

REMOVAL OF POLYPHENOLIC COMPOUNDS FROM AQUEOUS PLANT EXTRACTS
USING POLYAMIDE MINICOLUMNS

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Received March 23, 1998

Summary

An improved method for the removal of polyphenolic compounds from aqueous extracts of plants is presented. The method removes >99% polyphenolic compounds from 5mg of extract. The method is simple, robust and reproducible. We examined the removal of polyphenolics from 5 different aqueous extracts of Chinese medicinal herbs.

Key words: tannins, polyphenols, polyamide, Chinese herbs

Introduction

Tannins comprise a group of secondary metabolites widely distributed in the Plant Kingdom (1). Apart from their traditional use in the tanning of leather, tannins have been found to affect a wide number of biochemical reactions due primarily to their high affinity for proteins (1). For example, they have been shown to inhibit reverse transcriptase (2,3), DNA polymerase (4) and the enzymes topoisomerase I and II (5,6). The almost ubiquitous presence of polyphenolic compounds, especially tannins, in plant extracts makes the isolation and characterization of other compounds from these sources very difficult. The rapid removal of tannins from small samples prior to screening for activity in various mechanistic assays and high throughput screening assays is therefore highly desirable. Our laboratory is concerned with the isolation of compounds from aqueous extracts of Chinese medicinal herbs which are inhibitory to HIV-1. Many of the herbs used are often rich in tannins. Tannins have previously been found to be potent inhibitors of HIV-1 reverse transcriptase and the binding of HIV-1 gp120 to the CD4 T-cell receptor (2,7,8). This problem can be circumvented by the use of polyamide to which polyphenolic compounds readily bind (2,9-11). Wall et al. recently compared a number of

methods for tannin removal, including the use of polyamide resin, and suggested that passage through Sephadex LH-20 was the best method for tannin removal from aqueous extracts (11). However, for the rapid removal of polyphenolics from small samples of aqueous plant extracts the polyamide method described here is highly effective and does not require the extensive post-chromatography concentration of the Sephadex method. The method described here is very flexible as to the sample volume and concentration that can be applied to the column. The time required to prepare a tannin-free extract is just a few minutes and the entire procedure can be performed in a standard bench-top centrifuge.

Materials and methods

To prepare the polyamide minicolumn a hole is pierced in the base of several plastic 1.5ml micro-centrifuge tubes with a 20 gauge needle. A small wad of glass wool is placed in the bottom of each tube and tamped firmly in place with a glass rod. A suspension of 200mg polyamide CC6 powder (Macherey-Nagel GmbH, Duren, Germany), pre-rinsed thoroughly to remove unreacted monomer, is pipetted into the tube to form a bed about 1cm deep. It should be noted that the polyamide powder does not swell appreciably in water and the granular particles do not pack together well, requiring some care during sample application and elution to avoid disturbing the bed surface. The minicolumn is placed in a 13 x 100mm glass test tube inside a bench top centrifuge (IEC Clinical, IEC, Needham Heights, MA, USA) and spun for one minute at low speed (c. 1000 x g) to remove excess water and to pack the bed firmly. The tannin-containing sample (up to 1ml 25mg/ml) is applied to the surface of the column and allowed to percolate. The sample is washed through the minicolumn with 1ml water followed by low speed centrifugation for one minute. The minicolumn is then eluted with 1ml 50% (v/v) aqueous methanol and 1ml absolute methanol, each wash being followed by a one minute low speed centrifugation. The tannin-free eluate is combined and lyophilized and stored for further use. The herbs were obtained from local vendors. Aqueous extracts were prepared as described previously (7). Tannic acid, (+)catechin and Folin-Ciocalteu reagent were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Results and discussion

To test this method of polyphenol removal varying amounts of tannic acid were applied to the minicolumn and the amount present in the eluate was quantitated with a previously described assay for total polyphenolic compound content utilizing Folin-Ciocalteu reagent (12).

Assay for tannic acid

The polyphenol assay method was linear over the range 0-100 μ g (Figure 1). The detection limit of the assay was 2.5 μ g.

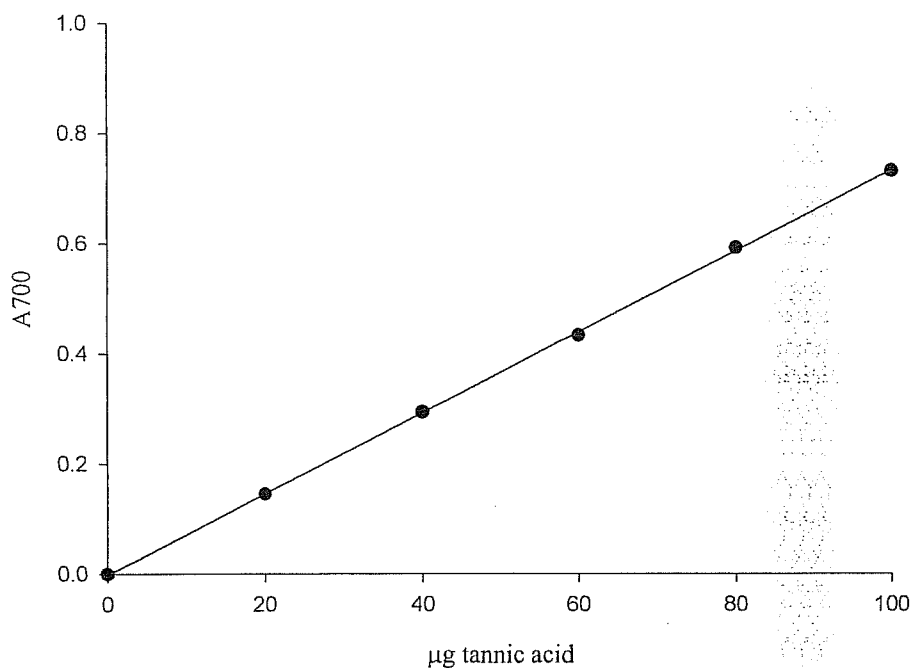


Figure 1.

Standard curve for tannic acid assayed with Folin-Ciocalteu reagent.

All values are the average of duplicate measurements that did not vary by more than $\pm 2.5\%$.

Effect of sample volume on tannin removal.

Tannic acid (5mg) in differing volumes (0.25-1ml) was applied to the minicolumn and eluted as described above. The minicolumn effectively removed the majority of the applied sample irrespective of the sample volume (Table 1).

Determination of minicolumn binding capacity.

The maximum capacity of the minicolumns for polyphenols was examined. Increasing amounts of tannic acid in a 0.5ml sample volume were applied to the minicolumn and the amount of tannic acid in the eluate was quantified as before. The results are shown in Table 2. As sample size increases the amount of tannic acid detected in the eluate increases. The majority of tannic acid was removed from up to 25mg applied sample. The column becomes 50% saturated at about 70mg tannic acid per 200mg polyamide. Sample size should be kept well

Table 1.

Effect of volume size on removal of polyphenolic compounds

Volume (ml)	% tannic acid removed
0.25	98.9 \pm 2.5
0.5	99.3 \pm 1.0
0.75	98.6 \pm 2.4
1.0	98.6 \pm 2.4

Values are mean \pm S.D. (n = 3)

Table 2

Effect of sample size on removal of polyphenolic compounds.

Tannic acid applied (mg)	% removed
5	99.3 \pm 1.0
10	97.3 \pm 0.3
17.5	92.5 \pm 0.5
25	87.1 \pm 0.3
100	27.6 \pm 0.6

Values are mean \pm S.D. (n = 3)

below this value for optimum removal. Simple phenolic compounds, those containing 2 or 3 phenolic hydroxy groups, bind less strongly to the polyamide resin and are eluted by the methanol wash. This was confirmed when 40% of the simple tannin (+)catechin was recovered from the minicolumn (Table 3).

Removal of polyphenolic compounds from aqueous plant extracts.

The polyamide minicolumns were also tested with samples extracted from plants. Aqueous extracts of *Salvia miltiorrhiza*, *Chrysanthemum morifolium*, *Paeonia suffruticosa*, *Prunella vulgaris*, and *Arctium lappa* were prepared as described earlier (7). An 80% (v/v)

Table 3

Removal of polyphenolic compounds from aqueous plant extracts.

Sample	% polyphenolics removed
<i>Prunella</i>	85.5 ± 0.9
<i>Arctium</i>	80.6 ± 3.4
<i>Chrysanthemum</i>	80.2 ± 1.7
<i>Paeonia</i>	69.9 ± 1.3
<i>Salvia</i>	64.2 ± 1.0
(+) catechin	60.6 ± 1.0

Values are mean ± S.D. (n = 3)

ethanol precipitate was prepared that was enriched in polysaccharide. It was suspected that this precipitate also contained polyphenolic contaminants. A 0.75ml (10mg/ml) sample of each herb extract was applied to separate minicolumns and eluted as described above. A significant amount of binding, seen as a pronounced brown discoloration of the resin, was seen. After washing the minicolumn, and collecting the eluate, the brown-coloured bound material was removed with several washes of 0.1M NaOH. The brown colour became more intense in the alkaline solution - a common qualitative test for the presence of tannins (13). The results are shown in Table 3. The woody stems of the plants gave pronounced tannin reactions in the untreated samples which were significantly reduced following passage through the minicolumns. The much lower extent of removal of polyphenolic compounds from the plant samples compared with the tannic acid controls indicates the greater variety of polyphenolics present in plants and the presence of compounds containing only 2 or 3 phenolic hydroxy groups which bind the resin less strongly.

The use of polyamide minicolumns is thus ideal for the almost complete removal of up to 5mg polyphenolic compounds from aqueous sample volumes of up to 1ml, although it is recommended that the sample volume and concentration be kept as small as practicable. The method is rapid (performed in minutes), reliable (complete removal of polyphenolics), robust (varying sample size and concentration does not affect performance) and cheap. It is now routinely used in our laboratory as a means of removing potentially inhibitory polyphenolic compounds from our herb preparations prior to screening for anti-viral compounds.

Acknowledgements

This work was funded by an Earmarked Research Grant provided by the Research Grants Council of the Hong Kong Government.

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