Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 951–957

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Evaluation of quality control strategies in *Scutellaria* herbal medicines

Susanne P. Boyle*, Paul J. Doolan, Clare E. Andrews, Raymond G. Reid

School of Pharmacy and Life Sciences, Robert Gordon University, Aberdeen, AB10 1FR, United Kingdom

ARTICLE INFO

Article history:
Received 15 May 2010
Received in revised form
11 November 2010
Accepted 13 November 2010
Available online 21 November 2010

Keywords:
Scutellaria
Herbal medicines
Quality control
Antioxidant coefficient
Chromatographic fingerprint

ABSTRACT

The statutory regulation of herbal medicines is under review within the United Kingdom (UK) and by 2011 all herbal medicines will require either a Product Licence or a Traditional Herbal Registration. The species *Scutellaria baicalensis* has been shown to possess anti-inflammatory, anti-viral and anti-tumor properties and is one of the most widely used Chinese herbal extracts in Eastern and Western medicines. The bioactivity of this herbal medicine is due to the radical scavenging activities of the flavone components of which there are more than 60.

This research has characterised 5 key flavones in 18 extracts of *Scutellaria* using a combination of HPLC with DAD and MS detection. Employing an internal standard approach, the validated HPLC method afforded good sensitivity and excellent assay precision. Assays for the ferric reducing antioxidant power (FRAP) and total phenol determinations enabled determination of the antioxidant coefficient (PAC) of each *Scutellaria* extract.

The potential usefulness of employing multivariate statistical analysis using a combination of the key parameters collected namely, FRAP activity, total phenol content, levels of 5 flavone biomarkers and the PAC as a means of quality evaluation of the *Scutellaria* herbal extracts was investigated. The PAC value was predicted by soft independent modelling of class analogy (SIMCA) as being the most discriminatory parameter and applying this ranking the herbal extracts were grouped into 3 clusters. The second most influential parameter in determining the clustering of the samples was the level of baicalin in each extract.

It is proposed that the PAC value alone or in combination with a chromatographic fingerprint of key biomarkers [e.g. baicalin or (baicalin + baicalein)] may be useful indicators to adopt for the quality control of *S. baicalensis*.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Ancient traditional Chinese Medicine relies on the theory of yin-yang balance in order to diagnose and treat diseases. Popular for more than 2000 years it has been proposed recently that yin-yang balance is in fact the antioxidation–oxidation balance [1].

The genus *Scutellaria* consists of over 350 species worldwide. The species *Scutellaria baicalensis* is one of the most widely used Chinese herbal medicines in Eastern and Western medicines and has the potential for commercial production [2,3]. Chinese medical herbalists use *S. baicalensis* (Skullcap) by decoction (boiling plant material) or as tinctures. Skull cap has been used for the relief of fever [4], in the treatment of viral and bacterial infections, as an anti-cancer agent [2], for inflammatory disorders and in the treatment of hepatitis [5].

Flavonoids have been shown to be one of the principal components contributing to the bioactivity of Skull cap [4]. Flavonoids are low molecular weight secondary plant phenolics [6] of which there are 12 different sub-classes defined by variations around the heterocyclic C-ring (Fig. 1). More than 8000 different flavonoid compounds exist many of which possess strong antioxidant activity [1]. The antioxidant effects may be due to metal chelating activities resulting in an inhibition of lipid peroxidation or direct radical scavenging activities. The radical scavenging potential of a compound is related to its reducing capacity (proton donor potential) which in the case of flavonoids is primarily dependent on the number and location of B ring hydroxyls.

Structure–activity relationships indicate that the significant structural elements contributing to the antioxidant properties of flavonoids include the presence of 2,3 unsaturation in conjugation with a 4-oxo group in the C ring, a 5 hydroxy group in the A ring and the number and orientation of hydroxyl groups in the B ring [7,8].

More than 60 flavonoids have been identified from different sources of *Scutellaria*. The principal flavonoid components contributing to the bioactivity of Skull cap have been reported as: baicalin, baicalein, wogonin, wogonoside, apigenin and chrysin [3]. Wogonin, baicalein and baicalin possess the 2,3-unsaturation and the 4-oxo in the C-ring and 5-hydroxyl group in the A ring (Fig. 2).

* Corresponding author. Tel.: +44 (0)1224 262529; fax: +44 (0)1224 262555.
E-mail address: crovie38@yahoo.co.uk (S.P. Boyle).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.11.019

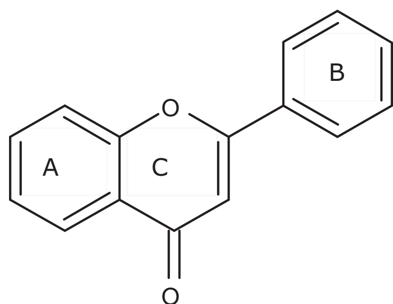
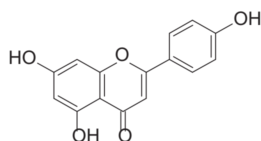


Fig. 1. Basic flavonoid skeleton.

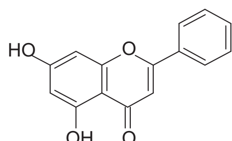
In a comparative study examining the antioxidant activity of 20 plant extracts with reported anti-inflammatory properties, *S. baicalensis* was one of the most potent inhibitors of radical induced and enzymatic lipid peroxidation [9]. The flavones, wogonin, baicalein and baicalin have significant anti-inflammatory effects which are due in part to the down-regulation of a number of inflammation-associated genes such as lipoxygenases, cyclo-oxygenases and inducible nitric oxide synthase [10,11]. The increasing reports of efficacy of such herbal compounds coupled with the general public's desire for natural medicinal products has driven a review of both quality evaluation procedures and the licensing of practitioners of herbal medicine.

In the UK, the quality control of herbal medicines is regulated under Section 12(1) of the Medicines Act 1968 and the EU Directive for Traditional Herbal Medicinal Products (2004/24/EC). The UK Medicines and Healthcare Products Regulatory Agency (MHRA) [12] is considering reform of Section 12(1) to introduce statutory regulation of herbal medicines and the Register of Chinese Herbal Medicine, which represents more that 450 practitioners, has noted "the urgent need for the statutory regulation of herbal medicine in the UK" [13]. Given the importance of the flavone components of *Scutellaria* on the anti-inflammatory and anti-cancer properties reported it seemed reasonable to consider the flavone content and/or associated bioactivity of *Scutellaria* extracts as potential parameters for quality control (QC) testing.

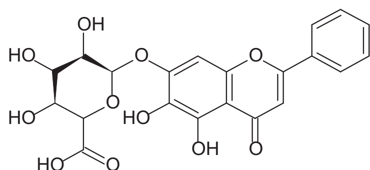
The aim of this research was to examine the potential of the FRAP antioxidant capacity assay in parallel with chromatographic fingerprinting techniques as a quality evaluation measure for herbal compounds with reported antioxidant activities. The research focussed primarily on identifying potential QC indicators for extracts of *S. baicalensis* with a single sample of *Scutellaria amoena* being included for genus comparison.



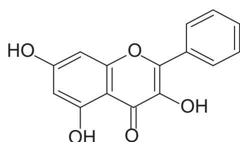
Apigenin



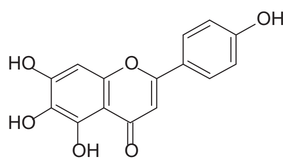
Chrysin



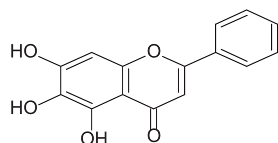
Baicalin



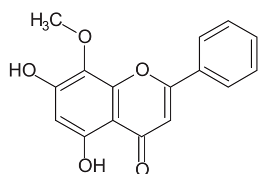
Galangin



Scutellarein



Baicalein



Wogonin

Fig. 2. Principal constituents reported for *Scutellaria baicalensis*.

2. Materials and methods

2.1. Chemicals

Scutellarein, baicalin, and wogonin were purchased from Apin Chemicals Ltd (UK) and guaranteed ≥99% purity by HPLC assay. Baicalein and chrysin was purchased from Apin Chemicals Ltd (UK) with guaranteed purity of ≥95% and 90% respectively by HPLC assay. No further confirmation of purity was necessary. Acetic acid, hydrochloric acid, Folin & Ciocalteu's phenol reagent and sodium carbonate anhydrous were purchased from Fisher Scientific (UK). Ferric chloride, ferrous sulphate and sodium acetate-3-hydrate were purchased from BDH (Dorset). 2,4,6-tripyridyl-s-triazine (TPTZ) was purchased from FLUKA. Gallic acid was purchased from Sigma–Aldrich. All solvents employed for chromatographic analysis were of HPLC grade.

The *S. baicalensis* samples ($n = 17$) and one sample of *S. amoena* were obtained commercially from herbal practitioner outlets in Thailand (Bangkok, Khon Kaen), Singapore and the UK (London, Aberdeen, Glasgow and Edinburgh).

2.2. Instrumentation

HPLC analysis was performed on a Shimadzu SPD-M20A Prominence quaternary HPLC system with an SIL-20AC Prominence auto-sampler, a Prominence CTO-20AC column oven, a continuous vacuum degasser, combined with an on-line Shimadzu SPD-20A Prominence diode array detector.

LC–MS analysis employed an Agilent 1100/1200 series LC system with autosampler, on-line diode array detection and a 6130 Quadrupole MS with an API-ES interface.

UV spectrometric determinations employed a Shimadzu UV-160A spectrophotometer.

2.3. Chromatographic conditions for LC–DAD analysis

Separation of the flavonoid reference standard mix and *Scutellaria* sample extracts employed a Zorbax SB C18 (5 μM) column

(150×4.6 mm i.d.) maintained in a column oven at 30 °C. A gradient analysis was employed utilising (A) 0.1% formic acid in water and (B) methanol at a flow rate of 1 ml/min and a total analysis time of 45 min. A brief isocratic phase (10% B) was followed by a linear gradient to 40% B over 30 min followed by a column wash stage and then re-equilibration to the starting conditions. Detection was by diode array analysis with a monitoring wavelength of 278 nm and scans collected over the range of 200–450 nm both at 2 nm bandwidth.

2.4. Chromatographic conditions for LC–MS analysis

The mobile phase and gradient conditions employed for LC–DAD analysis (Section 2.3) were utilised for LC–MS determinations. The MS spray chamber was set up with a gas temperature of 350 °C, drying gas at a rate of 13 L/min, nebuliser pressure of 60 psig, quad temperature of 300 °C and VCap of 3000 V for positive and negative ionisation modes. The API ionisation mode was employed with a 70 V fragmentor voltage and positive and negative scans collected over the mass range of 60–500 amu.

2.5. Redox reaction assay of Scutellaria extracts

The ferric reducing antioxidant power (FRAP) assay was performed according to the method of Benzie and Strain [14]. Assay reagents included 10 mmol/L TPTZ in 40 mmol/L HCl, 20 mmol/L aqueous $FeCl_3{\cdot}6H_2O$ and 300 mmol acetate buffer (pH 3.6). Working FRAP reagent was prepared by mixing acetate buffer, TPTZ solution and $FeCl_3{\cdot}6H_2O$ in the proportions 25:2.4:2.5, by vol. The time to maximal response for each sample under investigation was determined and then triplicate determinations were performed. The FRAP reactivity was determined by reference to a ferrous sulphate calibration line.

2.6. Total phenol determination

The total phenol content of the *S. baicalensis* samples was determined by reference to a gallic acid calibration line and expressed as gallic acid equivalents (GAE) according to the method of Singleton et al. [15]. Assay reagents included 10% (v/v) Folin–Ciocalteu, 7.5% sodium carbonate and 150 μg/ml gallic acid.

2.7. Derivation of antioxidant coefficient

The antioxidant coefficient (PAC) calculated as the ratio of FRAP value to total phenol content enabled a facile comparison of antioxidant efficiency of the various *Scutellaria* samples.

2.8. Reference standards and Scutellaria extracts

A standard mix containing scutellarein, baicalin, baicalein, wogonin and chrysin was prepared in methanol and diluted with HPLC grade water to yield the working solution.

Sample extracts for HPLC and spectrometric analysis were prepared using a modification of Horvath et al. [3]. Samples were weighed in triplicate (0.1 g) and dissolved by sonication (60 min) in methanol:water:formic acid (72:29:1). The sample was allowed to cool and filtered and then 0.4 ml filtrate and 0.2 ml of IS standard solution was diluted to 10 ml final volume with extraction solvent.

2.9. Validation of HPLC assay

The HPLC assay was validated in accordance with FDA guidelines for Validation of Analytical Procedures (1996) [16] and key parameters addressed included linearity of assay, precision and sensitivity. The linearity of HPLC assay was determined over the range 0–2 μg/ml for wogonin, 0–6 μg/ml for chrysin and 0–4 μg/ml for scutellarein, baicalin and baicalein monohydrate. The internal standard was included at a concentration of 40 μg/ml and regression lines of peak area ratio versus concentration of each flavone established.

2.10. Multivariate statistical analysis as a predictor of antioxidant capacity

The potential usefulness of multivariate statistical analysis to differentiate between the herbal extracts was investigated using Pirouette Version 4.0 software (supplied by Infometrics Seattle, USA). A spreadsheet which collated the concentrations of individual components from the herbal extracts together with the FRAP, total phenol determinations and PAC values was created and the results were firstly subjected to hierarchical cluster analysis (HCA) to test for clusters. With clusters being formed then the data was subjected to principal component analysis (PCA). PCA is a pattern recognition technique in which uncorrelated variables are grouped into sets and are built as simple linear combinations of the original variables. Pre-processing options which take account of the magnitude of scale were fully investigated using PCA and soft independent modelling of class analogy (SIMCA). SIMCA is a combination of HCA and PCA which is used to create a model for the shape and position in space from the data which can then be used for prediction of unknown samples [17,18].

3. Results

3.1. Chromatographic separation of the standard mix

Employing the optimised gradient conditions, separation of the five flavonoid reference standards and the internal standard was achieved with a 45 min analysis time (Fig. 3). The order of elution of the reference standards was baicalin (16.52 min), scutellarein (17.05 min), baicalein monohydrate (23.32 min), wogonin (29.54 min) and chrysin (30.15 min). The internal standard, 3 hydroxyflavone, eluted at a retention time of 36.89 min. The repeatability of analysis for all 6 analytes was determined across 8 days and found to be quite precise with %RSD of <0.5 for all analytes.

Quantification of baicalin, scutellarein, baicalein, wogonin and chrysin was performed by internal standard least squares regression with at least five calibration concentrations being determined. Lines of regression were established on a daily basis with the correlation coefficients determined for each flavone typically being $\geq$0.99. The inter-day precision of linearity was investigated and the variation in assay response was low, typically less than 2.5% RSD.

3.2. Limits of detection (LOD)

The limits of detection (S/N of 3:1) for the 5 key biomarkers were determined by serial dilution of the calibration standards and were found to be baicalin (0.8 ng/ml), scutellarein (8.0 ng/ml), baicalein (10.4 ng/ml), wogonin (2.4 ng/ml) and chrysin (7.0 ng/ml).

3.3. Characterisation of principal components in S. baicalensis

HPLC analysis of each of the *S. baicalensis* sample extracts produced a profile of peaks and where possible peak identity was determined by consideration of 3 factors, namely comparison of retention time with that observed for the reference compound, similarity of the on-line diode array spectra to that of the reference compound and consideration of the mass spectra of each HPLC peak. A typical HPLC–DAD separation is illustrated in Fig. 4. Identification of the flavones in each herbal extract was achieved by

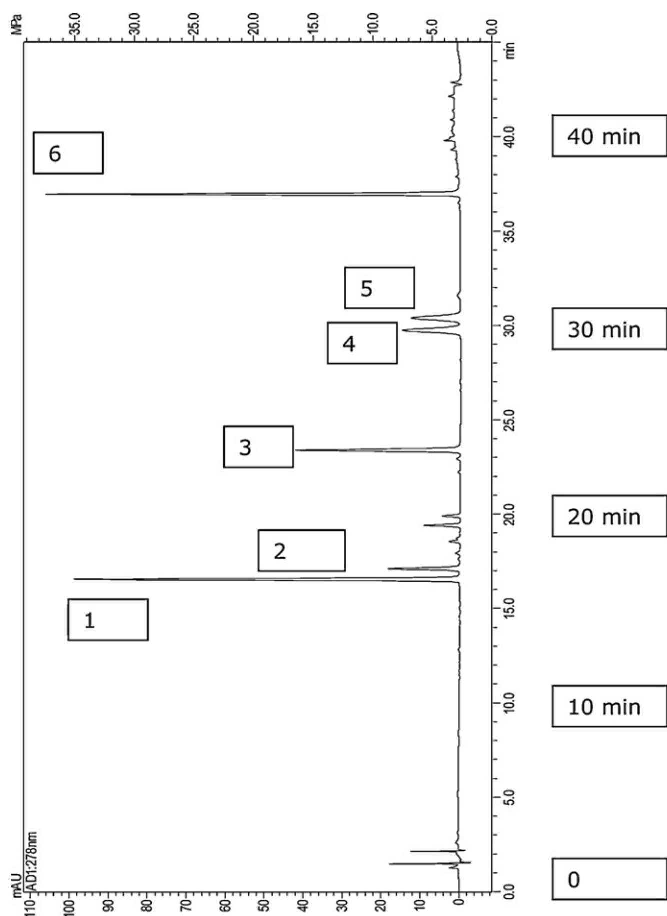


Fig. 3. Chromatographic separation of flavone standard mix. Chromatogram illustrating resolution of standard mix employing mobile phase conditions indicated in Section 2 and on-line diode array detection with a monitoring λ of 278 nm. Key: 1 = Baicalin (t_R = 16.52 min), 2 = Scutellaria (t_R = 17.05 min), 3 = Baicalein (t_R = 23.32 min), 4 = Wogonin (t_R = 29.54 min), 5 = Chrysin (t_R = 30.15) and 6 = Internal Standard (t_R = 36.89).

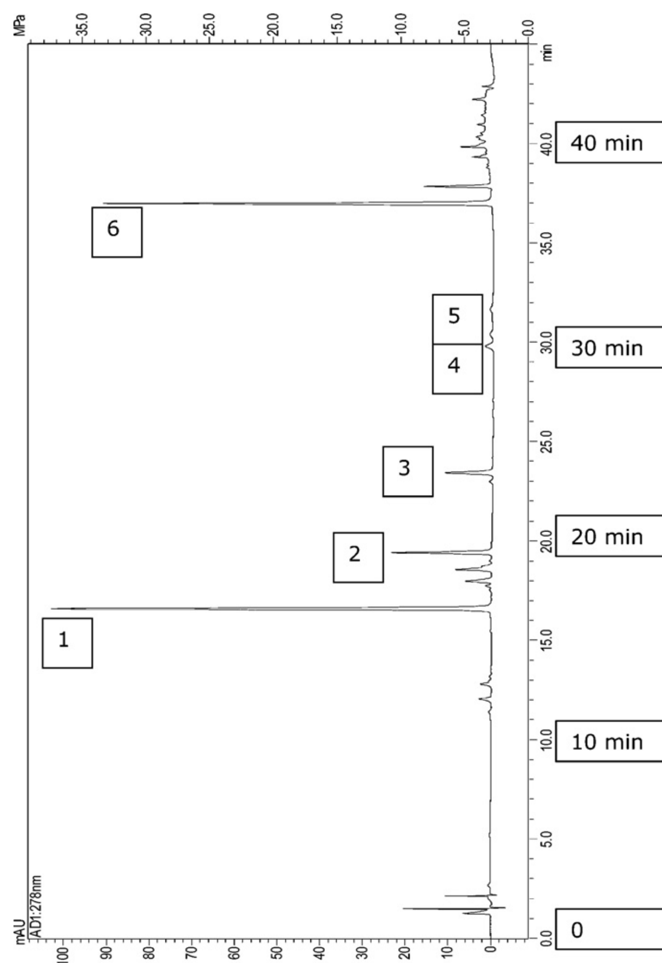


Fig. 4. Chromatographic separation of authentic sample of *Scutellaria baicalensis*. Chromatogram illustrating resolution of an extract of *Scutellaria baicalensis* sample 1 employing mobile phase conditions indicated in Section 2 and on-line diode array detection with a monitoring λ of 278 nm. Key: 1 = Baicalin, 2 = Scutellaria, 3 = Baicalein and 4 = Wogonin, 5 = Chrysin and 6 = Internal Standard.

comparison of retention times, UV–vis and mass spectral characteristics to those of commercially purchased reference standards.

Baicalin was the principal HPLC peak and was detected in each of the *S. baicalensis* extracts analysed. Typically it had a t_R of 16.52 min with a λ maxima of 283 nm, a shoulder at 320 nm and a $[M+H^+]$ at 447 on the mass spectrum. The HPLC peak with a retention time of 17.05 min, had a λ maxima of 335 nm and a shoulder at 286 nm and the mass spectrum showed a $[M+H^+]$ at 287 all characteristics consistent with scutellarein. Similarly, baicalein (t_R = 23.3 min) which was detected in all samples had a λ maxima of 275 nm and a shoulder at 325 nm and the mass spectrum showed a $[M+H^+]$ at 271 on the mass spectrum. Wogonin (t_R = 29.45 min) was characterised by a λ maxima of 280 nm and the mass spectrum showed $[M+H^+]$ at 285. Chrysin (t_R = 30.15 min) had a λ maxima of 270 nm and a shoulder at 315 nm and the mass spectrum was characterised by $[M+H^+]$ at 255.

Triplicate herbal extracts were analysed and individual flavonoid concentrations quantified (Table 1) by reference to the calibration lines.

The flavone present in greatest levels in all 18 samples was baicalin with baicalein being the next most predominant of the 5 flavones identified. There was marked variation in the levels of the 5 flavones of interest in the *Scutellaria* samples tested with the sum content of all five flavones being greatest in sample 18 (3965 μg/g SB) and lowest in sample 16 (107.7 μg/g SB). Indeed sample 16 was quite anomalous and if excluded from the set the mean sum content

Table 1
Principal components determined in *Scutellaria baicalensis* samples 1–18.

Sample	Baicalin	Scutellarein	Baicalein	Wogonin	Chrysin
	Concentration in μg/g *Scutellaria baicalensis* (SB)				
1	3023.2	0	292.4	53.2	2.3
2	1530.3	0	186.4	54.6	2.0
3	2606.6	33.9	137.8	0	101.4
4	1244.4	31.6	753.1	154.7	43.3
5	1240.3	33.9	918.8	210.0	53.4
6	2777.3	40.6	603.2	129.7	29.2
7	3020.3	0	516.6	88.6	12.9
8	2413.4	12.2	73.2	24.9	0
9	2528.8	1.3	510.1	119.2	53.8
10	1224.7	38.6	529.9	155.9	65.4
11	1740.2	73.5	801.1	190.2	81.5
12	1957.4	1.2	1110.8	234.8	80.0
13	1619.6	17.6	1329.5	255.7	90.0
14	2257.9	17.7	304.2	85.3	56.9
15	2862.5	14.8	642.6	151.2	73.9
16	55.7	4.4	20.6	2.1	24.9
17	2828.4	12.3	495.1	119.3	51.8
18	3224.5	23.5	530.4	131.2	55.4

Data are the mean of triplicate extracts quantified by HPLC–DAD.

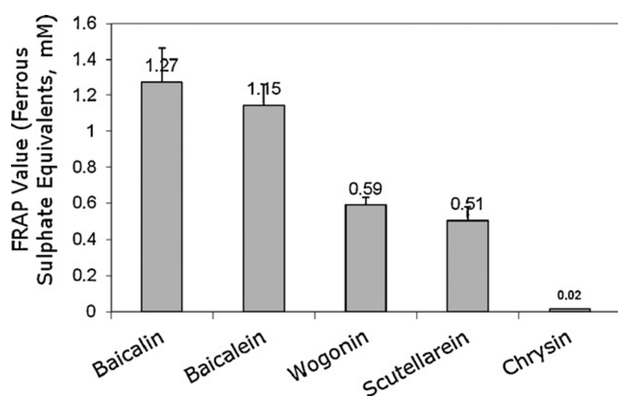


Fig. 5. FRAP values for the flavone standards. The data are the mean of triplicate FRAP assay determinations (±SD) for 1 mM stock solutions of each flavone.

of the 5 flavones in the 17 other *Scutellaria* samples was 3011.7 μg/g SB.

3.4. FRAP antioxidant activity of Scutellaria samples

The antioxidant reducing potential of the five key reference compounds and each of the herbal sample extracts was then assessed by the FRAP assay. The inter-assay linearity of the FRAP assay was determined across an 8-day period. A high level of precision was demonstrated with the mean value of assay response of 0.59 (%RSD = 3.10, n = 8) and a mean R^2 of 0.992 (%RSD = 0.58, n = 8). The relative antioxidant capacity of each of the 5 flavones was determined (Fig. 5).

When applied to the reference stock solutions, the intra-assay precision was high (≤1.3% RSD, n = 3). The FRAP values for the individual compounds varied from 1.27 mM FRAP activity for baicalin to 0.02 mM FRAP activity for chrysin.

The inter-assay precision was similarly high when the FRAP assay was applied to triplicate extracts of the *Scutellaria* samples and there was significant variability between the FRAP activity measured for each of the 18 herbal extracts (Fig. 6a). Sample 12 was the most potent with a mean FRAP value of 21.1 mM/g *S. baicalensis* and sample 16 the least potent with no detectable FRAP activity. In parallel with the FRAP activities a measure of total phenol content of each of the extracts was performed. The precision of this assay was very high with RSD typically less than 3% for duplicate determinations of triplicate extracts. Variation in the total phenol content of the extracts was observed (Fig. 6b) but generally the phenolic content of samples 12 and 16 mirrored the FRAP activities and overall a positive correlation (r = 0.77) between FRAP activity and total phenol content was observed for the sample set (n = 18).

3.5. Determination of the antioxidant coefficient

The antioxidant coefficient (PAC), essentially a ratio between FRAP activity (mM) and total phenol content (mM), was determined for each of the *Scutellaria* extracts (Table 2) and samples 6 and 16 were found to have the highest and lowest PAC values respectively, a slightly different ranking than that determined solely by FRAP analysis. There was a weak positive correlation (r = 0.61) between PAC value and the baicalin content of the *Scutellaria* samples and a stronger correlation (r = 0.75) was observed for PAC and the sum of (baicalin + baicalein) content. Importantly when the correlation between the PAC and the content of the five biomarkers of interest was examined there was no significant change in the correlation (r = 0.77) suggesting that the baicalin and baicalein concentrations determined by HPLC are useful indicators of the PAC value in this herb.

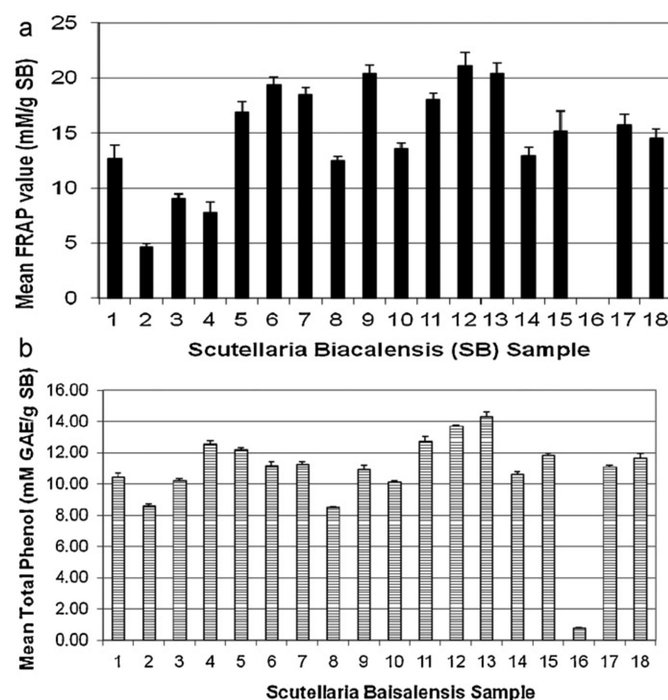


Fig. 6. a. Ferric reducing antioxidant potential of *Scutellaria* extracts. Data are the mean (n = 9) ± SD. Triplicate extracts were assayed in triplicate. b. Total phenol content of *Scutellaria* extracts. The data are the mean (n = 6) ± SD. Triplicate extracts were assayed in duplicate.

The PAC ratio is calculated from the mean FRAP and mean total phenol determinations described in Fig. 6a and b.

3.6. Principal component analysis (PCA) and SIMCA projections

The potential usefulness of employing PCA using a combination of the key parameters collected namely, FRAP activity, phenol content, levels of 5 biomarkers and the PAC as a means of quality evaluation of the *Scutellaria* herbal extracts was investigated. Numerous data sets were generated using the various pre-processing options and the potential usefulness of each as a QC indicator considered. In most cases PCA was able to form 2 or 3 individual groups. In all cases using PCA it was evident that sample 16 was different from the others. This method could not separate the different *Scutellaria* species, but it did manage to differentiate the

Table 2
Antioxidant coefficient (PAC) values of *Scutellaria* samples.

Scutellaria sample	PAC value
1-SB authenticated sample (China)	1.22
2. SB (China)	0.54
3. SA (China)	0.89
4. SB (Khon Kaen)	0.62
5. SB (Bangkok)	1.39
6. SB (Aberdeen)	1.74
7. SB (Aberdeen)	1.64
8. SB (London)	1.47
9. SB (Bangkok)	1.87
10. SB (Bangkok)	1.35
11. SB (Bangkok)	1.43
12. SB (Bangkok)	1.54
13. SB (Khon Kaen)	1.43
14. SB (Glasgow)	1.22
15. SB (Glasgow)	1.29
16. SB (Glasgow)	0
17. SB (Edinburgh)	1.42
18. SB (Singapore)	1.25

where SB = *Scutellaria baicalensis* and SA = *Scutellaria amoena*.

Table 3
Discriminatory ranking of parameters by SIMCA.

Parameter	Discriminatory value
Baicalein	63.7
Scutellarein	4.1
Baicalein	9.2
Wogonin	23.6
Chrysin	6
Phenol	47.9
FRAP	5.3
PAC	1753.4

poor quality sample. With SIMCA analysis and no pre-processing of data the PAC value is predicted as being the most discriminatory parameter (see Table 3).

When this discriminatory ranking was applied to generate a SIMCA projection the samples were clustered into 3 separate groups (see Fig. 7). Once more sample 16 that of lowest potency and lowest flavone content, was separated from all other samples (located in red cluster of SIMCA projection). The pink cluster contains *Scutellaria* samples 2, 4, 5, 10, 11, 12 and 13 whilst the green cluster contains samples 1, 3, 6, 7, 8, 9, 14, 15 and 18. Whilst samples 2, 3 and 4 have relatively low PAC values (<0.9) they are separated into separate clusters. Closer examination of the clustering of samples between the pink and green sets suggests that another factor influencing the separation is the level of baicalin in each sample. The level of baicalin in the *Scutellaria* samples clustered into the pink grouping is lower than 2000 μg/g SB whilst the level in the samples clustered in the green set is in the range of 2258–3022 μg/g SB.

4. Discussion

The current weak regulation of herbal remedies in the UK is under review and it is anticipated that by 2011 manufactured herbal medicines will be required to have either a Traditional Herbal Registration or a Product License. Whilst the World Health Organisation [19] has adopted fingerprint analysis as a strategy for the assessment of the quality of herbal medicines, it is recognised that this approach has limitations. For example synergistic interactions between multiple component parts of the herbal medicine may be ignored.

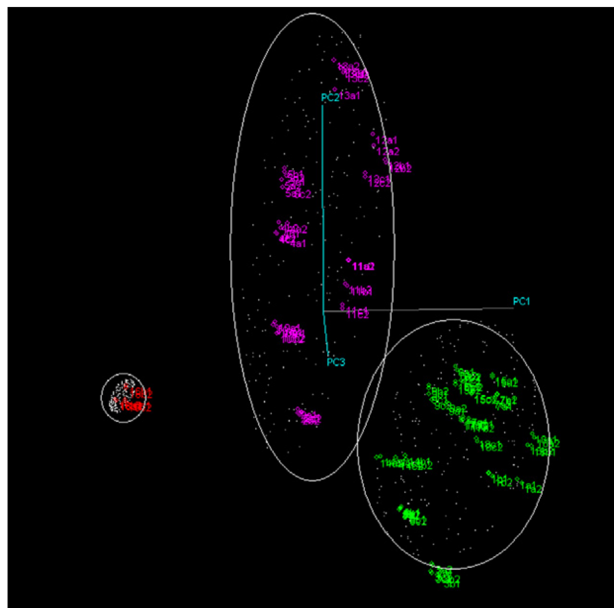


Fig. 7. SIMCA projection.

This research investigated the potential usefulness of three measurements as parameters of quality evaluation; namely individual component determination by HPLC, total phenol content and FRAP antioxidant capacity. Identification of the flavones in each herbal extract was achieved by comparison of retention times, UV–vis and mass spectral characteristics to those of commercially purchased reference standards and by reference to previously published literature [3]. The precision of analyte retention time was high and enabled direct comparison of HPLC chromatograms following analysis of reference standards and *S. baicalensis* and one *S. amoena* extracts.

For herbal medicines in which antioxidant activity is believed to be an important mode of action, there exist a number of relatively simple spectrometric assays to determine either total phenol content or antioxidant capacity. The total phenol assay of Singleton and Rossi [20] provides a molar response, typically expressed as gallic acid equivalents, which is roughly proportional to the number of phenolic hydroxyl groups in a given compound with the reducing capacity being enhanced when two phenolic hydroxyls are oriented ortho or para [21]. This proved to be a simple precise assay with the only slight drawback being the 60 min incubation step. The FRAP assay measures antioxidant capacity on the basis of the ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions and the reaction is linearly related to the molar concentration of the antioxidant(s) present [22]. The assay is commonly used for the routine analysis of single antioxidants and total antioxidant activity of plants [23]. It proved to be a precise, quick and simple assay and unlike the Trolox Equivalent Antioxidant Capacity assay [24] the reagents required may be prepared relatively quickly with no need for overnight stabilisation.

The principal components identified in the *S. baicalensis* and *S. amoena* extracts by combined LC–DAD and LC–MS analyses were baicalin, baicalein, scutellarein, chrysin and wogonin (Fig. 4) and of these the baicalin, baicalein and wogonin were the most potent reducing agents (Fig. 5). These results concur with previous studies of the antioxidant and free radical scavenging components of this plant [25]. However, in this study a weaker positive linear correlation (r = 0.77) between the FRAP activity and total phenol content of the *Scutellaria* extracts was found. This is important since it highlights the need to consider the principal chemical components within an herbal extract and their relative bioactivity rather than relying solely on a crude determination of total phenol content. This observation concurs with the findings of Ou et al. [1], in which they found no linear correlation between total phenolics and the antioxidant activity determined by the oxygen radical absorbance capacity assay. Thus the usefulness of such parameters in isolation as a measure of quality evaluation is questionable and a more robust measure of quality control for herbal compounds with antioxidant properties may be the antioxidant coefficient (PAC) [26], or where possible PAC coupled with chromatographic fingerprinting.

For the purpose of chromatographic fingerprinting a set of 5 flavones were considered, namely baicalin, baicalein, wogonin, scutellarein and chrysin. Chromatographic resolution of these flavones and the internal standard, 3-hydroxyflavone, was achieved in 45 min. The order of elution concurred with previously published reports [3,27] and the limits of detection were comparable to those of Gao et al. [27]. There was marked variation in the level of each of the 5 flavones of interest found in the 18 herbal samples analysed but in all cases, baicalin was the predominant biomarker and when assayed in isolation this flavone had the greatest FRAP antioxidant capacity (Fig. 6a). The sum content of all five flavones was greatest in sample 18 (3965 μg/g SB) but sample 6 with a sum content of 3580 μg/g SB had the highest PAC value (Table 2).

The PAC value provided a useful evaluation of the antioxidant efficiency of the various *Scutellaria* sample extracts and there was a positive correlation (r = 0.75) between PAC and the sum of (baicalin + baicalein) content determined by HPLC. Thus sample

2 and sample 8 are similar in terms of the total phenol content (approximately 1.4 mg/g SB) but the FRAP activity of sample 8 is approximately 3 fold greater than that of sample 2. This is reflected in the relative PAC values of 1.47 and 0.54 respectively. In these samples the levels of wogonin and chrysin are relatively low (<55 μg/g SB) and the main flavone components identified are baicalin and baicalein. The sum content of baicalin and baicalein in samples 8 and 2 is 2486 and 1716 μg/g SB respectively. Thus PAC determination may be a useful QC parameter to adopt for evaluating the relative antioxidant capacity of herbal medicines. The determination is underpinned by relatively inexpensive and simple spectrometric assays and does not require the acquisition of more expensive HPLC hardware or the more technically challenging LC method development and validation. Factors such as these will be important to a sector which is in the early stages of implementing methods and procedures to support the statutory regulation of herbal products. Whilst there have been reports of elegant HPLC hyphenated techniques which enable the online determination of antioxidant-activity-integrated fingerprints [28] the most popular are those employing single reagents such as the $ABTS^{\bullet+}$ radical. However, once generated this stock has a relatively short shelf-life and in an industrial QC setting the preference is for more robust methods which have the potential for high efficiency of throughput and good inter-batch precision. Thus chromatographic fingerprinting employing DAD or DAD–MS detection coupled with a determination of antioxidant coefficient may offer a more practical solution for companies embarking upon the adoption of QC procedures for herbal antioxidant products.

The potential usefulness of employing principal component analysis and SIMCA projections as a QC indicator was also investigated. In most cases PCA was able to form 2 or 3 individual groups. With SIMCA analysis and no pre-processing of data the PAC value is predicted as being the most discriminatory parameter and applying this ranking the herbal extracts were grouped into 3 clusters. The usefulness of PAC as a discriminatory parameter is interesting in light of the differing magnitude of some of the other data analysed. This is an important finding in light of the relative simplicity of the associated FRAP and total phenol assays.

Closer examination of the clustering of samples between the pink and green sets suggests that another factor influencing the separation is the level of baicalin in each sample. This is a useful observation since it may indicate that there is little or no significant antioxidant synergy between the 5 biomarkers when present in the *Scutellaria* extracts. Baicalin is the most efficient antioxidant of the 5 biomarkers characterised, it is also the most prevalent flavone in all the *Scutellaria* samples investigated and was the most discriminatory parameter identified by SIMCA projections. From a consumer viewpoint baicalin has significant anti-inflammatory and anti-vial activities and is a potent apoptosis inducer of cancer cells supporting the adoption of the quantification of this flavone in parallel with a PAC determination as a strategy for the QC testing *Scutellaria* extracts.

5. Conclusion

In conclusion this study demonstrates the extent of variation in antioxidant capacity and biomarker content of commercially available *Scutellaria* herbal medicines. Such variation may explain the differing efficacies reported for particular herbal medicines and further highlights the need for some fundamental regulation of such herbal medicines to address issues of quality, safety and efficacy. It is proposed that the PAC value alone or in combination with a chromatographic fingerprint of key biomarkers (e.g. baicalin or baicalin + baicalein) may be useful standards to adopt for the quality control of *S. baicalensis*.

References

[1] B. Ou, D. Hng, M. Hampsch-Woodill, J. Flanagan, When east meets west: the relationship between yin-yang and antioxidation–oxidation, FASEB J. 17 (2003) 127–129.
[2] H.B. Li, F. Chen, Separation methods used for *Scutellaria baicalensis* active components, J. Chromatogr. B 812 (2004) 277–290.
[3] C. Horvath, P. Martos, P. Saxena, Identification and quantification of eight flavones in root and shoot tissues of the medicinal plant Huang-qin (*Scutellaria baicalensis Georgi*) using high-performance liquid chromatography with diode-array mass spectrometric detection, J. Chromatogr. A 1062 (2005) 199–207.
[4] J. Han, M. Ye, M. Xu, J. Sun, B. Wang, D. Guo, Characterisation of flavonoids in traditional Chinese herbal medicine Huang-qin by liquid chromatography coupled with electrospray ionization mass spectrometry, J. Chromatogr. B 848 (2007) 355–362.
[5] K. Ishimaru, K. Nishikawa, T. Omoto, I. Asai, K. Yoshihira, K. Shimomura, Two flavone 2′-glucosides from *Scutellaria baicalensis*, Phytochemistry 40 (1995) 279–281.
[6] K.E. Heim, A.R. Tagliaferro, D.J. Bobilya, Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships, J. Nutr. Biochem. 13 (2002) 127–129.
[7] C.A. Rice Evans, N.J. Miller, G. Paganga, Structure–antioxidant activity relationships of flavonoids and phenolic acids, Free Radic. Biol. Med. 20 (1996) 933–956.
[8] R.J. Williams, J.P. Spencer, C. Rice Evans, Flavonoids: antioxidants or signalling molecules? Free Radic. Biol. Med. 36 (2004) 838–849.
[9] G.R. Schinella, H.A. Tournier, J.M. Prieto, P.M. de Buschiazzo, J.L. Rios, Antioxidant activity of anti-inflammatory plant extracts, Life Sci. 70 (2002) 1023–1033.
[10] K.M. You, H.G. Jong, H.P. Kim, Inhibition of cyclo-oxygenase/lipoxygenase from human platelets by polyhydroxylated/methoxylated flavonoids isolated from medicinal plants, Arch. Pharm. Res. 22 (1999) 18–24.
[11] Y.C. Chen, S.C. Shen, L.G. Chen, T.J. Lee, L.L. Yang, Wogonin, baicalin and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide, Biochem. Pharmacol. 61 (2001) 1417–1427.
[12] MHRA, 2010. Herbal medicines, regulation and safety: United Kingdom Medicines and Healthcare Products Regulatory Agency. Available http://www.mhra.gov.uk/Howweregulate/Medicines/Herbalmedicines/index.htm (accessed April 8 2010).
[13] BBC News Channel, 'Guilty plea in Chinese herbal "cancer" case'. http://news.bbc.co.uk (accessed February 19 2010).
[14] I.F.F. Benzie, J.J. Strain, The Ferric Reducing Ability of Plasma (FRAP) as a measure of "Antioxidant power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76.
[15] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventos, Analysis of total phenol and other oxidation substrates by means of Folin–Ciocalteu reagent, Methods Enzymol. 299 (1999) 152–178.
[16] FDA Guidelines for Validation of Analytical Procedures, Q2B Validation of Analytical Procedures: Methodology, Center for Drug Evaluation and Research (CDER), ICH, 1996, pp. 1–12.
[17] B.K. Lavine, Applications, theory and instrumentation: chemometrics, in: R.A. Meyers (Ed.), Encyclopedia of Analytical Chemistry, John Wiley & Sons Ltd, Chichester, 2003, pp. 183–269.
[18] R.G. Reid, D.G. Durham, S.P. Boyle, A.S. Low, J. Wangboonskul, Differentiation of opium and poppy straw using capillary electrophoresis and pattern recognition techniques, Anal. Chim. Acta 605 (2007) 20–27.
[19] World Health Organisation, General Guidelines for Methodologies of Research and Evaluation of Traditional Medicines, 2000.
[20] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstate acid reagents, Am. J. Enol. Viticult. 16 (1965) 144–158.
[21] E.N. Frankel, A.L. Waterhouse, P.L. Teissedre, Principal phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low density lipoproteins, J. Agric. Food Chem. 43 (1995) 890–894.
[22] I.F.F. Benzie, Y. Wai, J.J. Strain, Antioxidant (reducing) efficiency of ascorbate in plasma is not affected by concentration, J. Nutr. Biochem. 10 (1999) 146–150.
[23] K. Schleisser, M. Harwat, V. Bohm, R. Bitsch, Assessment of antioxidant activity by using different in vitro methods, Free Radic. Res. 36 (2002) 177–178.
[24] N. Nenadis, S. Boyle, E.G. Bakalbassis, M.M. Tsimidou, An experimental approach to structure activity relationships of caffeic acid and dihydrocaffeic acids and related monophenols, JAOCS 80 (2003) 451–458.
[25] Z.H. Gao, K.X. Huang, X.L. Yang, H.B. Xu, Free radical scavenging activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi, Biochim. Biophys. Acta – Gen. Subj. 1472 (1999) 643–650.
[26] V. Katalinic, M. Milos, T. Kulisic, M. Jukic, Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols, Food Chem. 94 (2006) 550–557.
[27] J. Gao, A. Sanchez-Medina, B.A. Pendry, M.J. Hughes, G.P. Webb, O. Corcoran, Validation of a HPLC method for flavonoid biomarkers in skullcap (Scutellaria) and its use to illustrate wide variability in the quality of commercial tinctures, J. Pharm. Pharm. Sci. 11 (2008) 77–87.
[28] H.A.G. Niederlander, T. van Beek, A. Bartasiute, I.I. Koleva, Antioxidant activity assays on-line with liquid chromatography, J. Chrom. A 1210 (2008) 121–134.