However, there was no decrease in the rate of appearance of new peaks when air was excluded and no increase in rate when air or oxygen was passed through a fresh gossypol solution in methanol or ethanol.

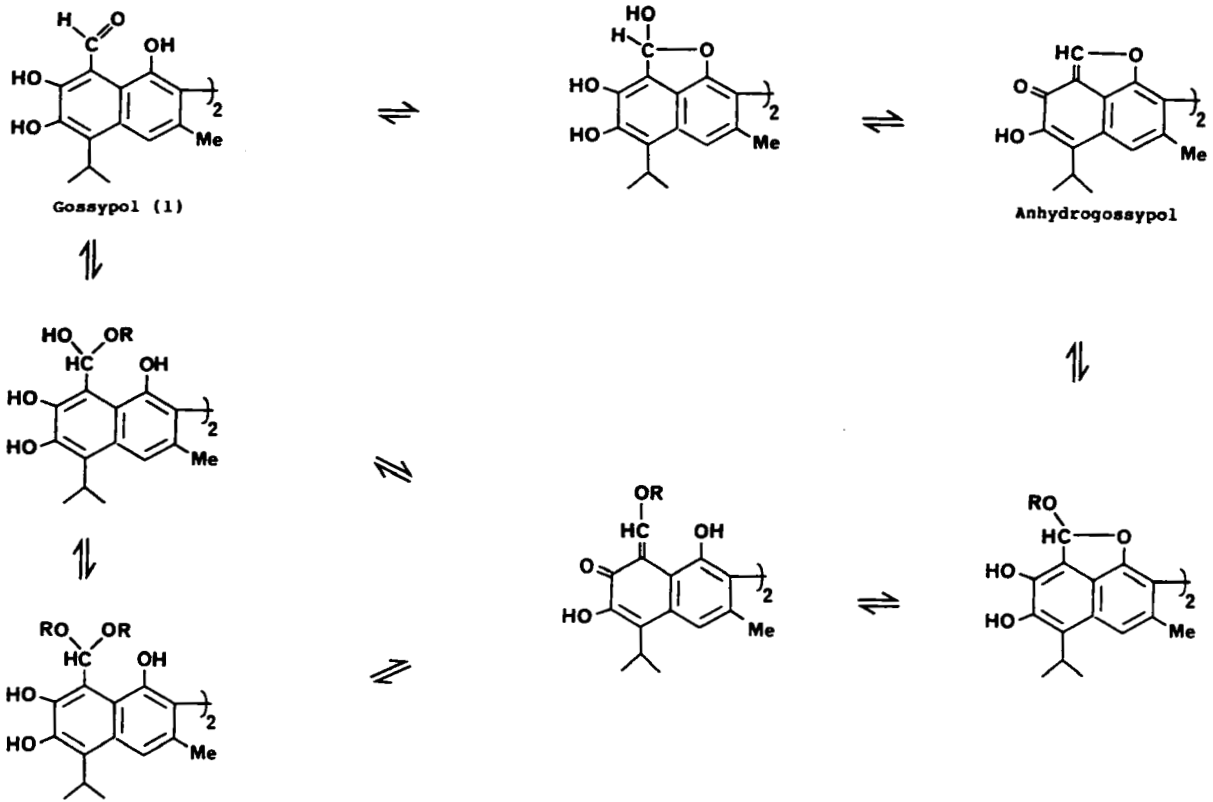

Figure 1 Reactions of gossypol (1) with alcohols (ROH)

Attempts were therefore made to isolate the newly formed compounds, by larger scale HPLC on a semi-preparative ODS-Hypersil column. An ethanol solution containing gossypol was stored for a few days and then concentrated under vacuum without heating. Fractions corresponding to each of the new peaks were collected from the preparative HPLC run and the MeCN-H_2O-AcOH solutions were freeze-dried to minimise decomposition reactions. The residues were then re-examined by HPLC and, surprisingly, were all found to consist of similar mixtures of gossypol and anhydrogossypol. It was apparent that the peaks formed during a few hours exposure of gossypol to ethanol represented gossypol derivatives which were converting to gossypol and its anhydride in the manipulations during or subsequent to preparative HPLC.

A reasonable explanation of these observations is that gossypol reacts with alcohols by formation of hemiketal and ketal drivatives of its various tautomeric forms. On isolation from an aqueous acid medium, these would tend to hydrolyse very readily, either by direct displacement of alcohol groups by water, or via elimination to give anhydrogossypol, which will gradually rehydrate to furnish gossypol (Figure 1: note that, for simplicity, each tautomer is represented as a symmetrical structure, whereas the mixed combinations of structures are all possible).

This conclusion was supported by the results of detailed studies of solutions of gossypol-acetic acid in methanol. A fresh solution of gossypol (Figure 2a) rapidly showed the appearance of four new peaks of shorter retention times and after two weeks only traces of the gossypol peak remained (Figure 2b, peak 5). On mixing some of this solution with an equal volume of

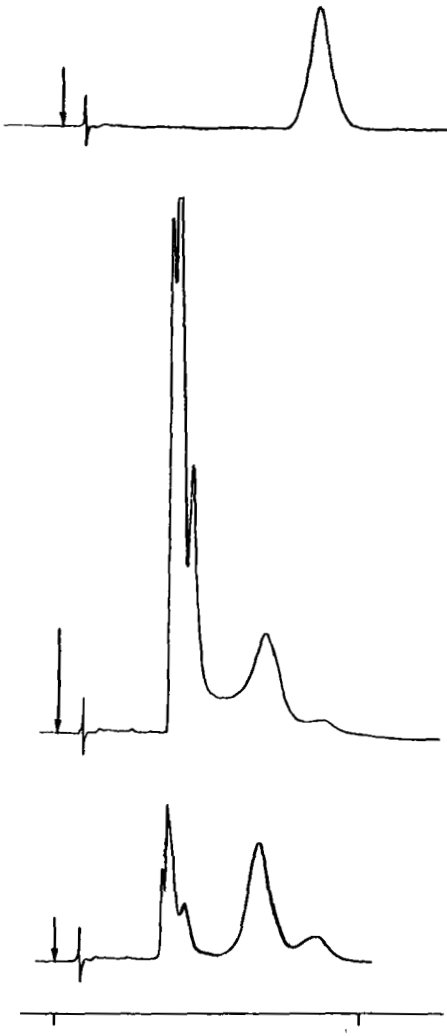

Figure 2 HPLC of gossypol solutions in MeOH

ODS-Hypersil 5 um, 12.5 x o.45 cm, eluted at 2.0 ml/min with MeCN-H_2O-AcOH 60:30:10 v/v/v, detector 290 nm

a) Fresh solution; b) 16-Day old solution;

c) (b) + mobile phase (1:1), stood 9 days

mobile phase (MeCN-H_2O-AcOH, 60/30/10 v/v/v), the size of peak 4 grew steadily over a period of several days, until it accounted for about half the total material visible (Figure 2c). A month-old solution of gossypol-acetic acid in methanol (similar on HPLC analysis to Figure 2b) was evaporated under vacuum and some of the residue immediately re-dissolved in acetonitrile. Its HPLC trace showed the main component to be peak 4, with some gossypol present and only small amounts of the three short-retained peaks seen in Figure 2b. Recrystallisation of the remainder of the residue from benzene-hexane yielded pure anhydrogossypol. When the residue was dissolved in mobile phase and examined by HPLC, it gradually transformed into gossypol during a period of hours. The four new peaks which appear in gossypol solutions in methanol are ascribed to readily hydrolysed ketal isomers. One of them, peak 4, has a similar retention time to anhydrogossypol, but its peak is much sharper than that of the anhydride, which tails considerably. Dissolution of an authentic sample of anhydrogossypol in methanol led to the appearance of new peaks of shorter retention times, identical to peaks 1-3 in Figure 2b.

The gossypol solutions in methanol were also examined by FD-MS. For a freshly-prepared solution (Figure 3a), the molecular ion (m/z 518) was the most abundant ion, with weaker ions at m/z 500 and 482, corresponding to losses of one and two water molecules: this result is similar to that reported previously for gossypol solutions loaded onto the FD emitter wire using non-alcoholic solvents [6]. However, a three-day old methanol solution showed a substantial increase in the proportion of the mono- and bis-dehydro ions (Figure 3b) and after 40 days standing the spectrum

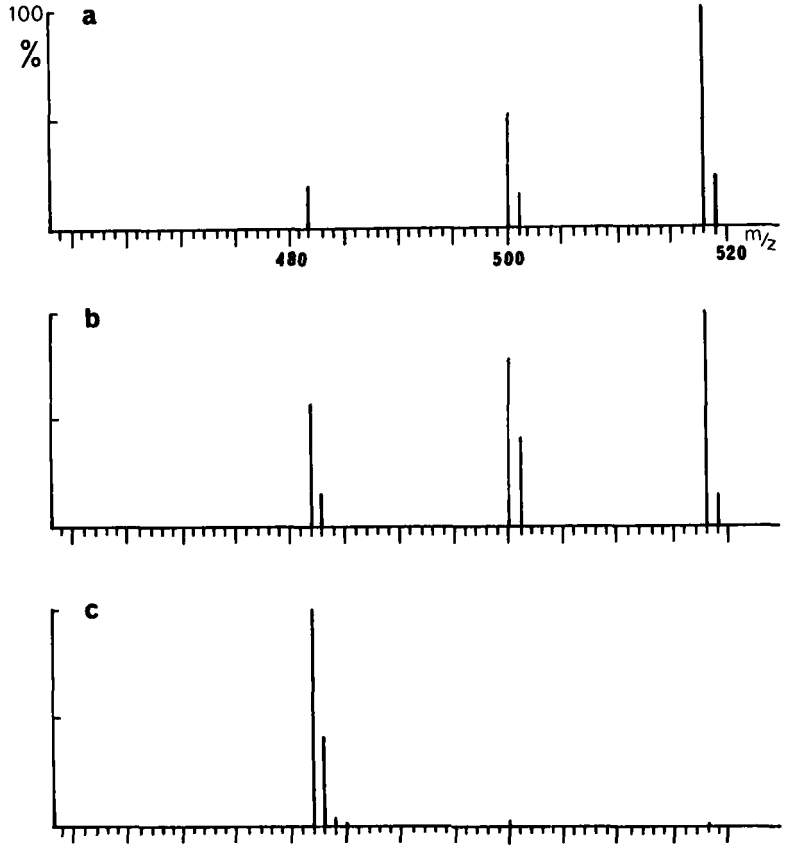

Figure 3 Field desorption mass spectra of gossypol solutions

a Fresh solution in MeOH

b 3-Day old solution in MeOH

c 40-Day old solution in MeOH

showed only an ion for anhydrogossypol (Figure 3c). This behaviour is again consistent with the formation of ketal derivatives of gossypol in the methanol solution: the ketals are easily dehydrated to anhydrogossypol during the drying-out and subsequent heating of the emitter wire.

A direct LC/MS study of a 20-day old solution of gossypol-acetic acid in methanol provided further confirmation of the conclusions. The TIC trace was very similar to the HPLC trace shown in Figure 2b and revealed only peaks of shorter retention time than gossypol and the EI-MS of each of these was identical to Figure 3c, having a molecular ion at m/z 482.

Solutions of gossypol-acetic acid in two other alcohols were also examined. In isopropanol, disappearance of the gossypol peak was somewhat slower and a very complex chromatogram with many overlapping peaks was obtained when a solution was kept for 2 months in the dark at 4^0. Gossypol re-appeared as the main constituent when this solution was mixed with mobile phase and stored for a further week. This behaviour is consistent with the conversion of gossypol into isopropyl ketals, which occurs slowly due to steric hindrance, and these hydrolyse back to gossypol in the presence of aqueous acid. A benzyl alcohol solution showed showed almost complete disappearance of gossypol after 5 days (Figure 4a), the new peaks now all appearing at longer retention times. This pattern remained essentially unaltered when the solution was kept for 2 months, but again the size of the gossypol peak increased greatly ang other peaks diminished after the solution was mixed with mobile phase (Figure 4b).

We conclude that solutions of gossypol in alcoholic solvents contain various ketal derivatives

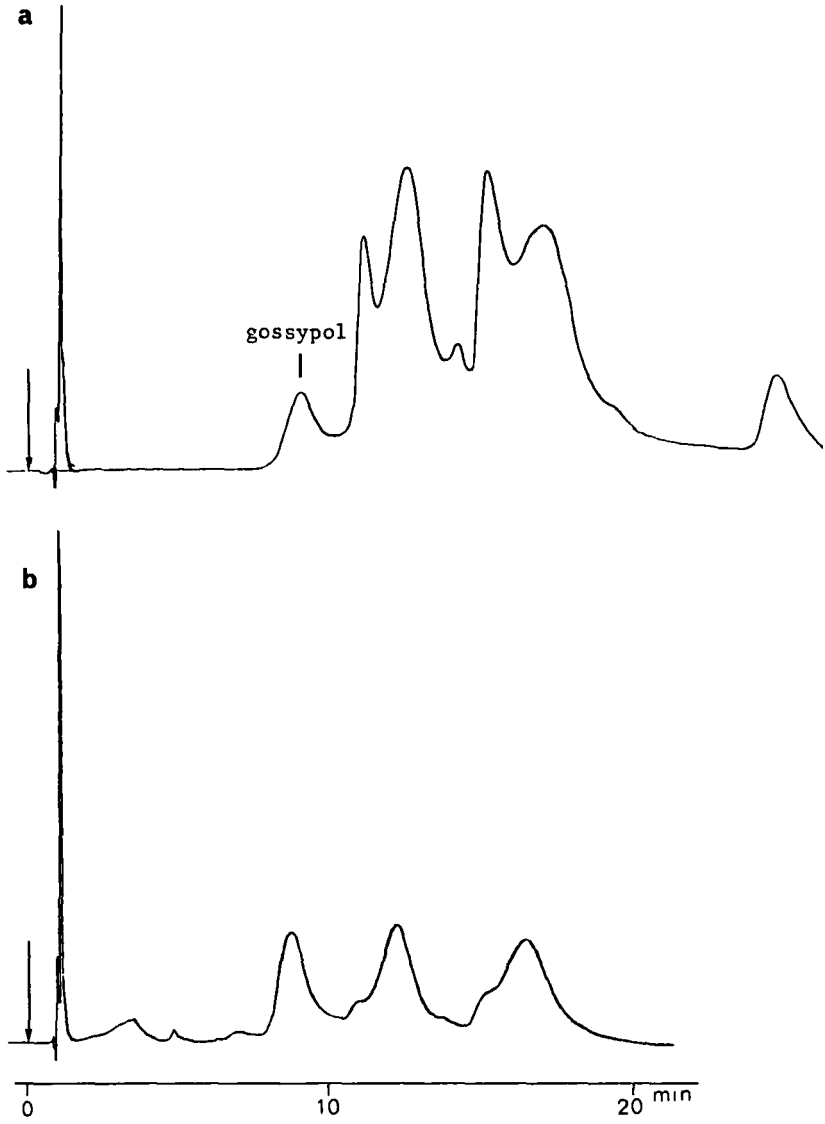

Figure 4 HPLC of gossypol solutions in benzyl alcohol
ODS-Hypersil 5 um, 12.5 x 0.45 cm, eluted at 2.0 ml/min with MeCN-H_2O-AcOH 60:30:10 v/v/v, detector 290 nm
a) 5-Day old solution;
b) 57-Day old solution + mobile phase (1:1) stood 1 day

(Figure 1), the proportion of these increasing with time until, in methanol or ethanol solutions a few days old, there is little or no "free" gossypol left. There would clearly be problems in using HPLC to monitor the purity or content of gossypol in stock solutions in alcohol which are prepared and stored for biological studies. The consequences of this for the utilization of such solutions are complex: it is evident from the attempts to isolate these alcohol adducts that they are very labile and readily revert to gossypol under aqueous conditions. Thus, solutions of gossypol in aqueous alcohol could be used for biological studies such as those involving enzyme or cell targets, since the equilibria depicted (Figure 1) will ensure that all the gossypol becomes available through dissociation. In principle, the same can be said of chemical studies applied to such solutions. However, it must be emphasised that rates of transport may be very different and, depending on the kinetics of the processes involved, competing reactions may also be seen from the various tautomeric ketal derivatives.

It is noteworthy that, in biological studies where the presence of alcohol or the existence of the ketal adducts is an undesirable complication, aqueous DMSO solutions may be a suitable alternative. Gossypol is very soluble in DMSO and the solutions are stable for months, as judged by HPLC analysis, when kept in the dark and refridgerated.

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