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Review

Separation methods used for *Scutellaria baicalensis* active components

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Abstract

*Scutellaria baicalensis* Georgi is one of the most widely used traditional Chinese herbal medicines. Its roots have been used for anti-inflammatory, antitumor, antiviral and antibacterial infections of the respiratory and the gastrointestinal tract, clearing away heat, moistening aridity, purging fire, detoxifying toxicosis, reducing the total cholesterol level and decreasing blood pressures. Baicalein, baicalin, wogonin and oroxylin A are its main active components. This review provides an overview of various separation, detection, and identification techniques employed for the quantitative and qualitative determination of these active components. Applications of high-performance liquid chromatography, high-speed counter-current chromatography, thin layer chromatography, capillary electrophoresis, and micellar electrokinetic capillary chromatography to the separation and determination of these active components are described. Examples of identification of these active components and their metabolites in complex matrices by high-performance liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry are also presented. The advantages and limitations of these separation and identification methods are assessed and discussed.

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Keywords: Reviews; *Scutellaria baicalensis*; Baicalein

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1. Introduction

*Scutellaria baicalensis* (S. *baicalensis*) Georgi (Huangqin in Chinese) is one of the most widely used traditional Chinese herbal medicines, and is officially listed in the Chinese Pharmacopoeia. Its roots have been used for anti-inflammation, anticancer, treating bacterial and viral infections of the respiratory and the gastrointestinal tract, cleaning away heat, moistening aridity, purging fire, detoxifying toxicosis, reducing the total cholesterol level and decreasing blood pressures. This herb also possesses cholagogic, diuretic, and cathartic actions. Some concentrated composite herbal preparations that contain *S. baicalensis* Georgi as a major ingredient in their prescriptions are widely used in countries [1–4]. *S. baicalensis* Georgi contains a variety of flavones, phenylethanoids, amino acids, sterols and essential oils. Its dried roots contain over 30 kinds of flavonoids, such as baicalin, baicalein, wogonin, wogonin 7-O-glucuronide, oroxylin A, and oroxylin A 7-O-gluconid.

Baicalin, baicalein, wogonin and oroxylin A are the main active components in *S. baicalensis* Georgi [5–8]. Baicalin is the most abundant component, and has anti-allergic [9], anti-inflammatory [10], anti-HIV [11,12], anti-tumor [13–15], antioxidant and free radical scavenging effects [16,17], and anti-SARS coronavirus effects [18]. Baicalein possesses anti-HIV [11,12], anti-tumor [15], antioxidant and free radical scavenging effects [16,17]. Wogonin has anti-respiratory syncytial virus activity [19], anti-hepatitis B virus [20], anti-tumor [15], antioxidant and free radical scavenging effects [17]. Oroxolin A has anti-respiratory syncytial virus activity [19].

The quantitative and qualitative determination of pharmaceutical components is critical in all stages of drug discovery. Biological analyses in support of ADME (absorption, distribution, metabolism and excretion), pharmacokinetic, and toxicokinetic studies are crucial in the drug selection and optimization processes [21]. A variety of separation techniques have been employed for the quantitative determination of *S. baicalensis* active components in various matrices. These techniques include high-performance liquid chromatography (HPLC), high-speed counter-current chromatography (HSCCC), thin layer chromatography (TLC), capillary electrophoresis (CE), and micellar electrokinetic capillary chromatography (MEKC). Fig. 1 shows chemical structures of six *S. baicalensis* active components. This review provides an overview of separation, detection, and identification methods employed for these active components in various types of samples.

2. Chromatographic methods

2.1. High-performance liquid chromatography

High-performance liquid chromatographic (HPLC) methods have been widely applied to the separation and determination of *S. baicalensis* Georgi active components in various matrices. Analyses of plant and raw medical material samples are discussed first, followed by medicinal preparations, and then biological fluids (blood and urine) samples.

2.1.1. Analysis of plant and raw medical materials

The active components must be extracted from plant or raw medical material samples prior to analysis by HPLC. Several extraction techniques have been developed [22–24]. Solid-phase extraction (SPE) is a commonly employed sample preparation method for drug compounds in complex matrices. SPE is used to selectively remove interfering matrix components, leading to reduced time required for chromatographic method development and improved assay selectivity, accuracy, and sensitivity. A method combining solid-phase extraction and reversed phase high-performance liquid chromatography was recently developed for simultaneous isolation and determination of flavones (baicalin, baicalein, chrysin, scutellarein) and some phenolic acids in aerial and underground parts of *S. baicalensis* Georgi [22]. The application of optimized enrichment conditions, elaborated on octadecyl and quaternary amine BakerBond microcolumns, led to the extraction of both groups of analytes with recoveries >95% and variation coefficients <5%. Supercritical fluid extraction (SFE) is another widely used technique for extraction of active components from plant or raw medical material samples, in which supercritical carbon dioxide is often used as an extraction solvent. The use of carbon dioxide has the following advantages: chemically inert, low toxicity, no pollution problem, and shorter concentration time. Under supercritical conditions, the solubility and diffusivity of the analytes in extraction fluid are increased, leading to improved extraction. For the extraction of polar or ionic compounds, organic solvents are added as modifiers or the compounds...
Scutellariae radix were determined by HPLC on a 10 μm ODS Hypersil column (25 cm × 4.6 mm i.d.) with gradient elution of acetonitrile and 0.1 M H₃PO₄ as mobile phase and detection at 280 nm [27]. The six main bioactive components, baicalin, baicalein, wogonin, wogonin glucuronide, oxoroxylin-A and oxoroxylin-A glucuronide in Scutellariae radix could be determined simultaneously by ion-pair high-performance liquid chromatography on a stainless-steel column (15 cm × 4 mm i.d.) packed with TSK gel LS-410 (5 μm) with aqueous 32% acetonitrile, containing 5 mM tetraperlylammonium bromide, as mobile phase, adjusted to pH 4 with H₃PO₄ [28]. Oxoroxylin-A and its glucuronide were separated for the first time. The method was one of the best methods for the simultaneous determination of main active components in S. baicalensis Georgi. In another study, flavonoid constituents of the roots of S. baicalensis Georgi were determined by HPLC on a column (20 cm × 6 mm) of Develosil ODS-5 at 50 °C, with 274 nm detection and tetrahydrofuran–dioxan– methanol–acetic acid–5% H₃PO₄·H₂O (145:125:50:20:2:322) or tetrahydrofuran–acetic acid–5% H₃PO₄·H₂O (95:10:1:444) as mobile phase [29]. By a combination of two mobile phases, total 11 flavonoids were separated in two runs. In addition, the content of the main flavonoids in the roots of S. baicalensis Georgi cultivated in Central Europe was evaluated by an HPLC method with gradient of acetonitrile in mobile phase [30]. The main components of the roots were baicalin (8.12% of dry root mass) and wogonin glucuronide (2.52%). The content of flavonoids was comparable with the content in plants cultivated in natural localities.

2.1.2. Analysis of medicinal preparations

Because baicalin is the main bioactive constituent of S. baicalensis Georgi, many reports have been published on the determination of baicalin by HPLC methods in different traditional Chinese medicinal preparations, such as No. 1 cold capsule [31], Qingxuewan [32], Yizhunxiang capsule [33], heat-clearing decoction [34], Fructus Sophorae pills [35], compound Houttuynia cordata granules [36], Qingshuiyuan granules [37], twelve baicalin-containing preparations such as pills, tablets and injection solutions [38], Biyankang tablets [39], and Huanglian Shangqing Wan [40]. HPLC methods have been also applied to the determination of baicalin and chlorogenic acid in Yinhuang oral liquid [41], baicalin and berberine in Wanshi Niuhuang Qingxin Wan [42], baicalin and puerarin in medicinal preparations [43], baikalin, sennosome A and glycyrrhizin in Niuuangjiedupian [44], baikalin, paeoniflorin and ferulic acid in Dang-Guei-San [45]. In these methods, reversed-phase columns such as C₁₈ are the most frequently used. Mobile phases usually consist of an aqueous phase with an acid and an organic solvent component, usually methanol or acetonitrile. The use of an acid modifier is to suppress ionization of the acidic groups and interactions of these groups with residual traces of metals in the stationary phase that are detrimental to peak shape.

Several bioactive components from S. baicalensis Georgi could be determined simultaneously in medicinal
preparations by HPLC [46,47]. Two components, baicalin and baicalein in processed Scutellariae radix and its pharmaceutical preparations (e.g. slices for infusion) were determined by HPLC on a column (15 cm × 6 mm i.d.) of ODS with THF-dioxane-methanol-n-propanol-acetic acid–5% H₃PO₄-H₂O (145:125:50:20:20:12:68) as mobile phase (1.5 ml min⁻¹) and detection at 274 nm [46]. Three components, baicalin, baicalein and wogonin in traditional decoctions and commercial extracts of Scutellariae radix were determined by HPLC with ethyl paraben as internal standard [47]. The analytes were separated on a 5 μm Inertsil ODS-2 column (25 cm × 4.6 mm i.d.) with acetoniitrile–0.005% phosphoric acid (9:16) as mobile phase (1 ml min⁻¹) and detection at 270 nm. The method was used to compare the absorption of baicalin, baicalein, wogonoside and wogonin between traditional decoction and a commercial powder preparation of Scutellaria radix [48]. The bioavailability of wogonin/wogonoside was about two times when compared with that of baicalein/baicalein from either traditional decoction or the commercial preparation. The flavone bioavailability from commercial preparation was significantly lower by 44.2 ± 0.1% for baicalin/baicalein and by 42.3 ± 0.1% for wogonoside/wogonin than those from traditional decoction.

For compound medicinal preparations, simultaneous determination of many bioactive constituents is needed for quality control. The six components, paoniflorin, cinnamic acid, baicalin, baicalein, wogonin and glycyrrhizic acid in medicinal preparation Chai-Hu-Kuei-Chih-Tang were determined by HPLC [49]. Commercial powdered sample was ultrasonicated with 70% methanol and filtered. The filtrate was analysed on a Cosmosil 5 C18-MS column (25 mm × 4.6 mm i.d.) with linear gradient elution of acetonitrile/aqueous 20 mM H₃PO₄ as mobile phase and UV detection. The seven constituents, gentiopicroside, mangiferin, palmatine, berberine, baicalin, glycyrrhizin and wogonin in Sann-Joong-Kuey-Jian-Tang were determined by HPLC with ethyl paraben as internal standard [47]. The analytes were separated on a 5 μm Inertsil ODS-2 column (25 cm × 4.6 mm i.d.) with acetoniitrile–0.005% phosphoric acid–acetonitrile (0%:90%:10%:90%:10%:90%:0%): flow-rate, 1.0 ml min⁻¹.

2.1.3. Analysis of biological fluids

Only a few reports have been published for the determination of the active components from S. baikalen-sis Georgi in biological fluids (blood and urine) samples. Baicalin in rabbits’ plasma was determined by a reversed-phase HPLC [53,54]. The sample (0.2 ml) was vortex-mixed with 4-nitrobenzoic acid (internal standard) and acetonitrile. The mixture was shaken ultrasonically and centrifuged. The supernatant was analysed on a 5 μm Cosmosil 5 C18-AR column (15 cm × 4.6 mm i.d.) with methanol/acetonitrile and H₂O as mobile phase with gradient elution and diode array detection from 200 to 400 nm.

Fig. 2. Chromatogram of the marker substances gentiopicroside, mangiferin, palmatine, berberine, baicalin, glycyrrhizin and wogonin in Sann-Joong-Kuey-Jian-Tang. Column, Cosmosil 5 C18-MS, 150 mm × 4.6 mm i.d.; guard column, Cosmosil 5 C18-AR, 50 mm × 4.6 mm i.d., mobile phase, 0.03% phosphoric acid-acetonitrile (0 min, 90:10; 10 min, 87:13; 17–27 min, 77:23; 40 min, 62:38; 50 min, 55:45); flow-rate, 1.0 ml min⁻¹. (1) Standard decoction; (2) commercial preparation; (3) standard decoction without Gentianae scabrae radix. Reprinted from [50] with permission from Elsevier.

wogonin, wogonoside, orxylin-A glucoside, orxylin-A, paoniflorin, glycyrrhizic acid, glycyrrhetinic acid, liquiritin, isoliquiritigenin, liquiritigenin and ononin in the traditional Chinese medicinal preparation Huangqin-Tang by HPLC [52]. Huangqin-Tang samples were mixed and boiled with water, centrifuged then concentrated and analysed on a 5 μm Wakosil II SC18-AR column (15 cm × 4.6 mm i.d.) with methanol/acetic acid and H₂O/acetic acid as mobile phase with gradient elution and diode array detection from 200 to 400 nm.
Fig. 3. High-performance liquid chromatograms of the standard solution (A) and I-Tzu-Tang (B). AE: aloe–emodin, B: baicalein, BG: baicalin, CA: caffeic acid, E: emodin, FA: ferulic acid, GZ: glycyrrhizin, IS: internal standard (tetralin), OG: oroxylin A 7-\text{O-glucuronide}, SA: sennoside A, SB: sennoside B, W: wogonin, WG: wogonin 7-\text{O-glucuronide}. Reprinted from [51] with permission from Elsevier.

used included acetonitrile/H$_2$O/acetic acid for flavonoids, isopropanol/H$_2$O/acetic acid for racemic liquiritin, or $n$-hexane/ethanol/acetic acid for chiral analysis. Detection was at 279, 275, and 295 nm for davidigenin and liquiritin, wogonin and oroxylin A and baicalein, and for dihydrowogonin and dihydrooroxylin A, respectively. Four flavonoids, liquiritigenin, baicalein, wogonin and oroxylin A, were found both in the urine and Shosaiko-to. The glycosides in Shosaiko-to were absorbed after microflora hydrolysis. Two flavanones, S-dihydrowogonin and S-dihydrooroxylin A, were identified as the metabolites of wogonin and oroxylin A, respectively. Although UV detection is still the most widely used, electrochemical detection is employed for improved specificity and sensitivity. Baicalin and baicaelin in rat plasma were determined by HPLC with electrochemical detection [59]. Baicalin was extracted from plasma by SPE with 2,3-dihydroxynaphthalene as internal standard; baicaelin was extracted at low or high concentration by SPE or liquid–liquid extraction with quercetin as internal standard. The analyte was determined on a stainless steel Nova-Pak column (15 cm × 3.5 mm i.d.) of octadecylsilane C$_{18}$ (4 µm), operated at 50 °C, with a mobile phase (1.2 ml min$^{-1}$) of 0.1 M H$_3$PO$_4$–acetic acid–THF (800:80:57) for baicalin or a mobile phase (0.8 ml min$^{-1}$) of 0.2 M H$_3$PO$_4$–methanol–THF (500:250:34) for baicaelin. Following separation baicalin and baicaeline were oxidized at a vitreous C electrode to permit selective electrochemical detection. The method was more sensitive than the HPLC method with UV detection.

2.2. High-speed counter-current chromatography

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography that uses no solid support matrix. Therefore, it eliminates irreversible adsorptive loss of samples onto the solid support matrix used in the conventional chromatographic column. The method has been successfully applied to the analysis and separation of various natural products [60–62]. In our laboratory, HSCCC has been applied to the isolation and purification of several bioactive compounds from the Chinese medical plants, such as tanshinones and salvianolic acid B in Salvia miltiorrhiza Bunge [63,64], salidroside in Rhodiola sachalinensis A. Bor [65], shikonin in Lithospermum erythrorhizon Sieb. et Zucc. [66], chlorogenic acid in Flos Lonicerae [67], glycyrrhizin in Glycyrrhiza uralensis Fisch [68].

Baicalin was separated and purified from *S. baicalensis* Geoigi by HSCCC [4]. Crude baicalin was obtained by extraction with methanol–water (70:30) from *S. baicalensis* Georgi. The separation was performed in two steps with a two-phase solvent system composed of $n$-butanol–water (1:1), in which the lower phase was used as the mobile phase at a flow-rate of 1.0 ml min$^{-1}$ in the head-to-tail elution mode. The HSCCC chromatogram is shown in Fig. 4. A total of 37.0 mg of baicalin at 96.5% purity was yielded from 200 mg of the crude baicalin (containing 21.6% baicalin) with 86.0% recovery. The simultaneous separation and purification of several active components in *S. baicalensis* Geoigi by HSCCC in a single run is being investigated in our laboratory.

2.3. Thin layer chromatography

Thin layer chromatography (TLC) is a quick, convenient, and inexpensive technique widely employed for pharmaceutical analyses. The main advantage of TLC is its ability to assay many samples in parallel on a single TLC or high-performance TLC plate, leading to a large increase in sample throughput compared with the column techniques such as HPLC. However, it also possesses several limitations including lower separation efficiency and capacity compared with the column separation.

A method was developed for the separation of baicalin, baicaelin, wogonin, and oroxylin A in Scutellariae radix by high-performance TLC on phenylmethylsilyloxysilane-treated silica plates with methanol–phosphate buffer (pH 6.2) as mobile phase [69]. Quantitative determination of the
flavonoids was conducted with a dual-wavelength flying-spot scanner at 280 nm.

Thin layer chromatography has been widely applied to separation and determination of baicalin in medicinal preparations, such as, Qingkailing injection solution [70], Xiaochaihutang oral liquid [71], injections and tablets [72], fast-acting anti-diarrhoeal tablet [73], and antibiotics [74]. TLC methods have been also applied to the separation of baicalin and berberine sulfate in HHK injection solution [75], baicalin and emodin in Fangfeng Tongsheng pills [76], and baicalin and chlorogenic acid in Yinhuang tablets and injection solutions [77,78] and in Tongkangtai oral-medication solution [79].

Baicalein and wogonin in the Korean crude drug preparation Soshiho Tang were separated by TLC on Silica gel 60 with benzene–ethyl acetate (1:1) as mobile phase and detection with 20% H_2SO_4 as spray reagent and heating at 110°C [80]. In another method, baicalin, ibuprofen, rhein and berberine hydrochloride in Sanhuang tablets were determined by TLC scanning method [81]. The powdered sample was extracted by Soxhlet with 95% ethanol. The diluted extract was subjected to TLC on silica gel with benzene-ethyl acetate-acetic acid (150:50:3) as mobile phase and detection by scanning at 430 and 425 nm for determining ibuprofen and rhein, respectively; this TLC plate was then heated at 80°C for 10 min and developed with CHCl_3-ethyl acetate-methanol-formic acid (7:3:1:1) for separating berberine hydrochloride and baicalin. Detection was at 420 and 280 nm for berberine hydrochloride and baicalin, respectively.

3. Electromigration methods

3.1. Capillary electrophoresis

Capillary electrophoresis (CE) is based on different mobilities of ions in electric field, and is applied to the analysis of charged species. CE is a separation technique of high efficiency with low sample and solvent consumption. It has been widely used for separation of both large and small drug molecules. This method, however, has low sensitivity and low reproducibility compared with HPLC methods [21,82]. CE with electrochemical detection operated in amperometric mode can provide higher sensitivity and selectivity than CE with UV detection. Baicalin and baicalein were determined by capillary electrophoresis with electrochemical detection [83]. The two analytes could be well separated within 8 min in a capillary (40 cm × 25 μm i.d.) operated at a separation voltage of 12 kV, with 100 mM borate buffer (pH 9.0) as running buffer. The working electrode was a 300 μm diameter carbon disc electrode at a potential of 0.90 V. The method was employed for the differentiation of Scutellariae radix from Astragali radix. Scutellariae radix contains a great deal of baicalin and baicalein that are not present in Astragali radix, so both these crude drugs can be differentiated by determining their baicalin and baicalein contents. A similar method was applied to simultaneous determination of baicalein, baicalin and quercetin in Scutellariae radix and its preparations [84]. The similar method was also used to the determination of baicalein, baicalin, and chlorogenic acid in traditional medicinal preparation Yinhuang oral liquid [85]. CE with UV detection, however, is more widely employed than CE with electrochemical detection. Baicalin in prescriptions containing S. baicalensis Georgi was determined by high-performance capillary electrophoresis [86]. The sample solution with p-nitrobenzoic acid as internal standard was analysed on an uncoated quartz column (39.5 cm × 50 μm; effective length 34.8 cm), operated at an applied voltage of 17 kV, with 40 mM borax buffer (pH 9) as running buffer and detection at 285 nm. In another method, two constituents, baicalin and chlorogenic acid in Yinhuang granules were determined by CE [87]. The sample solution with
Micellar electrokinetic capillary chromatography (MEKC) is based on partition between bulk solution and micelle moving in opposite direction to analyte, and is used to determine neutrals. MEKC was employed as an alternative to HPLC for separations of some special samples such as enantiomeric mixtures and very polar compounds [21,82]. The analysis of plant extracts was carried out on a fused-silica capillary column (82 cm \( \times \) 50 \( \mu \)m i.d.; 72.5 cm to detector) for separation at 25 kV and detection at 254 nm. The run buffer contained 50 mM sodium cholate, 4.25 mM sodium borate and 40% acetonitrile. Separation of berberine, palmatine, baicalin, sennoside B, emodin, and sennoside A was achieved in 20 min. A similar method was applied to the determination of these six bioactive components in Hsiao-Cheng-Chi-Tang [90].

3.2. Micellar electrokinetic capillary chromatography

Micellar electrokinetic capillary chromatography (MEKC) is based on partition between bulk solution and micelle moving in opposite direction to analyte, and is used to determine neutrals. MEKC was employed as an alternative to HPLC for separations of some special samples such as enantiomeric mixtures and very polar compounds [21,82]. MEKC with UV detection has been widely employed for the determination of large numbers of samples and for quality control in pharmaceutical plants. The method can be used for the quality control of Scutellariae radix in a shorter analysis period than HPLC. In another study, the six flavonoids, baicalin, baicalein, wogonin 7-O-glucuronide, wogonin, oroxylin A 7-O-glucuronide, and oroxylin A in Scutellariae radix were determined by MEKC [92]. The extract with salicylic acid as internal standard was analysed on a fused-silica capillary (1 m \( \times \) 75 \( \mu \)m i.d.) at 20°C with an electrolyte of pH 9.7 containing 20 mM SDS, 10 mM sodium dihydrogen phosphate and 12.5 mM sodium borate, and an applied voltage of 30 kV and detection at 275 nm. A similar method was used for the simultaneous determination of these six flavonoids in Scutellariae radix by MEKC with UV detection at 254 nm [93]. For medicinal preparations, baicalin, chlorogenic acid and forsythin in Shuanghuanglian oral liquid were determined by MEKC on a bare fused-silica capillary (60 cm \( \times \) 50 \( \mu \)m i.d.) operated at an applied voltage of 15 kV, using borate buffer, SDS and acetonitrile as background electrolyte and UV detection [94]. The three compounds were completely separated within 15 min. In another method, four constituents, baicalin, baicalein, wogonin and wogonin 7-O-glucuronide in traditional Chinese medicinal preparations were determined by MEKC [95]. The sample solution with salicylic acid as internal standard was analysed on a fused-silica capillary (100 cm \( \times \) 75 \( \mu \)m i.d.; 95.4 cm to detection window) at 20°C with an applied voltage of 30 kV, an electrolyte of 20 mM SDS/10 mM sodium dicydrogen phosphate/12.5 mM sodium borate buffer and detection at 275 nm. Injection was performed at 3 psi for 1 s. A method was developed for the simultaneous determination of ten constituents, baicalin, wogonin 7-O-glucuronide, oroxylin A 7-O-glucuronide, baicalein, wogonin, oroxylin A, berberine, palmatine, coptisine and epiberberine in the scute-coptis herb couple by MEKC with a photoion detector operating at 270 nm [96]. The carrier composed of a buffer solution (5 mM sodium borate, 15 mM sodium dihydrogenphosphate and 50 mM sodium cholate) and acetonitrile (3:2) was found to be the most suitable electrolyte for this separation, whereby the contents of these compounds in the herb couple and herb couple containing Chinese herbal preparations could be determined within 30 min. The electropherogram is shown in Fig. 5. Furthermore, the twelve constituents, baicalin, baicalein, wogonin 7-O-glucuronide, oroxylin A 7-O-glucuronide, sennoside A, sennoside B, emodin, aloes-emodin, glycyrrhizin, ferulic acid and caffeic acid in a Chinese herbal preparation I-Tzu-Tang could be determined simultaneously by MEKC on a fused-silica capillary tube (70 cm \( \times \) 75 \( \mu \)m i.d.; 62.5 cm to detection window) at 30°C with an applied voltage of 20 kV, an electrolyte solution consisting of 18 mM SDS, 2 mM sodium cholate, 12.5 mM Na2B4O7 and 10 mM NaH2PO4 and detection at 254 nm [51]. Injection was performed at 2 psi for 1 s. The electropherogram is shown in Fig. 6. The contents of these components in an untreated I-Tzu-Tang extract could be determined in a shorter time by MEKC (14 min) than by HPLC (50 min) (Fig. 3). Moreover, all 11 components of I-Tzu-Tang could be determined by MEKC (Fig. 6B), but only eight components could be determined by HPLC owing to serious interference from some impurities (Fig. 3B). Compared with the HPLC method, the MEKC method is more attractive, especially owing to its shorter running time, and therefore should be useful for the determination of large numbers of samples and for quality control in pharmaceutical plants.

4. Hyphenation procedures

4.1. High-performance liquid chromatography–mass spectrometry

High-performance liquid chromatography–mass spectrometry is applied for the separation and identification of standard samples and for quality control in pharmaceutical plants.
of unknown mixtures in simple or complex matrices. The main advantages of HPLC–MS are its high speed and sensitivity compared with other hyphenated identification techniques such as HPLC–nuclear magnetic resonance (NMR) and HPLC–infrared. In most situations, HPLC–MS/MS or HPLC–(MS)\(^2\) is preferred over HPLC–MS for the structure elucidation of unknown mixtures, because multistage MS can provide additional information on fragmented ions, facilitating structural assignments [21].

The active components in the traditional Chinese medicine Xiefei decoction were separated and identified by liquid chromatography–mass spectrometry [97]. The analytes were separated on an YWC ODS-AQ column (5 cm × 2 mm i.d.) with linear gradient elution of H\(_2\)O/ammonium acetate/formic acid/methanol as mobile phase and atmospheric-pressure-ionization tandem MS detection operating in multiple reaction monitoring. Nine active ingredients, rhein, aloe-emodin, physcion, baicalin, wogonoside, berberine, coptine, jatrorrhizine and palmatine were simultaneously determined.
Wogonin in rat plasma was determined by liquid chromatography-tandem mass spectrometry \[98\]. The analyte with daidzein as internal standard was determined on a 5 μm Diamonsil C18 column (25 cm × 4.6 mm i.d.) with acetonitrile-H2O-formic acid (80:20:1) as mobile phase (0.8 ml min \(^{-1}\)) and tandem positive-ion atmospheric pressure chemical ionization MS detection operated in selected-reaction monitoring mode. In another method, wogonin and its major metabolite in rat plasma were determined by liquid chromatography-tandem mass spectrometry \[99\]. Only one conjugated metabolite with glucuronic acid was identified by chromatographic and electrospray multi-stage MS assay (Fig. 7). A derivatization reaction with 2-chlorethanol further demonstrated that the metabolite was wogonin 7β-D-glucuronide, not wogonin 5β-D-glucuronide. Other conjugated metabolites, e.g., sulfates and glucosides, were not detected. The plasma concentration of free wogonin was determined using atmospheric pressure chemical ionization source in the selected reaction monitoring (SRM) mode (Fig. 8). Incubation of the plasma samples with β-glucuronidase allows the quantitation of wogonin 7β-D-glucuronide.

4.2. Gas chromatography–mass spectrometry

Because of the high polarity, low volatility and poor thermal stability, gas chromatography was not widely employed for the determination of baicalin, baicalein or wogonin. As a result, few gas chromatographic methods were developed and reported in the literature. These active compounds in the crude extracts of \(S. baicalensis\) Georgii were identified by gas chromatography–mass spectrometry \[23\]. A 30 m × 0.25 mm HP-5MS (crosslinked 5% diphenyl–95% dimethylpolysiloxane) capillary column was used (0.25 μm film thickness) with helium as carrier gas. The molecular ion peaks of wogonin and baicalein were at \(m/z\) 284 and 270, respectively. As to baicalin, its molecular ion peak could not be observed at \(m/z\) 446. All crude extracts showed the similar patterns of total ion chromatograms. By comparison of MS fragments with library search software, wogonin was identified. But at the retention time of 20.03 min, fragments of \(m/z\) 270 had a relatively low intensity compared with those of \(m/z\) 284, 269, 139 and 241. By comparison with the peak intensities of the library database, these peaks may be shown to represent the combinations of many constituents, such as baicalin, baicalein and oroxylin A. Therefore, optimal conditions for separation of the coexisting baicalein and baicalin by GC–MS require further investigation.

5. Quantitation and validation

Often minor reagent substitutions, changes in laboratory temperature, or lot-to-lot variations in extraction cartridges and analytical columns affect analytical results. The use of internal standard method can compensate for sample losses occurring during extraction, clean-up and final analysis and for signal changes caused by variation in experimental conditions. An ideal internal standard should resemble the analyte as closely as possible in terms of chemical and physical properties and this requirement is fulfilled when a structural analogue is selected as an internal standard.

![Fig. 7. LC–MS² analysis of wogonin and its conjugated metabolites in plasma after an administration of 5 mg kg\(^{-1}\) wogonin to a Wistar rat. (A) Total ion current and selected ion monitoring chromatograms (A2, wogonin glucuronide; A3, wogonin sulfate; A4, wogonin glucoside; A5, free wogonin); (B) full scan MS–MS spectrum of peak at 6.18 min; (C) full scan MS² spectrum of peak at 6.18 min. Reprinted from \[99\] with permission from Elsevier.](image-url)
Good science and effective medical care demand inexpensive validated methods with high throughput which are capable of simultaneously analyzing multiple drugs in various matrices. The analytical methodology should be validated in terms of precision, accuracy, limit of detection, limit of quantitation, specificity, linearity and range, ruggedness and robustness [100].

In a method for the HPLC-UV determination of baicalin, baicalein and wogonin in Scutellariae radix, ethyl paraben was used as internal standard. The linearity ranges were 3.1–100, 2.5–80 and 2.5–80 μg ml$^{-1}$, respectively. The determination and detection limits were 2.5–3.1 and 0.2–0.3 μg ml$^{-1}$, respectively. The recoveries were 87.8–110% with the intra- and inter-run R.S.D. of 5.3% [47]. In another HPLC-UV method for the determination of baicalin in rabbit serum, p-nitrobenzoic acid was used as internal standard. The calibration graphs were rectilinear for up to 2, 1.2, 5 and 10 μg for ibuprofen, rhein, berberine hydrochloride and baicalin, respectively. The recoveries averaged >94% with the coefficients of variation of <4% [81].

When baicalin, baicalein and quercetin in Scutellariae radix were determined by CE with electrochemical detection, the linearity ranges were 0.0010–1.0, 0.0005–1.0 and 0.0005–1.0 mmol l$^{-1}$, the limits of detection were 0.548, 0.224 and 0.274 μmol l$^{-1}$, the recoveries were 95.24, 99.08 and 97.52%, and the R.S.D.s were 2.79, 0.94 and 4.76%, respectively [83]. The method was less sensitive than the HPLC with electrochemical detection [59], but it was more sensitive than the HPLC with UV detection [47]. For determination of baicalein in prescriptions containing S. baicalensis Georgi by high-performance capillary electrophoresis with UV detection, the linearity range was 10–640 mg l$^{-1}$. The recoveries were 100.3–100.6% with the R.S.D. of <5% [86]. For determination of baicalin and chlorogenic acid in Yinhuang granules by capillary electrophoresis with UV detection, the calibration graphs were linear from 160–960 and 80–960 mg l$^{-1}$, respectively. The recoveries were 99.8–102.1% with the R.S.D. of 0.87–3.09% [87]. When six flavonoids in Scutellariae radix were simultaneously determined by MEKC with UV detection, the calibration graphs were linear for 0.007–0.135 mg ml$^{-1}$ of baicalein, wogonin and oroxylin A 7-O-glucuronide, 0.013–0.234 mg ml$^{-1}$ of wogonin 7-O-glucuronide, 0.025–0.450 mg ml$^{-1}$ of baicalin and 0.005–0.09 mg ml$^{-1}$ of oroxylin A. The recoveries were 98.1–102.6% and the R.S.D.s were 0.9–2.4% [92].

For determination of wogonin in rat plasma by liquid chromatography-tandem mass spectrometry, the linearity range was 0.25–20 ng ml$^{-1}$. The limit of detection was 0.25 ng ml$^{-1}$ and the within- and between-day R.S.D.s were 2.2–19.1 and 5.9–7.3%, respectively [98]. The HPLC-MS [98] was the most sensitive, followed by the HPLC with electrochemical detection [59], and the CE with electrochemical detection [83]. The HPLC-UV [47] was the least sensitive.

6. Evaluation of the analytical results

Analytical methodology is an essential component not only in the developmental phase of a drug substance but also in the continuing evaluation of clinical efficacy. The HPLC
methods have been applied to pharmacokinetic studies. The pharmacokinetics of wogonin in rats was studied by HPLC after a dose of 5 mg kg\(^{-1}\) by intravenous administration [101]. A biphasic phenomenon with a rapid distribution followed by a slower elimination phase was observed from the plasma concentration time profile. In another report, the metabolism of baicalin in human was studied by HPLC after oral administration of Sho-Saiko-To [102]. Baicalin is one of the major components of Sho-Saiko-To. Baicalein 6-\(O\)-sulfate was identified as the metabolite of baicalin. Baicalein 6-\(O\)-sulfate was detected after 1 h, reached a maximum level at 5 h and then decreased to the level less than the quantitative limit (5 ng ml\(^{-1}\)). After intravenous administration of baicalin, absorption of baicalin itself was negligible, whereas the glucuronides/sulfates of baicalin were predominant in the plasma. When compared with intravenous bolus administration with dose correction, the absolute absorption was 40%. When baicalin was administered orally, glucuronides and sulfates of baicalin were exclusively circulated in the plasma. The relative absorption for baicalin was 65% when compared with baicalein. Profound differences of serum profile and pharmacokinetics were observed between oral baicalin and baicalein. Baicalein demonstrated significantly a time lag to achieve the peak concentration \(C_{\text{max}}\) and the lower peak serum concentration \((C_{\text{max}})\) of baicalein conjugated metabolites compared to baicalin, indicating baicalin was absorbed more slowly and to poorly than baicalein. In addition, the urinary pharmacokinetics of baicalin, wogonin and their glycosides in humans were studied after oral administration of Scutellariae radix [104]. Ten healthy male volunteers received a dose of 5.2 g commercial powder (comparable to 9 g crude drug), respectively. The mean cumulated renal excretion of baicalin glucuronides and sulfates were 43.1 ± 4.5 μmol (2.9% of dose) and 64.8 ± 6.3 μmol (4.3% of dose), respectively, whereas wogonin glucuronides and sulfates were 216 ± 2.0 μmol (5.9% of dose) and 207 ± 1.7 μmol (5.7% of dose), respectively. The renal excretion of conjugated metabolites of wogonin (11.6% of dose) was higher than that of baicalin (7.2% of dose). The baicalein sulfates was predominant than the corresponding glucuronides, whereas wogonin sulfates was comparable to the corresponding glucuronides.

The metabolism of active constituents in Huangqin-Tang, a prescription in traditional Chinese medicine, was studied by human intestinal flora [105]. Huangqin-Tang and all individual herbs in the decoctions were incubated with a human fecal suspension separately. The active constituents of Huangqin-Tang, including baicalin, wogonoside, oroxylin-A glucuronide, paeoniflorin, liquiritin, isoorientin and glycyrrhizic acid, were converted to their metabolites baicalein, wogonin, oroxylin-A, paeonimetabolin-I, liquiritigenin, isoorientigenin and glycyrrhetinic acid by human intestinal flora. The contents of the metabolites in Huangqin-Tang and in each single herb decoction increased significantly after incubation with intestinal flora. Comparing with single herb decoctions, the transformation of baicalin, wogonoside, oroxylin-A glucuronide, liquiritin and isoorientin in the compound prescription was promoted, however, that of paeoniflorin and glycyrrhizic acid was inhibited. All the results suggested that the glycosides of many medicinal herbs could be converted to aglycones by human intestinal flora, and the metabolism of most glycosides was improved in the compound prescription. In another report, the pharmacokinetics of multi-constituents in Huangqin-Tang decoction in rats were studied [106]. At different intervals after oral administration of the Huangqin-Tang decoction or a single herb decoction at a dose of 10 g kg\(^{-1}\), the concentrations of the constituents and their metabolites, baicalin, wogonoside, oroxylin-A glucuronide, baicalein, wogonin, oroxylin-A, paeoniflorin (PF), paeonimetabolin-I, liquiritin, liquiritigenin, glycyrrhizic acid and glycyrrhetinic acid, were detected in the rat plasma by HPLC. There were obvious differences in the pharmacokinetic parameters of most constituents (especially constituent wogonoside) between the compound prescription and single herb decoction. The constituents in the compound prescription had delayed absorption and elimination, a longer residence time in the body, and higher \(C_{\text{max}}\) and AUC (0-lim), than those in the single herb decoction. Therefore, they were more efficient and durable, making them promising to exerting pharmacological effects in vivo.

The degradation of thirteen flavonoid aglycones-wogonin, diosmetin, hesperetin, baicalein, morin, genistein, daidzein, quercetin, naringenin, luteolin, kaempferol, apigenin and neohesperidin were investigated in rabbit, rat and human fecal flora suspensions as well as in artificial intestinal juice, using HPLC [107]. The result indicated that all flavonoid aglycones except baicalein, diosmetin and quercetin were stable in artificial intestinal juice, whereas all were degraded in rabbit, rat and human fecal flora suspensions. Wogonin and diosmetin were among the less degraded ones for all three feces tested. The presence of a methoxy group on the A or B ring of the flavonoid seems to protect the structure from bacterial degradation. In addition, the bioavailability of baicalin–phospholipid complex was also studied by HPLC [108]. A complex of baicalin with soy phospholipid was prepared to improve the bioabsorption of baicalin. The concentration of baicalin in rat plasma reached a peak of 0.42 μg ml\(^{-1}\) at 5.3 h after oral administration, 600 mg kg\(^{-1}\). However, after intake of its phospholipid complex, a peak of 0.90 μg ml\(^{-1}\) occurred at a later time, 6.1 h. The elimination of the complex tended to be slower than that of the free drug. There was a significant difference in the mean area under the
S. baicalensis affects, such as anti-SARS coronavirus effect of baicalin [18]. Should be carried out to determine new pharmacodynamic efficacy of these active components and their metabolites in complex matrices. High-speed counter-current chromatography will be more widely used for the preparative separation and purification of S. baicalensis active components on a large scale.

7. Conclusions

S. baicalensis Georgi is widely explored for the treatment of inflammation, cancers, bacterial infections, and a variety of other diseases. A wide range of separation, detection, and identification methods have been developed and employed for quantitative and qualitative determination of its main active components, baicalin, baicalein, wogonin and oroxylin A. Different sample preparation techniques such as supercritical fluid extraction, pressurized hot water extraction and solid-phase extraction have been also utilized prior to analysis.

High-performance liquid chromatography with ultraviolet detection is the most frequently employed for the determination of these active components in various matrices. The development of HPLC–mass spectrometry coupling is a major advance, offering greater sensitivity than ultraviolet detection and greater selectivity through selected ion monitoring or by using tandem mass spectrometry and selected reaction monitoring mode. These techniques are efficient for identification of these active components and their metabolites in complex matrices because of their excellent specificity, sensitivity and speed. Capillary electrophoresis, micellar electrokinetic capillary electrophoresis, and thin layer chromatography are also often applied to the separation of these active components. High-speed counter-current chromatography has been used for the preparative separation and purification of baicalin from Scutellariae radix.

In the future, the automated high-throughput sample preparation in conjunction with parallel column HPLC with tandem mass spectrometry detection will play a key role in quantitative pharmaceutical and biological analyses of these active components. Combined use of HPLC–MS and HPLC–NMR will be the most efficient approach for identification of these active components and their metabolites in complex matrices.

8. Nomenclature

CE capillary electrophoresis
GC gas chromatography
HIV human immunodeficiency virus
HPLC high-performance liquid chromatography
HSCCC high-speed counter-current chromatography
MEKC micellar electrokinetic capillary chromatography
MS mass spectrometry
NMR nuclear magnetic resonance
PHWE pressurized hot water extraction
PLE pressurized liquid extraction
R.S.D. relative standard deviation
SARS severe acute respiratory syndrome
SDS sodium dodecyl sulphate
SFE supercritical fluid extraction
SPE solid-phase extraction
SRM selected reaction monitoring
TLC thin layer chromatography
UV ultraviolet

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