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Rapid determination of ten polyphenols in Kudiezi injection using ultra-performance liquid chromatography-tandem mass spectrometry in multiple reaction monitoring mode

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A simple and rapid method for simultaneous determination of ten polyphenols in Kudiezi injection has been developed and validated using ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) in multiple reaction monitoring (MRM) mode. The chromatographic separation was performed on an ACQUITY UPLC BEH C_{18} column (2.1 mm × 50 mm, 1.7 μm) with a gradient of acetonitrile and 0.5% formic acid (v/v). Ten polyphenols were rapidly analyzed within 10 minutes. Very low limits of detection (LODs) of 0.002–0.630 μg L^{-1} and limits of quantitation (LOQs) of 0.005–2.930 μg L^{-1} of the ten components were achieved. The linear calibration range extended from 2.73 to 660.40 μg L^{-1}. The developed method was validated in terms of good linearity (R^2 > 0.9990), precision (less than 3.71%) and accuracy (from 97.35 to 102.02%) for ten components. The developed UPLC-ESI-MS/MS method was simple and useful for rapid determination of the constituents in Kudiezi injection.

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Introduction

Ixeris sonchifolia (Bunge) Hance (Family Compositae), also called Kudiezi or Mantianxing in China, mainly grows in northeastern areas of China. It has been generally used as a folk medicine in China for its remarkable medical effects, such as invigorating the circulation of blood, dissipating blood stasis to relieve pain, *etc.*[1] A series of components have been isolated from the herb, including flavonoids, phenolic acids, triterpenes, sesquiterpene lactone, *etc*.[2–9] Some have been reported to have various activities, for instance, increasing the coronary artery flow, decreasing the oxygen consumption of the myocardium, improving the microcirculation, as well as anti-inflammatory, antibacterial, antioxidant, hypocholesterolemic and anticancer effects, and so on.[10–16] Kudiezi injection (extract of the whole herb), as a preparation of *Ixeris sonchifolia*, has been widely applied to treat cerebral infarction, coronary heart disease, effort angina and myocardial infarction for several years in the clinic.[17–19] Moreover, in recent years, side effects of Kudiezi injection have been described,[20] so it is necessary to develop simple, reliable and sensitive quality control methods to ensure its safe administration.

Some studies on the quantitative determination of major active components in *Ixeris sonchifolia* and Kudiezi injection have been reported. According to the properties of the analytes, high performance liquid chromatography with ultraviolet-visible detection (HPLC-UV) and high performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) have been used in those studies.[21,22] However, multi-component analysis by high performance liquid chromatography (HPLC) method was lengthy,[21] and it was not sensitive enough for trace component analysis in mixtures. UPLC coupled with mass spectrometry in MRM mode has been reported to be a powerful approach for the rapid analysis of constituents in TCM.[23,24]

In this study, a simple, rapid and accurate UPLC-ESI-MS/MS method for the simultaneous quantitative assay of ten polyphenols is developed to evaluate the quality of Kudiezi injection. Without preconcentration and chemical treatment, it provides a simple analytical method that could be adopted to simultaneously determine abundant and trace components in Kudiezi injection with wide linear ranges, short detection times, high recoveries and low detection limits for all ten analytes. Samples from different pharmaceutical factories could be distinguished on the basis of the contents of ten components using cluster analysis.

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Materials and methods

Materials and chemicals

The chemical reference substances of neochlorogenic acid (I), chlorogenic acid (II), cryptochlorogenic acid (III), isochlorogenic acid B (VII), isochlorogenic acid A (VIII) and isochlorogenic acid C (IX) were purchased from Chengdu Biopurify Phytochemicals Co, Ltd (Sichuan, China). Luteolin-7-O-β-D-glucoside (VI), luteolin (X) and caffeic acid (IV) were obtained from National Institutes for Food and Drug Control in China; chicoric acid (V) was purchased from Chengdu Deitian Creature Science Co, Ltd (Sichuan, China). The structures are shown in Fig. 1.

Acetonitrile, methanol and formic acid (FA) (HPLC grade) were purchased from Fisher Scientific (USA). Ultra-pure water was produced by a Milli-Q purification system (18.2 MΩ cm at 25 °C) (Millipore, Bedford, MA, USA). The 0.22 μm membranes used in the experiment were purchased from Waters Corporation (USA). The commercial products of Kudiezi injection were purchased by prescription from hospitals. Kudiezi injections were produced by Tonghua Huaxia Pharmaceutical Co, Ltd (Jilin, China) (A) and Shenyang Shuangding Pharmaceutical Co, Ltd (Liaoning, China) (B).

Instrumentation

A UPLC-ESI-MS/MS instrument (ACQUITY Xevo TQ–S, Waters, USA) was used to analyze the polyphenols in Kudiezi injection. The column (ACQUITY BEH C_{18}, 2.1 × 100 mm, 1.7 μm) was purchased from Waters Corporation.

Analytical methods

Standard solutions. Each reference compound was accurately weighed, dissolved in methanol, and serially diluted to produce the calibration curves, check linearity, and determine LODs as well as LOQs. All the standard solutions were stored at 4 °C prior to analysis.

Sample preparation. Each sample was accurately measured (1 mL), added into a 100 mL flask, and diluted with 10% acetonitrile. The dilution was mixed by ultrasonication at room temperature for 2 minutes. The solution was then filtered through a 0.22 μm membrane, then a 10 μL aliquot of the filtrate was injected into the UPLC-MS/MS system for analysis.

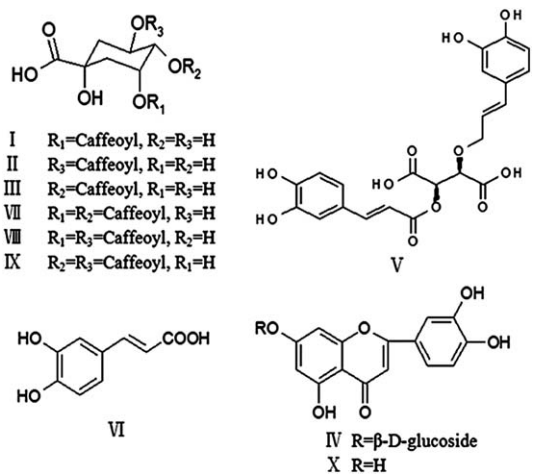

Fig. 1 The structures of ten active components.

Liquid chromatographic conditions. The flow rate of the mobile phase, composed of acetonitrile and Milli-Q water containing 0.5% FA, was 0.4 mL min^{-1}. The samples for analysis were kept at 10 °C in a sample room. The column was maintained at a temperature of 40 °C. The separation was started with 8% acetonitrile for 3 min, then the acetonitrile concentration was increased immediately to 17% at 3.1 min and maintained for 4 min, and then the acetonitrile concentration was increased to 100% within 2 min, finally it was dropped back to 8% and run for 1 min to equilibrate before the next injection. All samples were filtered through 0.22 μm filters prior to injection.

Mass spectrometry. The mass spectrometric analysis was performed using an ESI source in its negative ion mode. For all of the ten components, the precursor ions $[M - H]^-$ together with one product ion were selected. The detected precursor and product ions (m/z) of ten components, dwell time, cone voltage and collision energy are shown in Table 1.

The other important parameters were as follows: capillary voltage of 2.50 kV; source temperature of 150 °C; desolvation temperature of 400 °C; desolvation gas (nitrogen gas, >99.99%) of 800 L h^{-1}; cone gas (nitrogen gas, >99.99%) of 150 L h^{-1}; collision gas (argon gas, >99.99%) of 0.15 mL min^{-1}. MassLynx 4.1 and SPSS 16.0 were used for data acquisition and processing.

Standard calibration curves, LODs and LOQs. The mixed standards stock solution was prepared in methanol and stored at 4 °C. The solution was diluted with 10% acetonitrile to an appropriate concentration. Calibration curves were obtained by plotting peak areas *versus* six different concentrations of standard solutions. In order to validate the method, its sensitivity was tested by calculating LODs and LOQs according to the signal-to-noise ratios (S/N) of 3 and 10, respectively. The LODs and LOQs were obtained by serial dilutions of the standard solution.

Precision, repeatability and accuracy. The precision was evaluated on the basis of the results of six replicate injections of the mixed standard solution under the optimal conditions during a single day for intra-day variation, and on three consecutive days for inter-day variation. Six different working solutions prepared from the same sample (A) were assessed to assure the repeatability. The relative standard deviation (RSD) was chosen to evaluate the precision and repeatability.

The recovery test was used to evaluate the accuracy of the method. Three different concentration levels (approximately equivalent to 0.5, 1.0 and 1.5 times of the concentration of the sample) of the standards were added into a certain amount of the sample, which had been determined. The ten components were analyzed with the method described previously. The average recoveries were calculated according to the formula: recovery (%) = (detected amount − initial amount)/added amount × 100%.

Table 1 Precursor and product ions, dwell time, cone voltage and collision energy of components

Analyte	Precursor ions $[M - H]^-$ (m/z)	Product ions (m/z)	Dwell time (ms)	Cone voltage (V)	Collision energy (eV)
I	352.97	190.98	160	34	18
II					
III					
IV	178.90	134.96	160	38	16
V	473.11	311.05	106	8	12
VI	447.03	284.96	106	25	24
VII	515.10	353.05	106	28	18
VIII					
IX					
X	284.97	132.99	330	68	34

Results and discussion

Separation of the analytes

In order to achieve a satisfactory analytical method, the chromatographic conditions, including the mobile phrase (methanol/water and acetonitrile/water), flow rate (0.2, 0.3 and 0.4 mL min^{-1}), FA addition (0.1, 0.3 and 0.5%), injection volume and mode, the solvent type and volume of strong and weak wash solution, column temperature (30, 35 and 40 °C) and column type (Waters ACQUITY BEH C_{18}, 2.1 × 100 mm, 1.7 μm and Waters ACQUITY BEH C_{18}, 2.1 × 50 mm, 1.7 μm) were optimized after several trials. The resolution of chromatographic peaks can be improved significantly by the addition of FA. Meanwhile, the sample preparation conditions, such as dilution solvents (10, 50 and 100% acetonitrile) and dilution times (50, 100 and 1000 times) were investigated. 100 times dilution of Kudiezi injection should be prepared for analysis with 10% acetonitrile.

Mass spectrometric analysis

At the same time, the parameters of mass spectrometric analysis were optimized. The assay of ten polyphenols was attempted by UPLC-ESI-MS/MS in positive ion mode and negative ion mode. This study revealed that the signals obtained in the negative mode had a better sensitivity than those in the positive mode. Other conditions, such as capillary voltage (1.5, 2, 2.50 and 3 kV), desolvation temperature (350, 400 and 450 °C), flow rate of desolvation gas (600, 700, 800 and 900 L h^{-1}), flow rate of cone gas (150, 160 and 180 L h^{-1}) and flow rate of collision gas (0.1, 0.15 and 0.2 mL min^{-1}) were investigated one by one for optimization. Cone voltages and collision energy were optimized on the basis of the individual characteristics of target components (Table 1). The precursor ion $[M - H]^-$ together with one product ion was selected for every target component. Quantification was performed using electrospray ionization in multiple reaction monitoring (MRM) mode. I, II and III had the same precursor ion and product ion (352.97 → 190.98, $[M - H -$ caffeoyl$]^-$) because they are structural isomerides. Similarly, VII, VIII and IX had the same precursor ion and product ion (515.10 → 353.05, $[M - H -$ caffeoyl$]^-$). The ionization channels of 178.90 → 134.96 $[M - H - CO_2]^-$, 473.11 → 311.05 $[M - H -$ caffeoyl$]^-$, 447.03 → 284.96 $[M - H -$ glucoside$]^-$ and 284.97 → 132.99 $[M - H - C_7H_4O_4]^-$ were selected for quantitative analysis of VI, V, IV and X, respectively. Mass chromatograms of the detective ions are shown in Fig. 2. In this study, cross-talk effect produced using different channels in the mass system was not observed.

Method validation

The developed UPLC-ESI-MS/MS method was validated for parameters such as linearity, precision, accuracy and repeatability. For determination of the bioactive components, a calibration curve for each component was constructed for linearity. The results are presented in Table 2. Good linearity ($R^2 > 0.9990$) was found in the given concentration range, with relatively low LODs (0.002–0.63 μg L^{-1}) and LOQs (0.005–2.93 μg L^{-1}) for all of the ten analytes.

The relative standard deviation (RSD, %) was taken as a measure of precision for quantitative determination of ten components, with intra- and inter-day variation less than 1.93 and 5.17%, respectively. For all analytes, the RSDs of the repeatability experiments were less than 1.80% (Table 2).

Recovery of the reference substances from samples was generally used to evaluate the accuracy of the developed analytical method. In the recovery test, the proposed method was applied to the samples blended with the mixed standard solution at high, medium and low concentration levels. Each level was performed three times. The mixture was processed by the same dilution procedure as that used in the sample preparation, and analyzed using the same method. In this study, known amounts of standards were spiked into the samples and the recoveries were calculated to evaluate the matrix effects. Matrix effects were minimized by diluting the sample solution. The results were acceptable for ten analytes with recoveries ranging from 97.35 to 102.02%, with RSDs from 0.59 to 3.71%. All data are depicted in Table 3.

From the above, the developed method is accurate and sensitive enough for the quantitative determination of bioactive substances in Kudiezi injection.

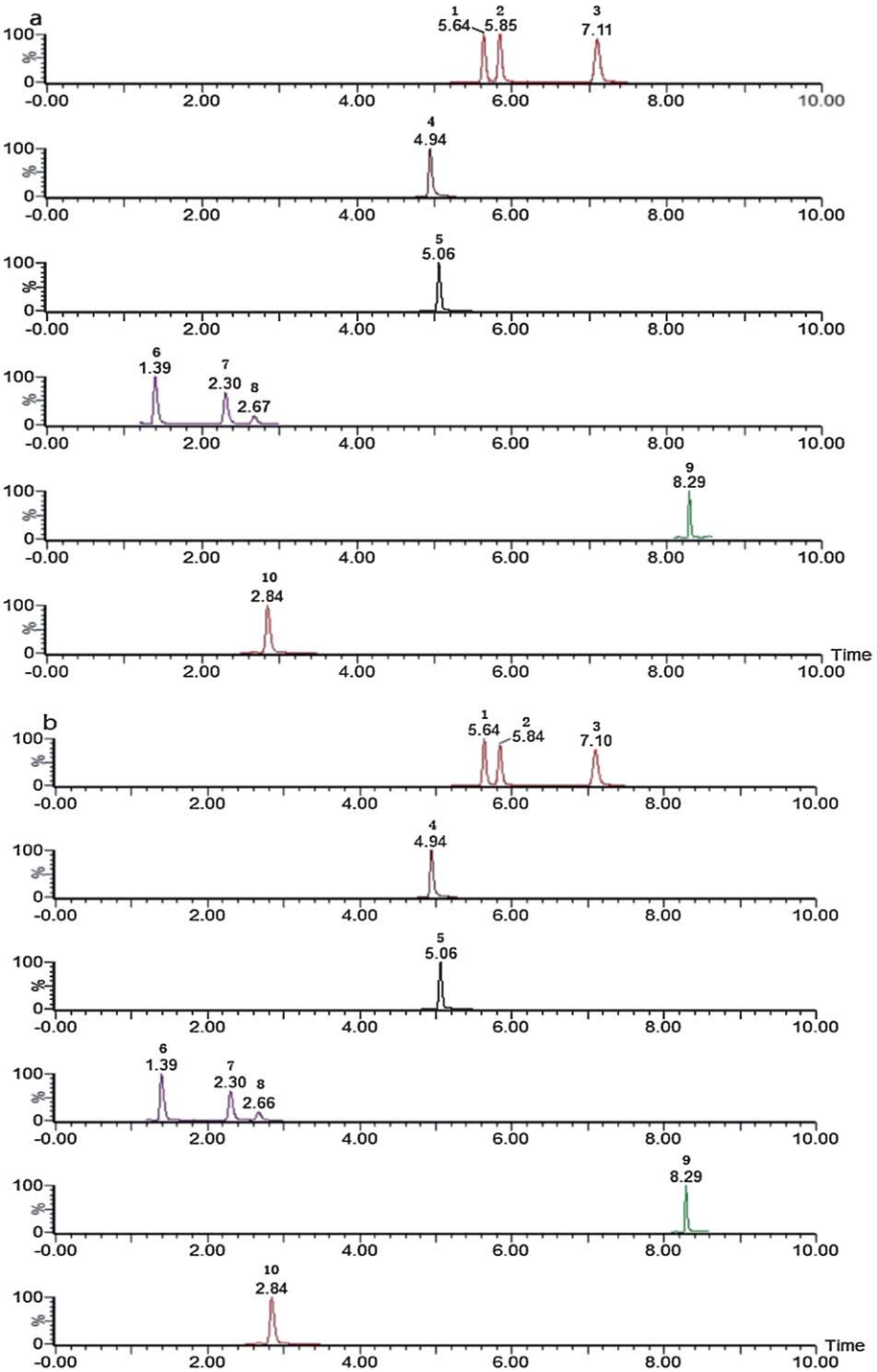

Fig. 2 Typical chromatograms of TIC using UPLC-ESI-MS/MS: (a) standard solution; (b) sample solution. The peaks numbered in the chromatograms denote: (1) VII; (2) VIII; (3) IX; (4) V; (5) VI; (6) I; (7) II; (8) III; (9) X; (10) IV.

Application

The UPLC-ESI-MS/MS method was subsequently applied to simultaneously determine ten bioactive markers in 7 batches of Kudiezi injection obtained from two pharmaceutical factories (A and B). Each sample was analyzed three times and the mean contents are shown in Table 4. The phenolic acid content in sample B was obviously higher than that in sample A. The content of the ten markers varied among the samples owing to different manufacturing processes and different origins of the crude drugs. Whether the differences in content of the ten polyphenols will cause a difference in clinical effect should be further investigated. Among the investigated

Table 2 Linearity of calibration curves, sensitivity, precision and repeatability of method

Analyte	Standard calibration curves					RSD (%)		
	Regression equation ($y = ax + b$)	R^2	Linear range (μg L^{-1})	LODs (μg L^{-1})	LOQs (μg L^{-1})	Intraday ($n = 6$)	Interday ($n = 6$)	Repeatability ($n = 6$)
I	$y = 1512.89x + 1423.41$	0.9999	12.69–406.00	0.63	1.27	0.41	3.03	0.39
II	$y = 1405.57x + 2917.23$	0.9997	10.32–330.40	0.52	1.03	1.19	3.50	0.58
III	$y = 548.43x + 11.81$	0.9998	5.86–187.60	0.59	2.93	1.20	3.75	1.67
IV	$y = 3550.63x + 17694.20$	0.9992	14.33–458.50	0.14	1.43	0.88	1.09	0.75
V	$y = 996.92x + 8348.75$	0.9991	20.82–666.40	0.010	0.052	0.67	4.70	1.38
VI	$y = 3599.89x + 29034.40$	0.9996	19.95–638.40	0.0020	0.0050	1.35	2.43	1.61
VII	$y = 1888.10x + 699.33$	0.9998	5.56–177.80	0.056	0.14	0.70	4.56	1.00
VIII	$y = 1806.03x + 1623.17$	0.9996	2.89–92.40	0.029	0.14	1.16	5.17	1.73
IX	$y = 2083.26x - 123.68$	0.9998	3.17–101.50	0.032	0.32	1.93	3.94	1.80
X	$y = 2388.12x - 2136.50$	0.9990	2.73–87.50	0.014	0.027	0.99	3.05	1.58

compounds, the content of V was the highest (62.937 μg mL^{-1}), followed by VI and IV. The amount of abundant and trace components (X, 0.343 μg mL^{-1}) could be simultaneously determined by the developed analytical method. The total content of polyphenols in the samples ranged from 115.069 μg mL^{-1} to 168.726 μg mL^{-1}. It is believed that the UPLC-ESI-MS/MS method could be helpful to improve the quality control of Kudiezi injection.

Cluster analysis

Hierarchical Cluster (SPSS 16.0) was used for classifying 7 batches of Kudiezi injection with the content of polyphenols.

Table 3 Recoveries of the ten components ($n = 3$)

Analyte	Initial amount (ng)	Added amount (ng)	Detected amount (ng)	Recovery[a] (%)	RSD (%)
I	38.19	18.20	56.45	100.31	1.41
		36.40	74.89	100.83	1.63
		54.60	91.50	97.63	2.50
II	30.16	14.90	45.01	99.71	1.41
		29.80	60.00	100.14	1.25
		44.70	74.00	98.10	1.41
III	21.51	10.50	31.80	98.03	1.17
		21.00	42.72	101.00	1.57
		31.50	52.83	99.43	2.05
IV	105.61	54.55	160.64	100.88	1.58
		109.10	216.13	101.55	1.85
		163.65	269.38	100.07	1.98
V	209.99	105.50	312.8	97.51	2.01
		211.00	425.26	102.02	3.64
		316.50	521.63	98.46	3.71
VI	87.97	44.11	132.58	101.13	0.59
		88.22	176.89	100.79	2.11
		132.33	220.09	99.83	0.75
VII	5.66	2.81	8.46	99.63	1.30
		5.62	11.38	101.75	0.78
		8.43	13.93	98.16	2.15
VIII	5.92	3.02	8.97	101.08	2.01
		6.04	11.87	98.52	2.53
		9.06	14.76	97.60	1.68
IX	6.58	3.25	9.84	100.32	2.73
		6.50	13.12	100.61	1.33
		9.75	16.26	99.362	1.66
X	4.16	2.10	6.25	99.53	3.01
		4.20	8.33	99.30	3.48
		6.30	10.29	97.35	1.05

[a] Recovery (%) = 100 × (detected amount − initial amount)/added amount; the data presented as average of three determinations.

Table 4 The mean contents of ten components in samples A and B ($n = 3$)

	Detected content (μg L^{-1})[a]						
	A				B		
Analyte	111220	111117	120308	120204	11122502	11092501	11081202
I	84.18	76.38	90.94	111.58	234.30	206.37	233.82
II	67.16	61.44	73.91	91.04	181.55	158.59	185.69
III	47.45	43.95	51.38	62.97	129.80	117.63	134.54
IV	180.44	211.22	197.45	194.58	179.17	182.66	144.63
V	534.26	533.86	574.23	663.64	629.37	615.31	619.69
VI	199.18	175.94	162.15	163.60	160.65	175.03	224.02
VII	13.78	11.32	17.52	22.74	45.20	49.25	45.82
VIII	15.15	11.846	19.55	25.13	45.82	48.76	45.24
IX	16.81	18.15	20.52	25.47	49.32	53.19	49.97
X	7.43	6.58	6.66	4.77	3.43	5.62	3.85
Total	1165.84	1150.69	1214.31	1365.52	1658.61	1612.41	1687.27

[a] The mean content of ten components in Kudiezi injection = 100 × detected content (μg L^{-1}).

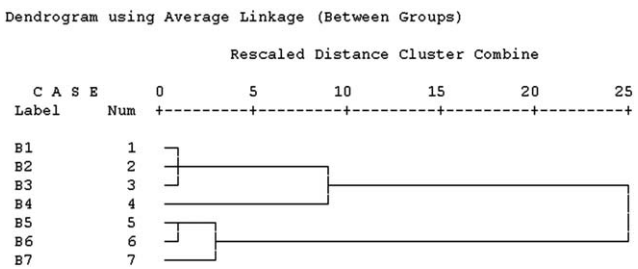

Fig. 3 The dendrogram of hierarchical cluster analysis for 7 batches of Kudiezi injection.

Between-groups Linkage and Squared Euclidean distance were chosen as cluster method and measure-interval in this method, respectively. 7 batches of Kudiezi injection were obviously classified into two groups in the dendrogram (Fig. 3). One cluster is B1, B2, B3 and B4, while another cluster is B5, B6 and B7. The two clusters of Kudiezi injections were produced by two different pharmaceutical factories. Although B4 was clustered in the first group, a slightly different content could be observed. As a result, the cluster method showed that the contents of ten polyphenols could be used to distinguish the samples from different pharmaceutical factories. Meanwhile, it could be used as a simple and rapid quality control method for samples from the same factories.

Conclusions

This is the first report on the simultaneous determination of ten polyphenols in Kudiezi injections from different manufacturers by a UPLC-ESI-MS/MS method. Ten active components were quantified by a validated method with acceptable linearity, LODs, LOQs, precision and accuracy. The high resolution obtained within an extremely short analysis time (within 10 minutes) made UPLC-ESI-MS/MS a rapid and sensitive method for quality control of Kudiezi injections.

Acknowledgements

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