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High-performance liquid chromatographic method for the comparison of tanning capacity of tannic acid batches used in the manufacture of pregnancy testing kits

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method was developed for the quantitative comparison of various batches of tannic acid from the same manufacturer used to aid the binding of human chorionic gonadotropin to sheep erythrocytes in the manufacture of pregnancy testing kits. The tannic acids were separated by reversed-phase HPLC on a C_{18} column using gradient elution with aqueous methanolic eluents at low pH. A portion of the chromatogram corresponding to the compounds involved in the tanninisation process was integrated and a linear relationship was established between this peak area and tannic acid concentration. The correlation coefficient was greater than 0.993 even in the absence of an internal standard. Tanning capacity was evaluated on the basis of the amount of tannic acid which remained following incubation with a known quantity of erythrocytes. The application of this procedure to three batches of tannic acid is demonstrated.

INTRODUCTION

Tannins comprise a heterogeneous group of polyphenols which can combine with skin proteins, *e.g.* gelatin, in such a way as to render them insoluble and resistant to putrefaction. They are of high molecular weight, containing sufficient phenolic hydroxyl groups to permit stable cross-links with proteins. Tannins are nowadays classified as "condensed"[1] or "hydrolysable"[2], depending on their structure. Hydrolysable tannins consist of gallotannins, ellagitannins and caffetannins. Of these, the gallotannins (which are composed of galloyl groups bound to a central polyol by a hydrolysable ester bond) are the most important. Besides the galloyl components, tannin extracts in general contain small amounts of free gallic, digallic and trigallic acids[3,4]. Condensed tannins consist of acacatechin, isoacacatechin and the gambin

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catechin tannins. Of the two classes, the condensed tannins are more widely distributed in higher plants.

Tannic acid is the usual form of tannin used in industry and is obtained by purification of hydrolysable tannins. Tannic acid is widely used in the pharmaceutical industry as an astringent, and in the brewing industry as a clarifier for wine and beer. It has many applications in the chemical industry, including leather tanning, ink compounding, as a reagent in analytical chemistry, and (of particular interest in the present study) as a coupling agent used to bind human chorionic gonadotropin (hCG) to erythrocytes in the manufacture of pregnancy testing kits.

The components of tannic acid have been separated by gel filtration[5], thin layer chromatography[6] and high-performance liquid chromatography (HPLC)[7–11]. Both normal-phase[7,811] and reversed-phase[9,10] HPLC techniques have been employed, and in both cases a gradient was required to separate gallic, digallic and trigallic acids from the tannic acid mixture. Quantitative analysis of tannic acids from different commercial sources was carried out by Verzele and Delahaye[8]. The normal-phase HPLC of tannic acid had a regular pattern of about six peaks with a general Gaussian shape pattern. They concluded from this that different sources of tannic acid could be identified on the basis of total peak pattern of the chromatogram and 2,3,4-trihydroxybenzoic acid was proposed as a suitable internal standard. Single peaks obtained by normal-phase HPLC could be further separated into two or three peaks by reversed-phase HPLC with a gradient of methanol–water containing phosphoric acid. Gallic, digallic, trigallic and ellagic acids have also been separated by reversed-phase HPLC[10]. In this case, on ODS column was used with an aqueous methanol mobile phase (adjusted to pH 2.5 with $HClO_4$) operated over a linear gradient ramp.

Tanning capacity (*i.e.* protein precipitating or binding power) is routinely determined by the "hide powder assay" prescribed by the American Leather Chemists Association[12–14]. This method, however, also determines non-tannins in an extract, and therefore gives purity percentages which do not always reflect the real tanning power[15]. As an alternative to the hide powder assay, tanning capacity may be evaluated by binding with bovine serum albumin (BSA), where it is expressed in terms of the number of milligrammes of protein bound to 1 mg of tannic acid[11]. Other methods used to study tannin–protein interactions include complexation with dinitrophenol-derivatised gelatin[16] and ^{125}I-labelled BSA[17]. In general, tanning power is considered to be related to molecular weight (optimum 3000), though it has also been reported that tanning capacity is improved if specific groups in a molecule are sterically well-positioned to permit binding to the protein surface. The latter hypothesis is used to explain the high tanning capacity of some tannic acids despite their low molecular weight.

There are, to date, no reports on the binding of tannic acid to erythrocytes although Schultz *et al.*[18] have described the use of haemoglobin in a quantitative assay for plant tannins. This method was based on the decrease in absorbance of haemoglobin at 578 nm following precipitation with tannins. They found that the tannin-protein binding reaction exhibited co-operativity, in that the first tannin bound facilitated binding of successive tannin molecules. The problem with this technique is that it necessitates the ready availability of large batches of fresh blood, as preserved human blood cannot be used due to the addition of anticoagulants.

The objective of the present study was to develop a suitable analytical method

for the quantitation of tannic acids and to use this method to compare the tanning capacity of new batches of tannic acid in the tanninisation of erythrocytes during the manufacture of pregnancy testing kits. Such a method might obviate the current requirement for expensive, time-consuming and sometimes inconclusive production trials in the quality control screening of new batches of tannic acid.

EXPERIMENTAL

Reagents and solvents

Tannic acid was supplied as pharmaceutical grade by Merck (Darmstadt, F.R.G.). Methanol (HPLC grade) was obtained from Fisons (Loughborough, U.K.). Disodium hydrogen phosphate, potassium dihydrogen phosphate and orthophosphoric acid (AnalaR grade) were purchased from BDH (Dorset, U.K.). Purified water was produced by passing distilled water through a Millipore (Milford, MA, U.S.A.) Milli-Q water purification system. Whole blood from sheep was decanted into an isotonic citrate buffer. The packed cell volume of a representative sample was measured following centrifugation and adjusted to a pre-determined value by addition of more citrate buffer or removal of supernatant as the case demanded. The erythrocytes were then separated, washed with more buffer and stored in isotonic saline under refrigeration until required for use.

Instrumentation and operating conditions

The tannic acids were separated on a Rosil (Alltech, Deerfield, IL, U.S.A.) C_{18} (5-μm) column, 15 × 4.6 cm I.D. Samples were introduced onto the column using a Perkin-Elmer (Beaconsfield, U.K.) Model LC600 autosampler with an injection volume of 10 or 100 μl. Detection was achieved by ultraviolet absorption at 280 nm using a Perkin-Elmer Model LC15 spectrophotometric detector and the resultant chromatograms were recorded using a Perkin-Elmer chart recorder at a chart speed of 5 mm/min. The mobile phase consisted of 0.5% aqueous phosphoric acid (A) and methanol–phosphoric acid [100:0.5, v/v] (B), delivered from a starting concentration of 30% B over a gradient ramp of 2% B per min for a run time of 23 min. The mobile phase components were filtered through a 0.45-μm membrane, degassed by helium sparging, and delivered by a Perkin-Elmer Series 2 dual pump at a flow-rate of 1.5 ml/min.

Procedure

Erythrocytes were tanninised by mixing 5 ml erythrocyte suspension with an equal volume of 1.0% tannic acid in 0.01 *M* phosphate buffer, pH 7.2, and incubating (in a standing position) the suspension at 56°C for 30 min. The supernatant was separated from the erythrocytes by centrifugation at 2000 *g* for 10 min. Erythrocyte-free tannic acid in phosphate buffer, pH 7.2, was treated in the same manner and used as a control.

Standard solutions in the concentration range 0.01–0.07% tannic acid in 0.01 *M* phosphate buffer were prepared for each batch of tannic acid resulting in a separate calibration curve for each new batch. Following incubation of the control and erythrocyte-treated samples, the supernatants were diluted 1 in 20 to bring them into the range of the calibration curve. The total peak area along the chromatogram from

11.5 to 23 min was integrated electronically and plotted as a function of concentration to yield a linear calibration curve. The peak area corresponding to the tannic acid remaining following treatment with erythrocytes was subtracted from the control peak area. The concentration of tannic acid bound to erythrocytes was determined through interpolation of this figure on the calibration curve and tanning capacity was expressed in terms of the percentage of the original concentration added which became bound to the erythrocytes.

RESULTS AND DISCUSSION

Development of chromatography

The chosen starting point was the mobile phase reported by Verzele and Delahaye[8]. A typical chromatogram resulting form this mobile phase programme is shown in Fig. 1A. Shown in Fig. 1B is a typical chromatogram which demonstrates the somewhat improved resolution of the main tannin band achieved with the modified mobile phase as indicated in this figure.

The stability of tannic acid solutions was determined by comparing chromatograms of a fresh 50% (v/v) solution in purified water and the same solution stored at 4-8°C over a 2-week period. Based on the fact that the number and magnitude of peaks remained constant, it was decided that the tannic acid solution was stable under these storage conditions. Tannic acid solutions prepared in buffer were stable for 5 h at room temperature. After this time, the early eluting peaks began to increase in intensity and there was a change in the peak pattern of the main band.

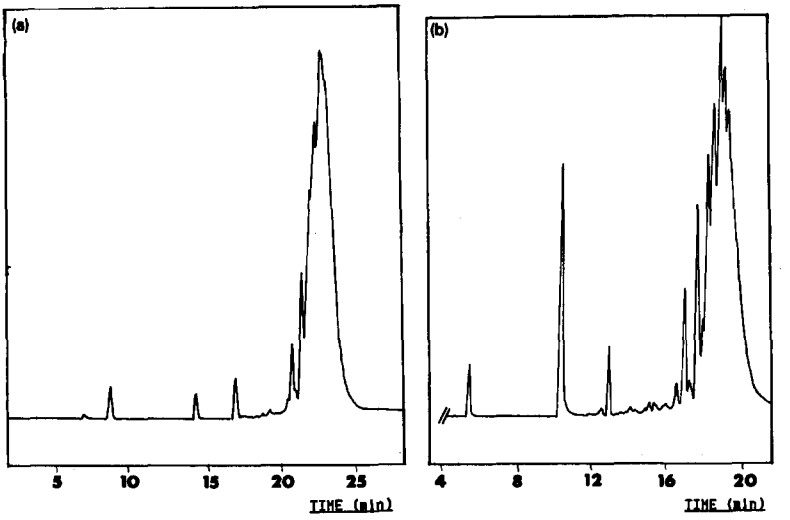

Fig. 1. Typical chromatograms of tannic acid constituents A, before and B, after mobile phase modification. (A) Mobile phase: methanol–water–orthophosphoric acid (10:90:0.5. v/v/v) increasing after 1 min to methanol–orthophosphoric acid (100:0.5, v/v) over a linear gradient ramp of 3% methanol–orthophosphoric acid/min. (B) Mobile phase: methanol–water–orthophosphoric acid (30:70:0.5, v/v/v) increasing after 1 min to methanol–orthophosphoric acid (100:0.5, v/v) over a linear gradient ramp of 2% methanol–orthophosphoric acid/min. Sensitivity = 0.256 a.u.f.s., injection volume = 10 μl, sample = 1 mg/ml tannic acid in purified water.

Selection of a tanninisation procedure

Initially, erythrocytes were tanninised by mixing with 0.01% tannic acid in phosphate buffer, pH 7.2, and incubating the suspension at 56°C for both 10 and 30 min. Following centrifugation, the supernatants of the two incubated suspensions, in addition to a suspension which had not been incubated, were tested for remaining tannic acid. Erytrocyte-free tannic acid solutions (in phosphate buffer, pH 7.2) were subjected to the same treatment and compared with the supernatants. No tannic acid was detected in the supernatant after 10 or 30 min incubation. In the incubated controls there appeared only a small change in the peak pattern along the chromatograms and a slight increase in the height of the early eluting peaks.

Since all of the 0.01% tannic acid solution had become bound to the erythrocytes after 10 min incubation, it was decided to increase the concentration of tannic acid to establish at what point it would be in excess of the erythrocytes binding sites. 0.1, 0.15, 0.18, 0.2, 0.25, 0.5 and 1.0% solutions of tannic acid were investigated and it was found that with the 0.5% solution, 12% (as compared with the corresponding erythrocyte-free control) remained after incubation, and that almost 60% remained using the 1% tannic acid solution. In order to allow for batch-to-batch variations of greater than 12% in tannic acid, it was decided to use the 1.0% solution in all subsequent tannisation procedures. Chromatograms of 1% tannic acid solutions following 30 min incubation both with and without erythrocytes are presented in Fig. 2.

Verzele and Delahaye[8] found that (i) high molecular weight polygalloyl glucose

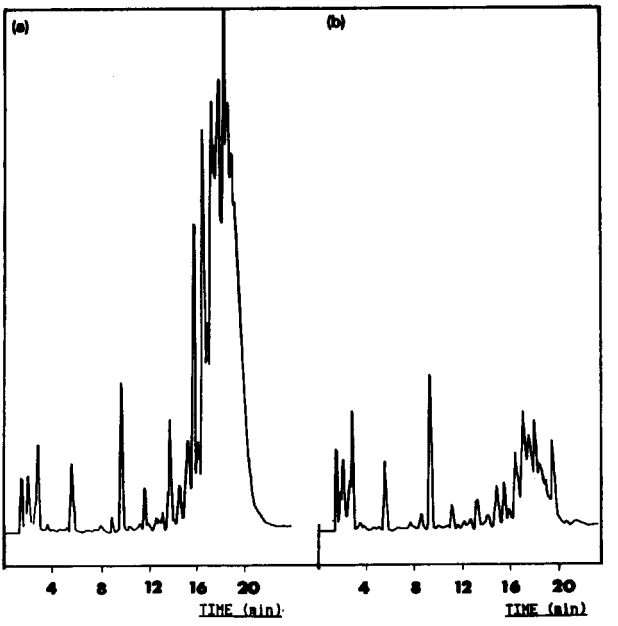

Fig. 2. Typical chromatograms of tannic acid constituents after incubation, A, with erythrocytes and B without erythrocytes. A = Control tannic acid; B = Erythrocyte-treated sample. Mobile Phase = methanol–water–orthophosphoric acid (30:70:0.5, v/v/v) increasing to methanol–orthophosphoric acid (100:0.5, v/v) over a linear gradient ramp of 2% methanol–orthophosphoric acid/min.

constituents preferentially bound to BSA, (ii) that low-molecular-weight polygalloyl glucose components bound preferentially to beer proteins, and (iii) that gallic acid and its oligomers, di- and tri-gallic acid, have no tanning capacity under these circumstances. In the present work it was found that all polygalloyl glucose constituents complexed with erythrocytes during tanninisation, since all peaks in the main peak pattern of the chromatogram reduced proportionally during tanninisation. Therefore, the total area of this band was used as measure of tanning capacity. The early peaks, corresponding to low-molecular-weight gallic acid-type consitituents, and which have no tanning capacity, were subtracted from the total area of the chromatogram. Furthermore, following processing, it was found that there was greater variation in the peak areas of the early-eluting peaks than in the main band within a given batch of tannic acid. Peak area integration was, therefore, commenced at 11.5 min following injection.

Evaluation of tanning capacity of three tannic acid batches

Quantitative measurements were based on the difference in tannin concentrations after tanninisation of erythrocytes compared to tannic acid control solutions which were subjected to the same incubation conditions. It was found that the chosen internal standard, 2,4,6-trihydroxybenzoic acid, co-eluted with the early eluting peaks, so attempts were made to extract the constituents responsible for these peaks since they are known not to partake in the tanninisation process. Peak removal was attempted using a cation exchanger but this approach resulted in a reduction of the peaks of interest as well as the interfering peaks. It was decided, therefore, to proceed with the analysis without an internal standard, and even under these circumstances there is a linear relationship between peak area and concentration with a correlation coefficient greater than 0.993. The correlation coefficients were compared for the total peak area in the chromatogram and for the corrected peak area, *i.e.*, the area of analytical interest along the chromatogram. There was no significant change in correlation when the corrected (as opposed to the total) peak area was integrated. The three batches tested had tanning capacities of 62%, 68% and 80% respectively. The 80% value was yielded by a batch manufactured in 1985 and the other two were manufactured in 1981. This suggests a fall-off in tanning capacity with time, though further investigations to confirm these findings have not yet been concluded.

CONCLUSION

Reversed-phase HPLC can be used to determine the reduction in tannic acids by incubation with erythrocytes. Using the described method, it was found that there appeared to be a decrease with time in the concentration of pollygalloyl constituents which are responsible for the tanninisation process. Potentially, this method can be used in the quality control screening of batches for the binding of antigens to erythrocytes.

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