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A validation protocol for the HPTLC standardization of herbal products: Application to the determination of acteoside in leaves of *Plantago palmata* Hook. f.s.

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Abstract

Formal validation, that is the study of the analytical performances of a method, is recognized as the best safeguard against the generation and publication of data with low reliability.

Although the topic of HPTLC validations has been largely investigated, there is still a need for a general validation method applicable whenever a blank matrix cannot be reconstituted, notably herbs and their extracts.

This work proposes two validation schemes aiming at generate linearity, accuracy and precision data in a minimal number of HPTLC plates, taking the standardization of *Plantago palmata* as an example with both UV and visible (post-chromatographic derivatization with a sulphuric acid–vanillin reagent) detections. A major problem associated with HPTLC determinations is underlined, namely the low range of linearity which makes spiking studies quite difficult as care must be taken to avoid overloading, whereas keeping the analyte detectable in blank extracts and avoiding spikes too close to endogenous levels. A second problem is the use of general post-chromatographic derivatization reagents that compromise the selectivity of the method by reacting with compounds that may not be resolved from the compound of interest. The use of such reagents is clearly not without danger, especially given the relatively low resolution of planar chromatography.

In conclusion, the retained validation protocol effectively yields the main validation data whereas allowing to pinpoint major analytical drawbacks. It was not possible to simultaneously validate aucubin and acteoside assays as both analytes are present at too different levels/detectabilities.

Keywords: Analytical validation; Aucubin; Acteoside; High performance thin-layer chromatography

1. Introduction

TLC and HPTLC are methods commonly applied for the identification, the assay and the testing for purity, stability, dissolution or content uniformity of raw materials (herbal and animal extracts, fermentation mixtures, drugs and excipients) and formulated products (pharmaceuticals, cosmetics, nutriments) [1]. These flexible and cost-effective techniques present the advantage of the simultaneous processing of standards and samples with versatile detection possibilities, including a great variety of post-chromatographic derivatization reagents. The validation of analytical methods is largely recognized as the best safeguard against the generation of unreliable data and is becoming an absolute requirement in many fields. Validation is the process by which it is established, by laboratory studies, that the performance characteristics of an analytical method meet the requirements for the intended applications [2]. Depending on the objective of the analytical procedure, the typical validation characteristics which can be considered through a statistical approach are accuracy, precision, specificity or selectivity, detection limit, quantification limit, linearity and ruggedness [3].

The concept of validation applied to densitometric determinations on high-performance thin-layer chromatography (HPTLC) indeed varies according to the goal of the analysis and the steps required for a formal validation have been thoroughly investigated [1,4–8], notably for purity testing [9], pharmaceutical dosage forms assay [10] and herbals fingerprinting [11]. The

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assay validations generally rely on the spiking of analytes to a reconstituted blank matrix.

For herbals and their extracts however, blank matrixes can be prepared only for the assay of contaminants (e.g. determination of aflatoxins in wheat [12]) or of components added to the raw extract (e.g. determination of caffeine in some drinks [13]).

For the assay of compounds endogenous to the plant, there is no possibility to reconstitute a blank matrix; some authors have overcome the difficulty by validating methods using standard solutions only [13], an approach yielding however much better results than those which may be forthcoming from a real sample [1]. In fact, such a validation requires spiking of non-blank matrixes, which introduces additional difficulties to the development of the validation scheme; this situation is not unlike those encountered in clinical biology for which more or less standard validation protocols are available [14,15]. However, due to the limited number of spots applicable on a single plate, such protocols cannot be transposed as is to TLC/HPTLC analyses. To our knowledge, no investigation of the steps required for a formal validation has been published and validation schemes tend to vary significantly from author to author [16–18]; performances are difficult to compare, reported data not always allowing to deduce the exact protocol implemented, notably the total number of plates used or of spots applied.

The determination of a phenylethanoid glycoside, acteoside, and of an iridoid, aucubin, in *Plantago palmata* Hook. f. (Plantaginaceae) leaves was selected as a model for developing a validation scheme to measure in only three plates the principal parameters of validation which are linearity, accuracy and precision. Both compounds are present in the plant at different concentration levels, can be extracted with the same solvent and analyzed along the same chromatographic runs. As they can be detected directly by their absorbance in UV, but also in the visible after spraying of a general chromogenic solution, they allow to compare both types of measurements.

Plantago palmata grows in the humid mountain regions of intertropical Africa at 1800–3000 m [19–21] and its leaves have a number of applications in traditional medicine, notably in Burundi, Rwanda and South Kivu (Congo): (i) crushed leaves treat abscesses, wounds, burnings, sting bites; (ii) leaves diluted with water enhance milk secretion and treat woman sterility, abortion menace, eye infections, hemorrhoids, dysentery, gonorrhea, ascaridiasis and hepatitis; (iii) decoctions are a remedy for ascites, hypertension, malaria and stomach ache; (iv) infusions are used for the treatment of pregnancy troubles, colibacillosis and for the improvement of health after disease [21]. The compounds investigated in the present study (Fig. 1) are part of the phenylethanoid glycosides and iridoids of *Plantago*, two groups of metabolites possibly related to their traditional uses and biological activities [18].

2. Material and methods

2.1. Plant

Plantago palmata Hook. f. (Plantaginaceae) seeds were harvested in the Democratic Republic of Congo and grown in a greenhouse (Experimental Garden Jean Massart, Brussels, Belgium); leaves and roots were collected after 3 months of culture, immediately immersed in acetone, dried and powdered. The plant was identified by Professor J. Lejoly in the Laboratory of Systematical Botany and Phytosociology, Free University of Brussels (ULB), Belgium, where a voucher specimen has been deposited. From the ethanol extract of leaves (yield: 8.72% on dried weight), aucubin (0.32%), gardoside (0.005%), 8-epi-loganic acid (0.13%), arborescoside (0.005%) and acteoside (verbascoside) (0.01%) could be isolated in pure form by medium-pressure liquid chromatography, courtesy of Dr. N. Ronsted (Department of Organic Chemistry, The Technical University of Denmark) and identified by NMR spectroscopy. This qualitative composition is similar to previously published data [22].

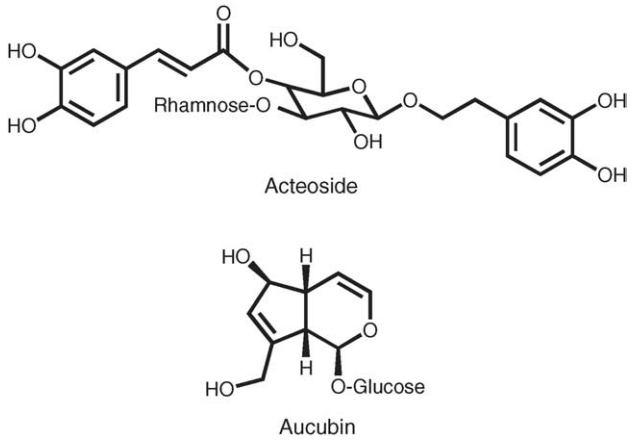

Fig. 1. Structures of acteoside and aucubin.

2.2. Chemicals

Aucubin and acteoside were obtained from Carl Roth, Karlsruhe, Germany. Silicagel 60F_{254} HPTLC plates and solvents were from Merck, Darmstadt, Germany. All other reagents were from Aldrich.

2.3. Extraction and HPTLC conditions

Forty milligram aliquots of leaves powder were spiked as indicated in Table 1 (methanolic solutions), added with methanol up to 10 ml, extracted by agitation (60 min) and centrifuged (2000 × *g*; 15 min); 10 μl of the supernatant were applied on HPTLC plates with a TLC sampler III piloted by the Wincats software 1.3.2 (Camag, Switzerland). Methanolic dilutions of a solution prepared by dissolving 3.75 mg of acteoside and aucubin in 5.0 ml methanol were similarly applied (range 0.3–1.2 and 0.15–1.05 μg/spot, respectively).

After development in ethyl acetate:water:acetic acid:formic acid (67:18:7.5:7.5) [23] and drying at 105 °C for 20 min (Camag TLC Plate Heater III), the plates were measured at 330 nm in reflectance mode with a TLC Scanner III (Wincats 1.3.2, Camag) (Fig. 2); they were then sprayed with 0.5 g vanillin dissolved in methanol:acetic acid:sulphuric acid (85:10:5), heated 20 min at