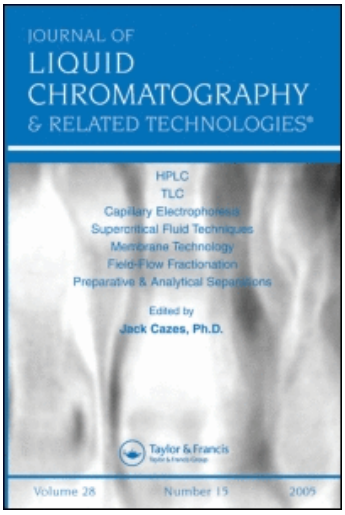

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ANALYSIS OF GOSSYPOL ENANTIOMERS IN HUMAN SERUM

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ABSTRACT

The concentrations of individual gossypol enantiomers in serum can be determined by derivatisation with phenylalanine methyl ester prior to extraction and reverse phase HPLC.

INTRODUCTION

Gossypol (1), a phenolic pigment extracted from the cotton plant, is orally active as an anti-spermatogenic agent in men and in male animals, [1,2]. It has also been demonstrated that gossypol is an active cytotoxic agent against tumour cells in vitro

[3-6] and against the parasite Trypanosoma cruzi which causes Chagas' Disease in South America [7]. Gossypol is optically active, exhibiting atropisomerism as a result of hidered rotation about the internaphthyl bond [8] and we have developed both analytical and preparative HPLC methods for the resolution of the enantiomers, using either chiral or achiral amines to form Schiff's bases which could be separated using HPLC columns containing chiral bonded phases or, in the case of Schiff's bases of certain chiral amines, using reverse phase HPLC [9-11]. Studies with the individual enantiomers have established that all of the antifertility effects are associated with (-)-gossypol [12,13] and that this enantiomer is also 4-12 times more potent than (+)-gossypol as a cytotoxic agent [3-6].

As a result of the potent anti-mitochondrial activity demonstrated for gosssypol in tumour cells in vitro [4,6,14,15] and the limited toxicity of gossypol observed in men participating in contraceptive trials [1,2], there is considerable interest in the development of gossypol and its derivatives as potential clinical anti-tumour agents. In connection with a clinical trial of racemic gossypol as an anti-tumour agent, it was required to monitor the levels of the individual enantiomers in serum from the cancer patients. Procedures for the extraction of racemic gossypol from serum and its determination by HPLC have been described [16-19]. However, there has hitherto been only one report [19] of the separate determination of the two isomers following administration of the racemate. This involved extraction of the isomer mixture, from which the total gossypol concentration was assayed by reverse phase HPLC using added gossypol dimethyl ether as internal standard, and additionally

derivatization of the extracted isomer mixture by heating with (R)-2-amino-1-propanol for 100 minutes, after which the isomer ratio was determined by reverse phase HPLC. We have now adapted a modification of our earlier procedure for the analytical resolution of racemic gossypol [10], making it suitable for the rapid analysis of concentrations of gossypol enantiomers in serum samples. In this paper, we report details of the methodology developed. Results of the clinical investigations will be discussed elsewhere.

MATERIALS AND METHODS

The HPLC equipment consisted of a Cecil CE1100 pump, Rheodyne 7125 injector fitted with a 50 ul loop and a Cecil 1200 variable wavelength detector set at 250 nm x 0.0002 AU fsd. Peak areas were measured with a Trivector Trio integrator. Columns (25 x o.45 cm id) were packed with 5 um ODS-Hypersil and eluted at 2.5 ml/min with MeCN:buffer:THF (76:22:2 v/v/v), the buffer consisting of 0.01M aqueous KH_2PO_4 adjusted to pH 2.35 with H_3PO_4. All solvents were either purchased as HPLC grade or were redistilled. Mobile phases were degassed ultrasonically before use.

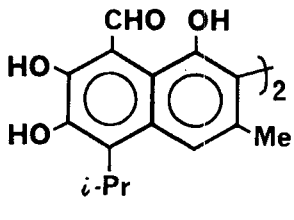
Serum samples from cancer patients were stored in a frozen state and were defrosted immediately before analysis. Blank serum samples were obtained from the same patients, prior to or at the time of oral dosing and were analysed with or without spiking with known quantities of racemic gossypol acetic acid.

0.40 ml of serum was mixed with 0.50 ml of a saturated solution of EDTA disodium salt and stood for 10 min. The solution was adjusted to pH 8.00 by addition of conc. NaOH solution, monitored using a micro-electrode. 50 ul of a solution of L-phenylalanine

methyl ester (1 g/ml in MeCN) was added and the mixture left for 10 min, then adjusted to pH 7.00 using conc. sulphuric acid and extracted with ether (2 x 2ml: vortex mixing and centrifugation to separate the layers) and the combined ethereal extracts evaporated under reduced pressure. The residue was reconstituted in 0.50 ml of acetonitrile and 50 ul of this solution injected on the HPLC column.

RESULTS AND DISCUSSION

Racemic gossypol (1) reacts with L-phenylalanine methyl ester to form the Schiff's base derivative (2) as a mixture of two distereoisomers. Preliminary investigations showed that, in mixed aqueous-organic solvents, the reaction proceeds rapidly to completion at pH 8. In order to minimise the binding of gossypol to serum proteins and maximise the recovery of the two enantiomers, serum samples were pre-incubated with the disodium salt of ethylenediamine-tetraacetic acid prior to addition of the amine. Following derivatisation, the diastereomeric Schiff's bases of gossypol are most efficiently extracted into diethyl ether at pH 7 for analysis by reverse phase HPLC.



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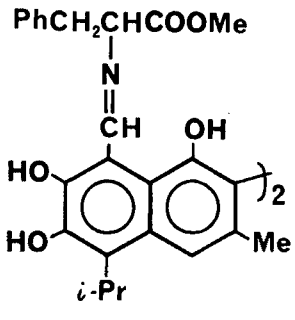

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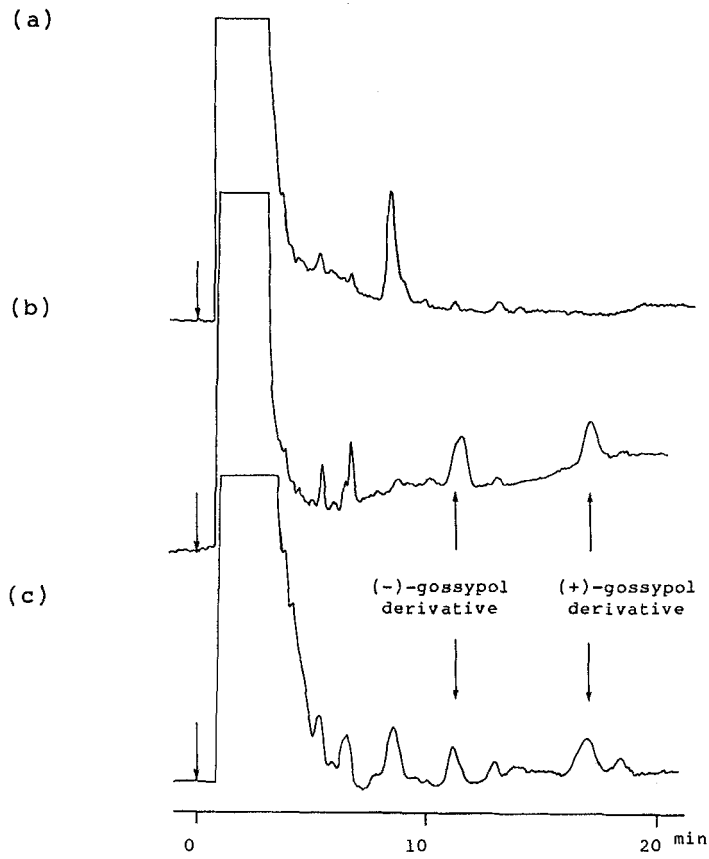

Figure 1 Analysis of gossypol isomers in human serum
(a) Serum from non-dosed, healthy volunteer
(b) Patient's serum sampled at time of oral dosing and spiked with 550 ng/ml of each enantiomer
(c) Patient's serum 1 day after oral dosing with racemic gossypol, showing 402 ng/ml (-)-gossypol and 529 ng/ml (+)-gossypol.
Column: 5 um Hypersil-ODS, 25 x 0.45 cm id. Mobile phase: MeCN:buffer:THF 76:22:2 v/v/v (buffer 0.01M KH_2PO_4 + H_3PO_4 to pH 2.35) at 2.5 ml/min; detector 250 nm x 0.002 AU fsd. All three sera samples were incubated with L-phenylalanine methyl ester before extraction, evaporation and reconstitution in 500 ul MeCN, from which 50 ul samples were injected.

Serum samples obtained from each patient prior to oral dosing were processed and analysed to confirm the absence of interfering peaks on HPLC. Samples from untreated subjects were spiked with a known amount of racemic gossypol for calibration of recoveries and response factors. When spiked serum samples were processed on the same day, the reproducibility of responses was found to be ± 4.1% SD (n = 6) for (+)- and (-)-gossypol. Over a period of time, there was some day-to-day variability in the extent of recovery (±10.5% SD), but good accuracy could be maintained when the spiked controls run on the same day were used to calibrate the values obtained for treated patients. Typical HPLC traces are shown in Figure 1. Detection limits were ca. 2 ng injected (corresponding to ca 30 ng/ml in serum) for each isomer of gossypol under the conditions employed. Detection limits for individual enantiomers were not reported in the earlier study, but the limit for underivatised racemic gossypol was ca. 25 ng/ml using an electrochemical detector [19].

Serum levels of total gossypol in cancer patients being dosed orally with tablets of gossypol acetic acid varied up to 1130 ng/ml (serum sample 24 h after patient given once weekly dose of 120 mg). As expected, the ratio of enantiomers also varied, in some cases being close to the 1:1 ratio administered but in several cases a substantial excess of (+)-gossypol was detected in serum. This result is consistent with the known tendency of (+)-gossypol to bind more tightly than the (-)-isomer to proteins [4]. (+)-Gossypol displays a serum half life which is almost twice as long as that for the (-)-isomer, e.g. 7.80 h for (+) versus 3.96 h for (-) in the rat [20]. A full report of the clinical findings will be presented elsewhere [21].

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