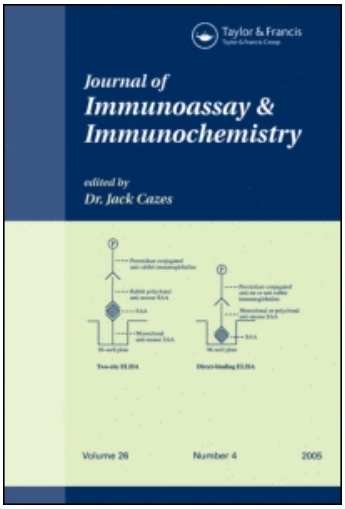

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Lehtola, Timo and Huhtikanqas, Aarre(1990) 'Radioimniunoassay of Aescine, a Mixture of Triterpene Glycosides', Journal of Immunoassay and Immunochemistry, 11: 1, 17 — 30
To link to this Article: DOI: 10.1080/01971529008053255
URL: http://dx.doi.org/10.1080/01971529008053255

JOURNAL OF IMMUNOASSAY, 11(1), 17-30 (1990)

RADIOIMMUNOASSAY OF AESCINE, A MIXTURE OF TRITERPENE GLYCOSIDES

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ABSTRACT

A radioimmunoassay (RIA) has been developed for the determination of picogram amounts of aescine, an anti-inflammatory and anti-oedematous glycoside mixture from unpurified extracts of Aesculus hippocastanum. Practically no interference is observed for various potentially crossreacting compounds. This RIA covers the range 100 pg - 50 ng, within which acceptable accuracy and precision are obtained.
(KEY WORDS: Aescine; Aesculus hippocastanum; triterpenoid saponin glycosides; radioimmunoassay; anti-inflammatory agent).

INTRODUCTION

Aescine [aescigenin-(2-methyl-3-acetoxy-butyrate)-(2-xylosido-4-glucosidoglucuronoside)] is a mixture of several acidic triterpenoid saponin glycosides found in the extracts of the horse chestnut tree, Aesculus hippo-

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castanum L. (Hippocastanaceae). The main glycoside is a compound in which the triterpene aglycone proaescigenin is esterified with angelic acid (C-21) and acetic acid (C-22) and glycosidically linked to C-3 of D-glucuronic acid which additionally binds two D-glucose units at C-2 and C-4 (1,2,3).

Aescine shows selective anti-inflammatory and anti-oedematous properties and is used e.g. in the treatment of traumatic edema of the brain (4,5,6) and muscular injuries (7,8). In clinical use, aescine is administered orally and intravenously, and gel formulations are widely used in percutaneous therapy (9,10,11).

Thin-layer chromatography methods have been described for aescine (1,2,3,12,) but the sensitivity of the assays have been only in the range of micrograms. For that reason the pharmacokinetics of aescine have been studied so far by the administration of tritiated aescine (9,10,11).

The rather high molecular weights and the relatively instable chemical structures associated with these glycosides do not favour common instrumental methods for quantitative analysis, but radioimmunoassay should, at least theoretically, allow reliable quantification of the compounds even from unpurified biological samples (13,14).

MATERIALS AND METHODS

Chemicals

Aescine, aescinol, acidic hydrolysate of aescine and total acidic and alkaline hydrolysate of aescine were obtained as a generous gift of Dr. Madaus GmbH & Co., Cologne, FRG. Other compounds used in crossreaction tests were obtained from Sigma Chemical Company, USA. [21,22-^{3}H] -aescine (specific activity 27 Ci/mmol) and the ACS-scintillation fluid were obtained from Amersham, England. All other chemicals used were standard commercial products of analytical grade.

Plant material

Seeds of Aesculus hippocastanum were obtained from Tägerwilen, Thurgau, Switzerland. Ethanolic water extracts of A. hippocastanum plants were diluted in phosphate buffered saline (PBS) (pH 7,2) for the preparation of RIA samples.

Antiserum

The aescine-immunogen for antiserum production was prepared by direct coupling of bovine serum albumin (BSA) to the carboxyl group of the glucuronic acid moiety by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) for the reaction (Fig. 1)(15,16,17).

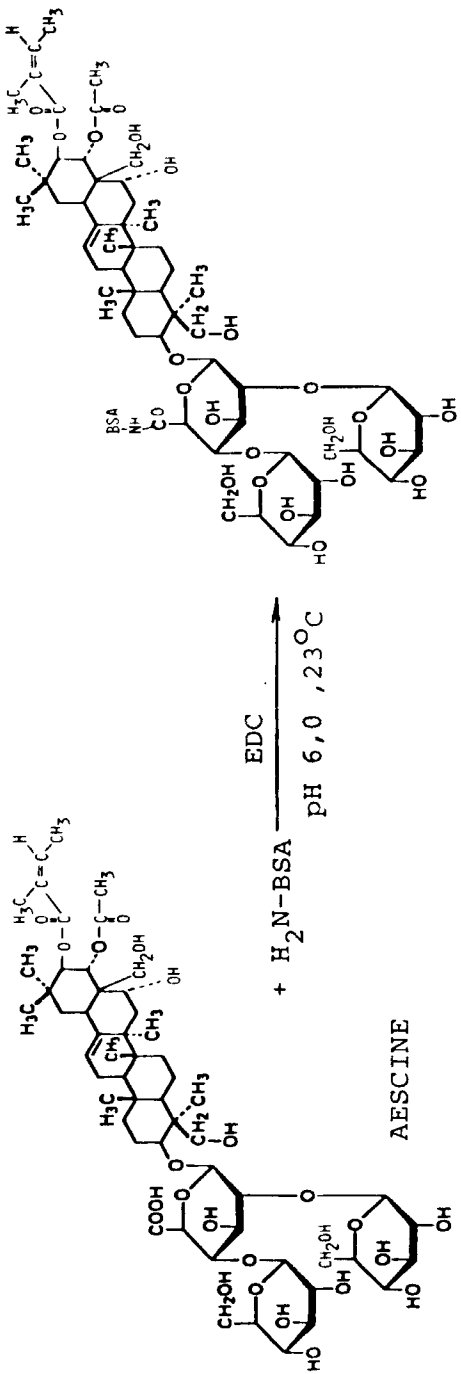

FIGURE 1. Synthesis of the aescine-BSA immunogen. H_2N-BSA = bovine serum albumin; EDC = 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

TABLE 1

Immunization Procedure of the Rabbits used for Aescine-Antibody Production

Time of injection (weeks)	Dose of antigen (mg)			
	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4
0	0,50	0,50	1,00	1,00
3	0,15	0,15	1,00	1,00
7	0,15	0,15	1,00	1,00
11	0,13	0,13	1,00	1,00
15	0,13	0,13	1,00	1,00
19	0,13	0,13	1,00	1,00

BSA (0,0074 mmol, 50 mg), water soluble EDC (0,27 mmol, 50 mg) and amorphous aescine (0,27 mmol, 300 mg) were added to 5 ml of phosphate buffer solution (pH 6,0). The reaction was allowed to proceed for three days at room temperature. The aescine-BSA immunogen was dialyzed against distilled water for four days at 4°C. The dialyzed and lyophilized immunogen was emulsified in Freund's complete adjuvant (18) and given in multi-site subcutaneous injections (19) to four New Zealand albino rabbits according to the protocol shown in Table 1. The rabbits were bled twenty weeks after the start of immunization, and the serum was stored in 1 ml aliquots at -20°C.

Radioimmunoassay

The RIA-procedure was based on polyethylene-glycol (PEG) precipitation (20) of the antigen-antibody complex from overnight incubation at 4°C, followed by liquid scintillation counting of ^{3}H-labelled free aescine in the supernatant (14).

After addition of 100 µl of a standard solution, a sample dilution or a solution of a compound used in the crossreaction tests, 100 µl of a 1 % solution of human gammaglobulin in PBS-buffer, 50 µl tritiated aescine in EtOH-PBS-buffer and 50 µl of the PBS-buffer-diluted aescine antiserum were added to the tubes. Anti-serum dilutions effecting 50 % binding of labelled antigen were used in the assay. The tubes were vortex-mixed and incubated overnight at 4°C. Bound and free antigen were separated by PEG-precipitation using 500 µl of a 25 % solution of PEG 6000 in PBS-buffer. After centrifugation (2000 g, 20 min) the supernatant was separated by decantation, mixed with 5 ml of ACS-scin-tillation solution and counted in a LKB-Wallac 1216 Rackbeta liquid scintillation counter (14,21).

RESULTS

Antibody production against aescine was clearly detectable in two of the four rabbits (Fig. 2). The present results refer exclusively to antiserum 1.

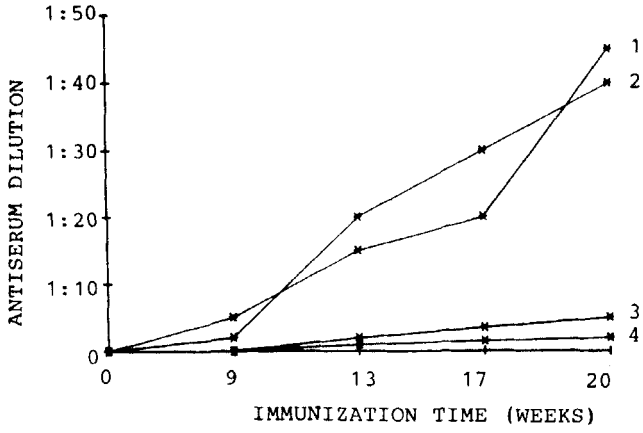

FIGURE 2. Titres of aescine-antiserums in rabbits 1,2,3 and 4 during the immunization procedure. Antiserum dilutions effecting 50 % binding of labelled antigen are indicated.

The titre, defined as the final dilution of the antiserum needed to bind 50 % of the added ^{3}H-aescine, was 1:270.

The total number of antibody binding sites, A_o= $3{,}6 \times 10^{-6}$ mol/l, and the corresponding value for the affinity constant, K_a=$4{,}6 \times 10^{9}$ l/mol, were derived from a Scatchard plot (22).

The standard curve for the present aescine-RIA and the structures of the compounds used in crossreaction tests, with amorphous aescine as the reference compound, are shown in Figs. 3 and 4, respectively. The crossreactivity data are summarized in Table 2.

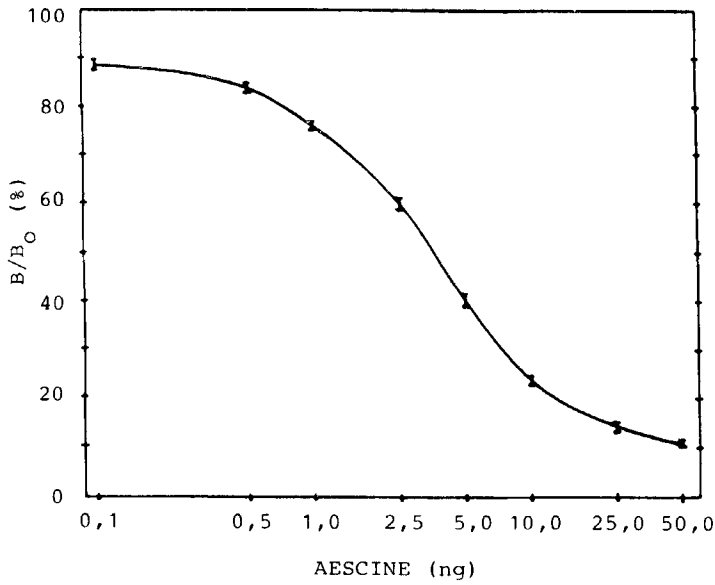

FIGURE 3. Standard curve for aescine radioimmunoassay. B and B_O are the binding percentages in presence and absence, respectively, of unlabelled aescine. Bars indicate the standard deviation (n=5).

The assay sensitivity, defined as the concentration obtained at twice the standard deviation of the zero-binding value, was approximately 100 pg / 100 µl sample. The measuring range of the assay extends from 100 pg to 50 ng of aescine.

Linearity was investigated by the sample dilution method (23). The regression equation for the extract dilutions was y=3,86+1,05x, with a coefficient of correlation of r=0,997 (n=9).

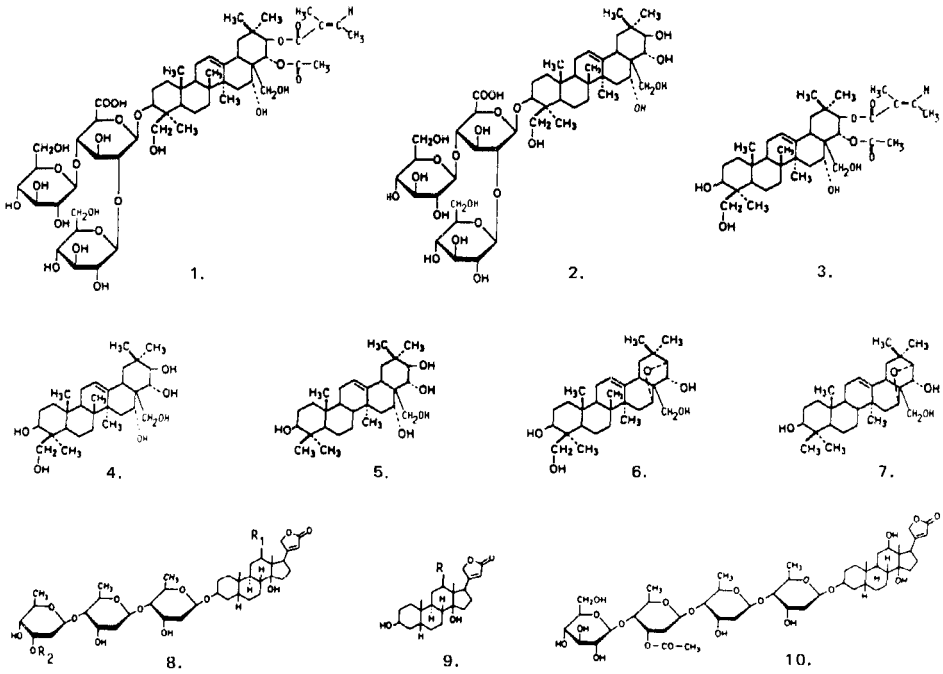

FIGURE 4. Structural features of the crossreacting compounds. 1. Aescine; 2. Aescinol; 3. Acidic hydrolysate of aescine; 4. Proaescigenin; 5. Barringtogenol C; 6. Aescigenin; 7. Barringtogenol D; 8. α-acetyldigoxin: R_1=OH, R_2=$COCH_3$; Digitoxin: R_1=R_2=H; Digoxin: R_1=OH, R_2=H; 9. Digitoxigenin: R=H; Digoxigenin R=OH; 10. Lanatoside C.

Known amounts of aescine (1 ng/ml-200 ng/ml) were added to a plant extract pool containing 6,75 ng/ml of aescine. The recovery of aescine ranged from 89,8 % to 110,1 % with an average of 98,8 $\pm$ 9,0 % (n=12).

Within-assay coefficients of variation for 10 successive analyses of each of three different dilutions (12,1 ng/ml, 25,8 ng/ml and 81,8 ng/ml) were 5,9 %

TABLE 2

Crossreactivity of the aescine-antiserum

Name of compound	Crossreaction (%)
Aescine	100
Aescinol	99,7
Acidic hydrolysate of aescine	0,02
Total acidic and alkaline hydrolysate of aescine (63 % proaescigenin, 19 % barringtogenol C, 14 % aescigenin, 4 % barringtogenol D)	0
α - acetyldigoxin	0
Digitoxigenin	0
Digitoxin	0
Digoxigenin	0
Digoxin	0
Lanatoside C	0

The crossreactivities were determined at 50 % inhibition of the binding of labelled aescine.

(n=30), 4,2 % (n=30) and 7,6 % (n=30), respectively. Between-assay coefficients of variation for 4 analyses of three dilutions were 7,5 % (n=12), 6,9 % (n=12) and 7,5 % (n=12), respectively.

DISCUSSION

The differences in the antiserum titres (Fig. 2) are at least partly due to the different antigen doses

used for the individual rabbits. Doses of 1,00 mg may have induced tolerance in the rabbits 3 and 4.

The specificity of an immunoassay method is generally determined by the site of linkage between hapten and protein moieties in the immunogen conjugate (24). The hapten-antibody binding specificity is largely determined by the hapten functional groups most distant from the linkage site and thus highly exposed to the immune system of the animal (25). The antiserum used in the present investigation was obtained with an immunogen in which the substituents of the triterpenoid moiety of aescine remained essentially intact.

Aescinol, the main aescine metabolite in human serum and urine (10,11), crossreacted 99,7 % in this assay. Since free aescinol is not contained in the extracts of A. hippocastanum, its high crossreactivity can be overlooked when analyzing plant extracts. With a crossreaction percentage of 0,02 %, the diester aglycone proaescigenin from acidic hydrolysis of aescine does not interfere in the assay. Alkaline hydrolysis of this compound yields proaescigenin, the main constituent (63 %) of the total acidic and alkaline hydrolysate of aescine. Proaescigenin and other compounds found in the total hydrolysate, barringtogenol C (19 %), aescigenin (14 %) and barringtogenol D (4 %) have no affinity for the antibodies, because the

total hydrolysate does not crossreact in the assay. Similarly, no interference was observed for other potentially crossreacting compounds tested (Table 2).

The present RIA-method allows an easy detection of picogram amounts of aescine from unpurified extracts of Aesculus hippocastanum.

Especially when considering the analytical sensitivity required for pharmacokinetic investigations of this type of compounds, immunoanalytical methods (14, 26) should offer plenty of potential. However, the present method has only limited value for pharmacokinetic applications since aescinol as the main aescine metabolite is associated with a practically 100 % crossreactivity in the assay.

ACKNOWLEDGEMENTS

This study was supported by Dr. Madaus GmbH & Co., Cologne, FRG and Savo Foundation for Advanced Technology, Kuopio, Finland. The skilful technical assistance of Mrs Helly Rissanen is gratefully acknowledged.

REFERENCES

1. Wulff, G. and Tschesche, R. Über die Struktur der Rosskastaniensaponine (Aescin) und die Aglykone verwandter Glykoside. Tetrahedron 1969;25: 415-436.

2. Wagner, J., Sclemmer, W. and Hoffmann, H. Über Inhaltsstoffe des Rosskastaniensamens. Arzneim.-Forsch. (Drug Res.) 1970;20: 205-209.

3. Tschesche, R. and Wulff, G. Chemie und Biologie der Saponine. In: Herz, W., Griesenbach, H. and Kirby, G.W., eds. Fortschr. d. Chemie d. Organ. Naturstoffe. Vienna-New York: Springer-Verlag, 1973:462-606.

4. Diemath, H.E. Zur Behandlung und Nachbehandlung Schädel-Hirnverletzter. Wien. Klin. Wochenschr. 1981;93: Suppl. 131.

5. Felix, W. Zur ödemprotektiven Wirkung von Aescin. In: Fischer, H., ed. Chronische Veneninsuffizienz Pathogenese und medikamentöse Therapie. Ergebnisse der Angiologie. Stuttgart-New York: Schattauer-Verlag, 1984:157-164.

6. Hemmer, R. Zur Therapie des Hirnödems beim Schädel-Hirn-Trauma. Unfallchir. 1985;88: 93-96.

7. Pabst, H. and Kleine, M.-W. Prävention und Therapie von Sportverletzungen. Erfahrungen mit einem Aescin-haltigen Gel. Fortschr. Med. 1986;16: 44-48.

8. Crielaard, J.M. and Franchimont, P. Behandlung von Muskelverletzungen in der Sporttraumatologie. Lokale und orale Kombinationstherapie mit Reparil. Therapiewoche 1988;38: 2505-2509.

9. Lang, W. and Mennicke, W.H. Pharmakokinetische Untersuchungen mit tritiiertem Aescin an Maus und Ratte. Arzneim.-Forsch. (Drug Res.) 1972;22: 1928-1932.

10. Lang, W. Untersuchungen zur perkutanen Absorption von ^{3}H-Aescin bei Maus und Ratte. Arzneim.-Forsch. (Drug Res.) 1974;24: 71-76.

11. Lang, W. Studies on the percutaneous absorption of ^{3}H-aescin in pigs. Res. Exp. Med. 1977;169: 175-187.

12. Przerwa, M. and Arnold, M. Untersuchungen zur Durchlässigkeit der Haut. Arzneim.-Forsch. (Drug Res.) 1975;25: 1048-1053.

13. Lehtola, T., Huhtikangas, A. and Virtanen, R. Application of radioimmunoassay to the quantitation of hyoscyamine in plant material. Planta Medica 1982; 45: 237-239.

14. Huhtikangas, A., Lehtola, T., Lapinjoki, S. and Lounasmaa, M. Specific radioimmunoassay for vincristine. Planta Medica 1987;53: 85-87.

15. Wurzburger, R.J., Miller, R.L., Boxenbaum, H.G. and Spector, S. Radioimmunoassay of atropine in plasma. J. Pharmacol. Exp. Ther. 1977;203: 435-441.

16. Virtanen, R., Kanto, J. and Iisalo, E. Radioimmunoassay for atropine and l-hyoscyamine. Acta Pharmacol. Toxicol. 1980;47: 208-212.

17. Lounasmaa, M., Jokela,R., Huhtikangas, A., Lapinjoki, S., Heiskanen, J., Lehtola, T. and Naaranlahti, T. Synthesis of protein complexes of bisindole alkaloids from Catharanthus roseus. Heterocycles 1986;24: 1355-1360.

18. Freund, J. The effect of paraffin oil and mycobacteria on antibody formation and sensitization. Amer. J. Clin. Pathol. 1951;21: 645-657.

19. Collins, W.P., Mansfield, M.D., Alladina, N.S. and Sommerville, I.F. Radioimmunoassay of plasma testosterone. J. Ster. Biochem. 1972;3: 333-348.

20. Shaw, W., Schulman, L. and Spierto, F.W. The carrier effect of gamma globulin when a polyethyleneglycol separation technique is used in digoxin radioimmunoassay. Clin. Chem. Acta 1975;60: 385-389.

21. Lehtola, T., Lavikainen, O. and Huhtikangas, A. Radioimmunoassay of trospiumchloride, a quaternary tropane derivative. J. Immunoassay 1988;9: 297-307.

22. Scatchard, G. The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 1949;51: 660-672.

23. Nickel, S. and Staba, E. Suitability of antidigoxin antiserum for digoxin in plant extracts. Lloydia 1977;40: 230-235.

24. Nieschlag, E., Loriaux, D.L. and Lipsett, M.B. Radioligand assay for Δ^5-3 β-hydroxysteroids. Steroids 1972;19: 669-679.

25. Gross, S.J., Campbell, D.H. and Weetall, H.H. Production of antisera to steroids coupled to proteins directly through the phenolic A-ring. Immunochemistry 1968;5: 55-65.

26. Lapinjoki, S., Veräjänkorva, H., Huhtikangas, A., Lehtola, T. and Lounasmaa, M. An enzyme-linked immunosorbent assay for the antineoplastic agent vincristine. J. Immunoassay 1986;7: 113-128.