HYPERICUM THE LAST SCIENCE DISCOVERY

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Part Four

THE DEVELOPMENT AND UPDATING OF ANALYTIC METHODS

1. INTRODUCTION

In agreement with general considerations, namely that naphtodiantrones (hypericins) are the components responsible for the therapeutic activity of St. John's wort, *the official (1) and unofficial (2) analytic methods of the drug and its preparations were based on the determination of these substances until 2000.*
This is still justified as the hypericins are the most immediately accessible components from the analytical point of view and are the markers of qualities which, by their presence, guarantee the presence of other active substances (3).
However, when it was first understood, in the 1990s, that the antidepressant activity was to be attributed to the mixture of the substances forming the total extract and not only to the hypericins (see above, in Part One), analytical research also turned to methods that guaranteed recognizing and assessing the greatest number of components, that is, towards HPLC methods (High Performance Liquid Chromatography).
In Germany, the BfArM has also recently modified the rules for granting sales authorization for preparations of St. John's wort, not accepting the expression of the titre in hypericins in the informative texts (product information, illustrative leaflets, packaging) but requiring that the total hydroalcoholic extract (methanol or ethanol) is shown and the contents of which in mg, in the daily dose of the preparation must correspond, on the basis of the drug/extract ratio, to 2-4 g of drug; on the other hand, the calculation and the *declaration of the titre in hypericins as markers of standardization of the aforementioned extract* is still recognized (4).

Lastly, it should be remembered that, *for the preliminary comparison of drugs and extracts of different origin, experts prefer the fingerprints of the chromatography chromatograms on a thin layer (TLC)* to HPLC chromatograms, in which the retention times of the complex mixtures sometimes give rise to uncertainties (13). The TLC plates "allow the different samples to run in parallel side by side, comparing them" and the relative methods are described in all the pharmacopoeial monographs which discuss St. John's wort (6), cfr. (18).

2. THE METHODS FOR THE ANALYTIC TESTING OF THE DRUG AND OF EXTRACTS

2.1 THE OFFICIAL METHODS

2.1.1. THE DRUG

The pharmacopoeial quantitative analytic methods for the drug are based on the determination of St. John's wort by the colorimetric method, that is, using the red colouring of St. John's wort. The original DAC86 colorimetric method (in the 1991 modification) extracts substances deemed "inert" using Soxhlet extractor apparatus, with dichloromethane before the determination of hypericins. This treatment has been criticised because it is unreliable (2), therefore *European Pharmacopoeia* (Ph. Eur. 2002, page 1353, "Hypericum" monograph), adopting the DAC method, has eliminated the pretreatment with dichloromethane and replaced methanol by tetrahydrophurane as the extraction solvent (Swiss Pharmacopoeia VIII), as follows:
"*Sample solution*. Introduce into a 100ml round-bottomed flask 0.800 g of pulverized drug (granulometric code: 500) 60 ml of a mixture of 20 volumes of *water* and 80 volumes of *tetrahydrophurane*, and a magnetic stirrer. Boil the mixture to fall out in a bain-marie at 70°C. Centrifugate (for 2 minutes at 700g) and decant the supernatant into a 250 ml flask. Take the residue with 60ml of a mixture of 20 volumes of *water* and 80 volumes of *tetrahydrophurane* and repeat the extraction and centrifugation as stated. Evaporate the extracts until dry. Take the residue with 15ml of *methanol* in an ultrasound bath and transfer it to a 25 ml volumetric flask. Wash the 250 ml flask with *methanol*, and bring up to volume the solution of the residue in the flask. Centrifugate again and filter 10 ml of the centifugated amount with a 0.2µm syringe with a porous frit. Pour 5.0 ml of the filtered amount into a 25 ml flask and bring up to volume with *methanol*. Using methanol as the white, measure the absorbance of the sample solution at 590 nm against the white and calculate the percentage of total hypericins, expressed as hypericin, with the following expression:

$$\frac{A \times 125}{m \times 870}$$

where: 870 = specific extinction of hypericin
A = absorbance read at 590 nm
m = weight of drug in grams

The value obtained must not be below 0.08%.
This value, in the 1986 DAC (1991 modification) and in the USP 25/NF 20 (St. John's Wort and Powdered St. John's Wort monographs) which described a similar analytic method, must not be below 0.04%.
The aforementioned percentages are deemed rather cautious, as the cultivation methods would allow, today, obtaining the drug at 0.1% of naphtodiantrones with the possibility of even reaching 1.5% (5).

2.1.2 THE EXTRACT

The methods shown above can be used both to test the drug and the extracts, taking into consideration the normal and opportune modifications relative to the drug/extract ratio which are always implemented in similar assessments. Nevertheless *European Pharmacopoeia (Ph. Eur.) publishes in Pharmeuropa* (6) the following draft monograph, with a particular assay for the extract, which represents an innovation as it adopts HPLC methods.

" DEFINITION: extract produced from St. John's wort (1438).
Titre:
- *from 0.2 to 0.3 percent of total hypericins*, made up of the sum of hypericin ($C_{30}H_{16}O_8$; M_r 504.5) and pseudohypericin ($C_{30}H_{16}O_9$; M_r 520,5) (anhydrous extract)
- *from…to…percent of hyperforin*

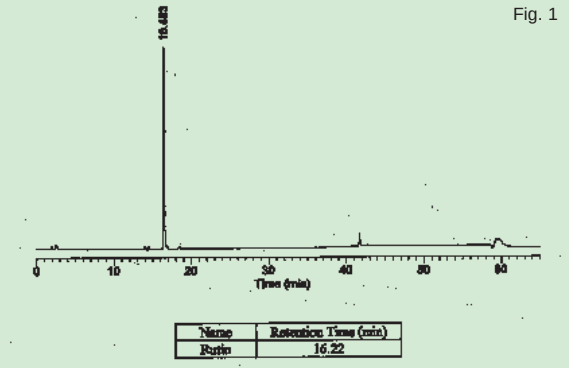
PREPARATION
The extract is produced with ethanol 70 percent V/V, operating in such a way that all the concentration and drying operations are carried out in a vacuum.
QUANTITATIVE ANALYSIS
(Assay)
Liquid chromatograph
Sample solution. In a graduated 25 ml flask, introduce 25.0 mg of the extract under examination and add about 10 ml of a mixture of 1 volume of *water R* and 9 volumes of *methanol R*. Treat with ultrasounds, dilute to 25 ml with the same mixture of solvents and mix. Filter the solution through a membrane filter with a porosity of 0.45 mm.
Standard solution. Dissolve an accurately weighed quantity of *rutin R* in *methanol R* in order to obtain a solution with a known concentration of about 0.025 mg/ml.
Precolumn: 0.5 mm filter
Column:
- *dimensions :* length = 0.25 m, diameter = 4.6 mm

Stationary phase: octadeylsilica gel for chromatograph (5 mm, 30 nm)
Temperature: 30°C
Mobile phase:
- *mobile phase A:* dilute 3 ml of *phosphoric acid R* at 1000 ml with *water R*
- *mobile phase B: acetonitrile for chromatograph R*
- *mobile phase C: methanol R.*

Flow rate: 1.0 ml/min
Revelation: spectrophotometer at 270 nm
Injection: 20 ml; inject the reference solution (fig. 1) and the sample solution (fig. 2)
Relative retention with respect to rutin (the retention time of which is about 16.17 min): pseudohypericin = about 2.53; hypericin = 2.68; hyperforin = 2.85
Reliability of system:
reference solution:
- *number of theoretic plates:* minimum 80,000 calculated for the peak of rutin
- *symmetry factor:* maximum 2.0 for the peak of rutin
- *repeatability*: maximum relative standard deviation: 2 percent after 5 injections

Time (min)	Mobile phase A (percento V/V)	Mobile phase B (percento V/V)	Mobile phase C (percento V/V)
0	100	0	0
0-10	100 → 85	0 → 15	0
10-30	85 → 70	15 → 20	0 → 10
30-40	70 → 10	20 → 75	10 → 15
40-55	10 → 5	75 → 80	15
55-56	5 → 100	80 → 0	15 → 0
56-65	100	0	0

Fig. 1



Chromatogram of the reference solution

Calculate the percentage of hypericin with the expression:

$$25x \frac{C \times P}{m \times 1{,}61} \times \frac{r_a}{r_s}$$

C = concentration of rutin in milligrams per millilitre, in the reference solution
P = known percentage of rutin
1,61 = response factor of hypericin relative to that of rutin
m = quantity of extract, in milligrams, in the sample solution
r_a = area or height of peak of hypericin in the chromatogram of the sample solution
r_s = area or height of the peak of rutin in the chromatogram of the reference solution

Calculate the percentage of pseudohypericin with the expression:

$$25x \frac{C \times P}{m \times 1{,}61} \times \frac{r_b}{r_s}$$

1.55 = response factor of pseudohypericin relative to that of rutin
r_b = area or height of the peak of hypericin in the chromatogram of the sample solution

Calculate the percentage of hyperforin ($C_{35}H_{52}O_4$) with the expression:

$$25x \frac{C \times P}{m \times 1{,}61} \times \frac{r_c}{r_s}$$

0.57 = response factor of pseudohypericin relative to that of rutin
r_c = area or height of the peak of hyperforin in the chromatogram of the sample solution

The above draft monograph does not yet show the percentage content of hyperforin, for comprehensible reasons, including the particular oxidative alterability of the substance and also because this content is closely linked with the period when the drug is collected (see the table in Part One) which in the relative monograph "Hyperici herba" of European Pharmacopoeia (1) is only defined: "during flowering".
A test of the percentages of hyperforin in commercial products (7) has shown contents of between 2.48% and 4.13% of the dry extract.
See also above, in Part Three under point 2.4 "The search fir the compound responsible for interactions".

2.2 UNOFFICIAL METHODS

2.2.1 THE DRUG

The first method of HPLC analysis of the drug was published in 1987 by Hölzl and Ostrowski (10), with remarkable results, and had the merit of acting as a basis for various other HPLC methods, mainly developed in Eastern Europe (11), (12), where St. John's wort was widely cultivated.
This method highlighted, amongst other things, the possibility of accurately determining the percentages of hyperforin in the drug and the difficulties of revealing hypericins which show very long retention times in contrast with their polarity in TLC. These problems still arise with the more updated HPLC methods (see above the European Pharmacopoeia method under point 2.1.2) if the analytical conditions described are not exactly followed.

Regarding the determination of the titre in "total hypericins", a criticism of the DAC analytic method 1986/91 for diantrones (point 2.1.1) comes from the German Consortium of Extract Producers (2) who deem the percentage limit of 0.04% too low, also observing that the protocompounds protohypericin and protopseudohypericin (fig. 3) cannot be determined if they are not transformed into hypericin and respectively Pseudohypericin by exposure to the light (8). They therefore deem it necessary to submit the analytic extractive solutions to artificial light for half an hour or to daylight for 4-5 hours, and therefore to submit them to the quantitative determination that is proposed with the HPLC method prepared for the purpose [(2), cfr. also (20)]; in this way the mixture of diantrones is transformed into what is analytically defined "total hypericins", that is, in the sum of the protocompounds and hypericins. A comparison, on the various samples of drugs, of the DAC method with the HPLC method (after exposure to the light) gives a ratio of the DAC/HPLC analytical results equal to 0.9, that is, HPLC values would be 10% higher.

Nr.	Substance	Retention time (min)
1	Isomer of chlorogenic acid	9,27
2	Chlorogenic acid	
3	Rutin	16,17
4	Hyperoxide	16,52
5	Isoquercitrin	16,92
6	Quercitrin	19,70
7	Quercetin	28,20
8	I3,II8 biapigenin	36,00
9	Pseudohypericin	40,93
10	Hypericin	43,37
11	Hyperforin	46,07
12	Adhyperforin	46,65

2.2.2 THE EXTRACTS

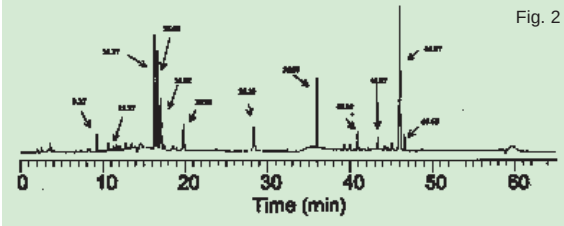
Comparing the analytical results obtained with the above HPLC method (2) for the extracts, with the DAC ones, the latter are on average higher by 30% because the liquid chromatograph succeeds in separating "aspecific inert substances, present in the extract, which are involved in the quantitative spectrophotometric determination, increasing their values". The ratio of the DAC/HPLC analytical values for the extracts increases to 1.30.
The HPLC (2) method, proposed for the drug and extracts by the Consortium of the German Producers, does not appear to have been adopted yet by any Pharmacopoeia.

3. TESTING THE BIOAVAILABILITY OF THE PREPARATIONS OF ST. JOHN'S WORT (7)

3.1 STANDARD PARAMETERS FOR TESTING BIOAVAILABILITY

As phytodrugs, like synthetic drugs, must correspond to characteristics of activity, safety and quality required by pharmaceutical legislation, the criteria of determination of bioavailability, where possible, also apply.

Fig. 2



Chromatogram of the champion solution

In the case of plant extracts, European Pharmacopoeia has presented a draft monograph (14), (which should be published in the First Supplement to the 4th edition 2002) in which they are divided into three categories, thus defined:
a) "standardized plant extracts": when the active ingredient is known
b) "quantified plant extracts": when the active ingredients are partially known
c) "other plant extracts" when the active ingredients are not known.
The extract of St. John's wort, according to present knowledge, is attributed to category b) considering hyperforin as its representative constituent.
The test of the bioavailability of a preparation in solid form (e.g. tablets, capsules, pills) is by the dissolution test (15) which, according to the EMEA standards (European Medicine Evaluation Agency) (16) is not compulsory for the extracts in category b); it provides valid indications on the time the active ingredients remain in the enteric tract where they are absorbed.
For synthetic drugs with rapid dissolution, 80% of the active ingredient has to be released in 20-30 minutes whilst, for phytodrugs, the release of 80% of the total extract or of its presumed active ingredient in 60 minutes is considered acceptable (17).
The authors of the following test (7), considering hyperforin and rutin as lipophile substances, which are hydrosoluble with difficulty and

estimating an average intestinal transit time of 4 hours, consider a release of 80% of hyperforin and rutin, contained in the preparation, in 120 minutes as valid.

3.2 EXPERIMENTAL PARAMETERS OF THE DISSOLUTION TEST OF HYPERFORIN (7) AND RUTIN (8)

Based on what is stated in Parts One and Three, hyperforin and rutin appear the two compounds, belonging to different chemical classes (phloroglucines and flavonoids) responsible for the therapeutic activity of St. John's wort extract. The release tests described (7), (8) were therefore based on these substances.

3.2.1 APPARATUS AND OPERATING CONDITIONS

Apparatus PADDLE, USP apparatus 2 (18); temperature: 37 ± 5°C; total dissolution media used: 500 ml in a closed recipient; rpm of stirrer: 100 rpm. Analytic samples of 5ml each time with syringe, at pre-determined times, and filtration through Teflon membranes with pores of 0.45 mm discarding the first 2 ml. Triple dissolution tests (one sample per container) carried out away from the light to avoid degradation of the active ingredient.
Dissolution media. The Fed State Simulated Intestinal Fluid (FeSSIF) containing 15 mmol/l of sodium taurocolate (NaTC) and 3.75 mmol/l of lecithin with pH 5.00 and osmolality of 670 mosm/kg, was found suitable for both substances and prepared as follows:
FeSSIF tampon: bring 8.08 g of NaOH , 17.3 g of glacial acetic acid, 30.4 g of potassium chloride to 2 litres with demineralized water and to pH 5,00 with NaOH 1N.
Dissolve in 450 ml of buffer 16.5 g of NaTC, add 59.08 ml of a solution of lecithin at 10% in chloroform (turbid emulsion), dispel the chloroform in Rotavapor at 40°C and bring to 2 litres with FeSSIF buffer at Ambient Temperature.

Preparation of sample. 10 doses of preparation are ground in an analytic mill and the quantity corresponding to one dose, introduced into a 50 ml flask, brought to the volume with methanol and subjected to ultrasounds for 10 mins. For the analysis, the contents of a flask was poured into a beaker and filtered in two dark glass HPLC vials, by a syringe with a 0,45 mm membrane filter.

3.2.2 METHOD OF HPLC DETERMINATION

Column. Merck LiChrospher 100 RP-8, 125 x 4 mm, 5 mm.
Eluant. Buffer phosphate mixture pH 2.1/ acetonitrile (without indication of ratios), with isocratic flow of 1 ml/min, at AT.
Injection. *20* ml of sample solution
Revelation. 270 nm
External standard. Hyperforin, dicyclohexylammonium salt; rutin

3.2.3 THE RESULTS

The results obtained with 4 of the best-known German commercial preparation are rather surprising, as the following Table 2 shows.

4. TEST OF STABILITY OF HYPERFORIN IN EXTRACTS AND PREPARATIONS (8)

Hyperforin is a photosensitive and oxidable compound, for which the rules of good production require that it keeps a titre equal to ± 10% of the declared value, for the whole period of stability contemplated.

The commercial products were kept in normal conditions of temperature, humidity and away from the light. Their titre in hyperforin (method 3.2.2., see above) was determined at the start (point 0) and after three, six and nine months.

5. CONCLUSIONS

The analytic test of the drug, extracts and preparations of St. John's wort is always based, both in the official methods and in literature, on the determination of the so-called "total hypericin" which represents hypericin and pseudohypericin together (see formulas in fig. 1 of Part One). After the recent evaluation according to which the anti-depressant activity is due rather to the overall action of the real components of the extract, with particular importance of hyperforin and some flavonoids (rutin), the titre in hypericins is given only a meaning of marker, whilst HPLC techniques are being developed, capable of quantifying the various components separately. This is also confirmed by the BfArM which, for the commercial preparations of St. John's wort, excludes the titre in hypericins as the expression of the contents in the active ingredient. Although the method described in USP 25 of 2001 still shows only an assay for hypericins, the European Pharmacopoeia first described a draft HPLC analysis for the ethanolic extract of St. John's wort in Pharmeuropa 2001.
An assay of bioavailability of commercial preparations can be made through the dissolution test for solid dosage forms, determining the release of hyperforin and rutin as compounds of reference.
The determination of the absence of degradation of hyperforin in commercial preparations, during the full period of stability as declared, is also recommendable.

Table 2. Percentage release of hyperforin and rutin in the dissolution test carried out in the conditions described

Minutes	Percentage release								
	sample 1		sample 2		sample 3		sample 4		
	hyperforin	rutin	hyperforin	rutin	hyperforin	rutin	hyperforin	rutin
30	0	0	30	30	60	100	10	40
60	0	0	35	50	80	100	20	55
120	10	20	60	85	90	100	32	75
180	18	30	72	100	96	100	42	80
240	20	45	85	100	100	100	45	82

The release of rutin always appears favoured compared to that of hyperforin.

6. LITERATURE AND NOTES

(1) To mention some of the commonest: DAC 1986, 3° Suppl. 1991, pag. 1
European Pharmacopoeia 4, (2001) pag. 1353
Pharmacopoeia Helvetica VIII
USP 25/NF 20 (2001), pag. 2611

(2) Gaedcke F., "Johanniskraut und dessen Zubereitungen", D.A.Z. **137,** 117 (1997)
The HPLC method, shown in the publication, is described as follows: "Stationary phase: RP-18, 125-4, 5 mm (e.g. Chromasil from MZ-Analytical); mobile phase: pH 2.1 buffer (NaH_2PO_4/H_3PO_4), methanol, ethyl acetate (618.4 g : 1893.4 g : 526.0 g); flow: 1.0 ml/min isocratic; temperature: 40°C; revelation: VIS 590 nm; injected volume: 10 ml; integration time: 10 min. Calibration against hypericin as external standard.
The artificial lighting was created by holding the containers of the analytic solutions at a distance of approximately 5 cm under a 36 W Lumilux Osram bulb for daylight, for 30 minutes

(3) Schilcher H. and Kammerer S. "Johanniskraut (Hyperici herba)" in Leitfaden Phytotherapie , Urban & Fischer Verlag, Munich 2000

(4) Dingermann T. (editor) et al., "Transparenzkriterien für pflanzliche, homöopathische und anthroposophische Arzneimittel", Karger Verlag, Basle, 2000 pag. 10.

(5) Hannig H-J. et al., "Erfahrungen beim grossflächigen Anbau von Johanniskraut – Anforderungen an die industrielle Verwertung" , Herba Germanica, **3,** 96 (1995)

(6) Anonymous, "St. John's Wort Dry Extract (Hyperici herba extractum siccum)", Pharmeuropa, vol. 13, No. 1, January 2001, pag. 226
"*Identification*: thin layer chromatography.
Sample solution. Disperse 0.25 g in 5 ml of methanol.
Standard solution. Dissolve 5 mg of rutin and 5 mg of hyperoxide in methanol and dilute to 5 ml with the same solvent.
Plate. Silica gel for TLC
Mobile phase. anhydrous formic acid, water, ethyl acetate (6:9:90 V/V/V)
Deposit on plate. 10 ml of sample solution and 5 ml of reference solution, in 10 mm strip
Run. 10 cm
Drying of plate. At 100°C - 105°C for 10 minutes
Revelation. Spray the plate with a solution of amino ethyl of diphenylboric acid at 10 g/l in methanol and then with a solution of Macrogol 400 at 50 g/l in methanol. Observe the plate After approximately 30 minutes under 365 nm. UV light.
Results. See table; other fluorescent areas may appear in the chromatogram of the sample solution

Upper end of plate	
	yellow-orange fluorescent area
	2 red fluorescent areas (hypericin and pseudohypericin)
	yellow-orange fluorescent area
	yellow-orange fluorescent area
	yellow orange fluorescent area
yellow-orange fluorescent area (hyperoxide)	yellow-orange fluorescent area (hyperoxide)
	yellow and blue areas, possible overlapping
yellow-orange fluorescent area	yellow-orange fluorescent area (rutin)
standard solution	**sample solution**

(7) Westerhoff K. et al., "Johanniskrautextraktpräparate" , D.A.Z. **142**, No. 3, 55 (2002)
This paper was then completed and incorporated in the following, extended also to biological tests.

(8) Wurglics M. et al., "Aktuelle Johanniskrautforschung", D.A.Z. 142, No. 10,
67 (2002). The classification of the extracts, according to European Pharmacopoeia, was published in full by the head of the project, Gerhard Franz in D.A.Z. **142**, No. 14, 47 (2002)
and will be part of a Supplement to the 4th Edition, to be published by the end of 2002.

(9) Cyclopseudohypericin, present only in traces, is not modified

(10) Hölzl J. and Ostrowski E., "Johanniskraut (Hypericum perforatum L.). HPLC-Analyse der wichtigen Inhaltsstoffe und deren Variabilität in einer Population", D.A.Z. **127**, 1227 (1987)

(11) Bombardelli E., Morazzoni P. "Hypericum perforatum" Fitoterapia, **66,** 43 (1995)

(12) Takel'ovà D. et al. "Quantitative Changes of Dianthrones Hyperforin and Flavonoids content in the Flower Ontogenesis of Hypericum perforatum", Planta Medica, **66**, 778 (200)

(13) Adam P.K. and Becker H., "Analytik biogener Arzneistoffe" Volume 4 in the Pharmazeutische Biologie series. WVG, Stuttgart 2000, pag. 143

(14) Lang F. and Stumpf H., "Considerations on future pharmacopoeial monographs for plant extracts" Pharmeuropa **11,** 268 (1999)

(15) Anonymous, "Dissolution Test for Solid Dosage Forms" European Pharmacopoeia 4, 194 (2002)

(16) Anonymous, "Test procedures and acceptance criteria for herbal drugs, herbal drug preparations and herbal medicinal products" EMEA/CVMP/815/500

(17) Tittel G., "Qualitätskontrolle von Phytopharmaka", Pharm. Ind. **59**, No. 11, 1002 (1997)

(18) Wagner H. and Bladt S., "Plant Drug Analysis", Springer Verlag, Berlin, 2nd Edition (1996)
pag. 70

(19) The Paddle apparatus is described in European Pharmacopoeia 4, (2001) pag. 194 "Dissolution Test for Solid Dosage Forms",

(20) Bilia A.R. et al., "Evaluation of the photo and thermal stability by RP-HPLC of commercial sample of dried extract of Hypericum perforatum" Joint Meeting "2000 Years of Natural Products Research", Amsterdam 26th-30th July 1999