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Liquid Chromatography–Atmospheric Pressure Chemical Ionization Mass Spectrometry as a Tool for the Characterization of Anthraquinone Derivatives from Chinese Herbal Medicine

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Abstract

We have examined the anthraquinone derivatives of a Chinese herb medicine 'Rhubarb' by using HPLC/MS/MS, equipped with an atmospheric pressure chemical ionization source.

The simultaneous determination of five ingredients in the sample extracts by HPLC/MS/MS was demonstrated. Product ion spectra of these components in the extracts were obtained and matched against known standards. Concentrations of these active ingredients were estimated by multiple reaction monitoring in the negative ion mode using a single HPLC run.

It has been estimated that medicinal herbs and their preparations account for 30–50% of the total consumption of medicines in mainland China today (Xiao 1983; Xiao & Chen 1987). In the USA and Europe, interest in low cost herbal therapies has significantly increased in the last decade (Canedy 1998; Greenwald 1998). Some herbal medicines and/or standardized extracts from herbs such as Ginkgo (*Ginkgo biloba*) and St John's Wort (*Hypericum perforatum*), Ginseng (*Panax ginseng* and *P. quinquefolius*), Aloe (*Aloe barbadensis*), Siberian ginseng (*Eleutherococcus senticosus*), Goldenseal (*Hydrastis canadensis*), Kava (*Piper methysticum*), Skullcap (*Scutellaria laterifolia*), and Valerian (*Valeriana officinalis*) have been shown to be safe and effective phytotherapeutic agents (Fugh-Berman & Cott 1999; Mar & Bent 1999).

One of the most important characteristics of herbal medicines is that multiple active substances exist in the crude materials and their extracts, thus characterization of these active constituents is necessary. Traditionally, TLC and HPLC have been used for the characterization of herbal medicine (Committee on Pharmacopoeia of the People's Republic of China 1995). However, the sensitivity and specificity of these techniques presents an increasing technical challenge (Willoughby et al 1998). Compared with TLC and HPLC, GC/MS is more sensitive and accurate. However, quantitative GC/MS methodology requires extensive sample cleanup followed by derivatization (Cui et al 1993, 1997, 1998). It was not until the incompatibility of linking liquid chromatography (LC) with mass spectrometry (MS) was overcome that LC/MS became a routine research tool in analytical chemistry (Henion et al 1983; Games 1987). As a newly developed analytical technique, LC/MS/MS combines high performance liquid chromatography with tandem mass spectrometry (Alexander et al 1987; Weidolf et al 1988). Due to its ability to detect co-eluting,
closely-related substances based on unique precursor/product ion combination (Covey et al 1986), the technique can be used to determine the identity and the content of low levels of drugs and their metabolites in biological fluids with high specificity and sensitivity (Barnes et al 1998; Muck 1999; Singh et al 1999). Analytical methods based on this technique have been demonstrated to be reliable for the determination of drugs and related substances or even characterization and QA/QC (quality assurance/quality control) of traditional herbal medicine (Wang et al 1998; Fuzzati et al 1999).

Rhubarb (*Radix et Rhizoma Rhei*) is the dried root and rhizome of *Rheum palmatum* L., *Rheum tanguticum* Maxim ex Balf or *Rheum officinale*

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Barll (Polygonaceae). It is officially listed in the Chinese Pharmacopoeia. Rhubarb is one of the oldest and best-known Chinese herbal medicines and is used as a laxative, antiphlogistic, and haemostatic in the treatment of constipation, gastrointestinal indigestion, diarrhoea, and jaundice (Tang & Eisenbrand 1992). The most important chemical constituents from Rhubarb are anthraquinone derivatives. The determination of aloe-emodin, rhein, emodin, chrysophanol and physcion has been carried out using TLC and HPLC (Yin & Guo 1995).

In this study, we have explored the application of HPLC-APCI (atmospheric pressure chemical ionization)/MS/MS techniques to the analysis of these anthraquinone derivatives in Rhubarb. We have attempted to establish flexible methods for the accurate and precise determination of the structures and concentrations of multiple substances in one traditional Chinese herbal medicine simultaneously.

Materials and Methods

Chemical standards and materials

Standard chemicals (rhein, emodin, aloe-emodin, chrysophanol and physcion) (Figure 1) were obtained from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Rhubarb was obtained from Ngai Hoi Dispensary, Kowloon, Hong Kong. Sample powders (0·5 g) were extracted with methanol (three times) by means of sonication at room temperature. After removal of the solvent, the extract obtained was dissolved and made up to 10 mL with methanol. After filtration through an Alltech syringe filter (0·2 μm; Beerfield, IL), a known amount of each extract was subjected to HPLC/MS/MS analysis.

LC/MS/MS

LC/MS/MS was performed on a PE-Sciex Model API-365 tandem triple quadruple mass spectrometer (Concord, Ontario, Canada) equipped with

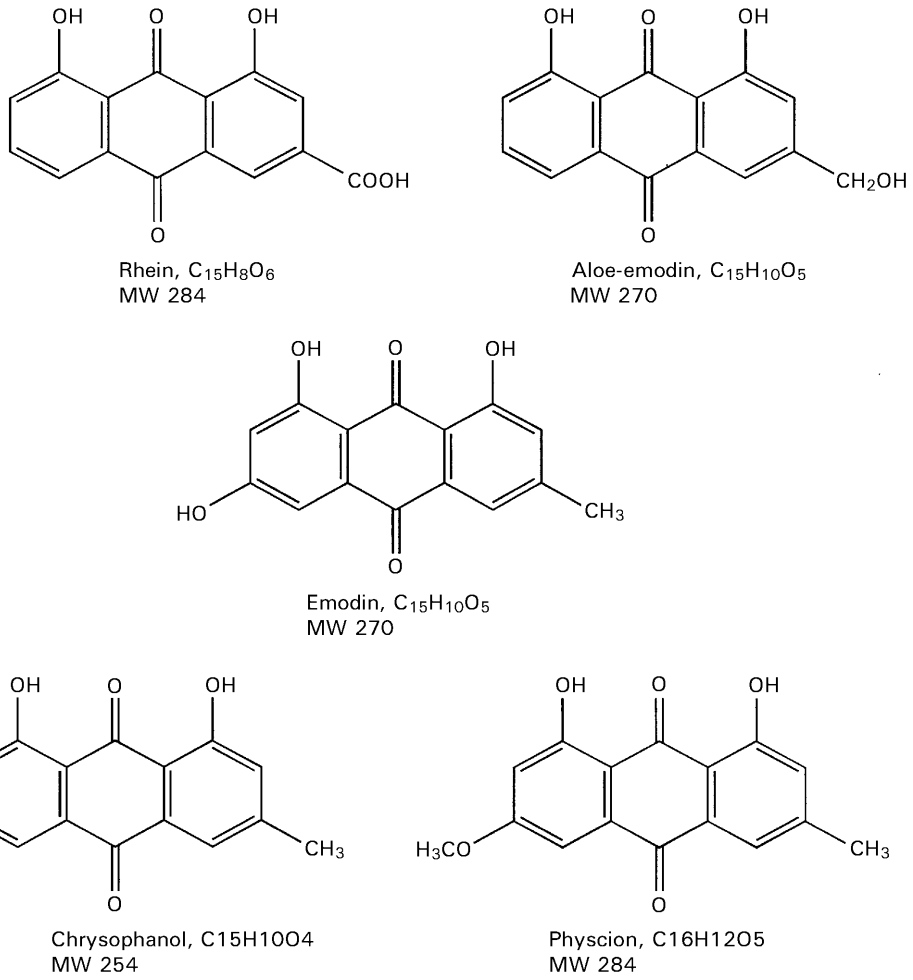

Figure 1. The chemical structures of anthraquinones in Rhubarb.

an APCI Heated Nebulizer Interface, PE 200 Micro LC Pump and PE 785A UV/vis detector. The mass spectrometer was mass calibrated with a polypropylene glycol solution. Multiple looping experiments were used to enhance separation and sensitivity. All samples were analysed in negative ion mode.

Separation was performed on a Supelcosil LC-PAH column (4·6 mm × 15 cm, 5 μm, Serial no. 080138AD) (Bellefonte, PA). The mobile phase composition was optimized as (A) acetonitrile (LAB-SCAN, Bangkok, Thailand) (containing 2% HOAc) and (B) de-ionized water (containing 2% HOAc) at a flow rate of 0·8 mL min^{-1} in a linear gradient over 35 min (Table 1). The sample injection volume was 20 μL.

Nitrogen gas at 70 psi was used as nebulizer gas. Auxiliary gas was air. The APCI temperature was maintained at 450°C. MS conditions were established by injecting pure standard solutions directly into the mass spectrometer with 2% HOAc acetonitrile flowing at 0·8 mL min^{-1} in single-quadruple, product ion and precursor ion scan mode in order to ascertain their precursor ions and to select product ions (for use in multiple reaction monitoring). Collision-induced dissociation experiments were carried out using nitrogen as the target gas. The mass of the precursor ion of interest was scanned in the first quadruple (Q1), m/z selected and collisionally activated in Q2, and the product ions were analysed in the third quadruple (Q3). After verifying the precursor ions of standard compounds, MS/MS was performed using different collision energies ranging from 40 to 60 eV to compare the fragmentation pattern of the targeted compounds. MS/MS instrument parameters were chosen that produced characteristic fragment ions to most of the compounds to be analysed. The most abundant product ion was chosen for determination by multiple reaction monitoring under the following experimental conditions: discharge needle current 2 μA.; orifice voltage −40 V; dwell time 100 ms (for each ion in multiple reaction monitoring), pause 2·0 ms. The ion pairs for multiple reaction monitoring are shown in Table 2.

Table 1. Solvent composition of the gradient of the HPLC analysis.

Time (min)	A(%)	B(%)	Curve
0·00	40	60	Linear
12·5	52	48	Linear
35	90	10	Linear

Quantitative analysis

External calibration curves for all five standard compounds were generated by regression of nominal concentrations against peak area of duplicate calibration standard solutions. Ratios of peak area of each analysed compound to that of a standard were computerized using PE Sciex's MacQuan software. The concentrations of unknown compounds from Rhubarb were determined by interpolation from the corresponding standard curves.

Results and Discussion

LC/MS/MS

The five precursor ions (rhein $(M\text{-}H)^-$, aloe-emodin $(M\text{-}H)^-$, emodin $(M\text{-}H)^-$, chrysophanol $(M\text{-}H)^-$ and physcion $(M\text{-}H)^-$) were confirmed through flow injection of small samples of standard solutions. Product ion mass spectra were obtained in three collision energies in order to characterize fragmentation patterns and to optimize instrument parameters that produced a useful abundance of fragment ions for each compound. Once a set of MS/MS instrument conditions had been selected by evaluating individual product ion spectra, the most abundant fragment ion (or group of fragment ions) for each analyte was chosen to complete the multiple reaction monitoring ion pairs. Table 2 lists the ion pairs in the multiple-reaction monitoring experiments. The product ion spectra of five components in the extract have been matched against

Table 2. List of ion pairs for the multiple-reaction monitoring experiments.

Name	Precursor ion (m/z)	Product ion (m/z)	Analytical MRM pair	Qualifier MRM pair 1	Qualifier MRM pair 2	Retention time (min)
Rhein	283·0	240·3, 210·9, 183·0	283·0/210·9	283·0/240·3	283·0/183·0	3·24
Aloe-emodin	269·0	238·6, 211·1, 182·6	269·0/211·1	269·0/238·6	269·0/182·6	12·7
Emodin	269·0	239·7, 195·0, 182·0	269·0/239·7	269·0/195·0	269·0/182·0	20·0
Chrysophanol	253·1	225·0, 195·0, 182·0	253·1/182·0	253·1/225·0	253·1/195·0	27·2
Physcion	283·0	239·7, 211·1, 183·2	283·0/239·7	283·0/211·1	283·0/183·2	33·3

MRM, multiple reaction monitoring.

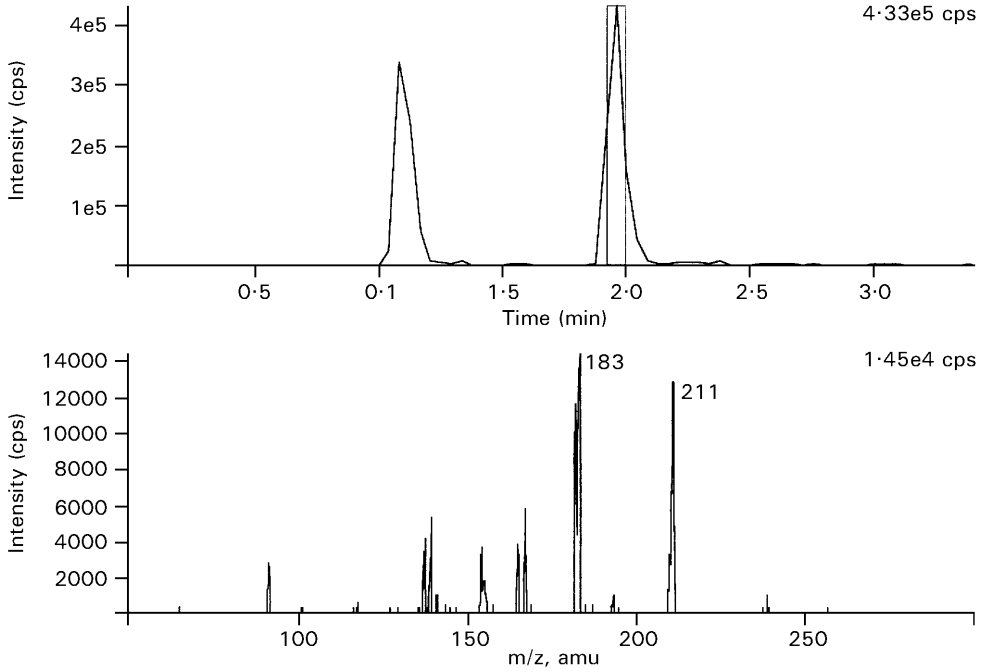

Figure 2. Product ion mass spectrum of rhein with deprotonated molecule as precursor ion.

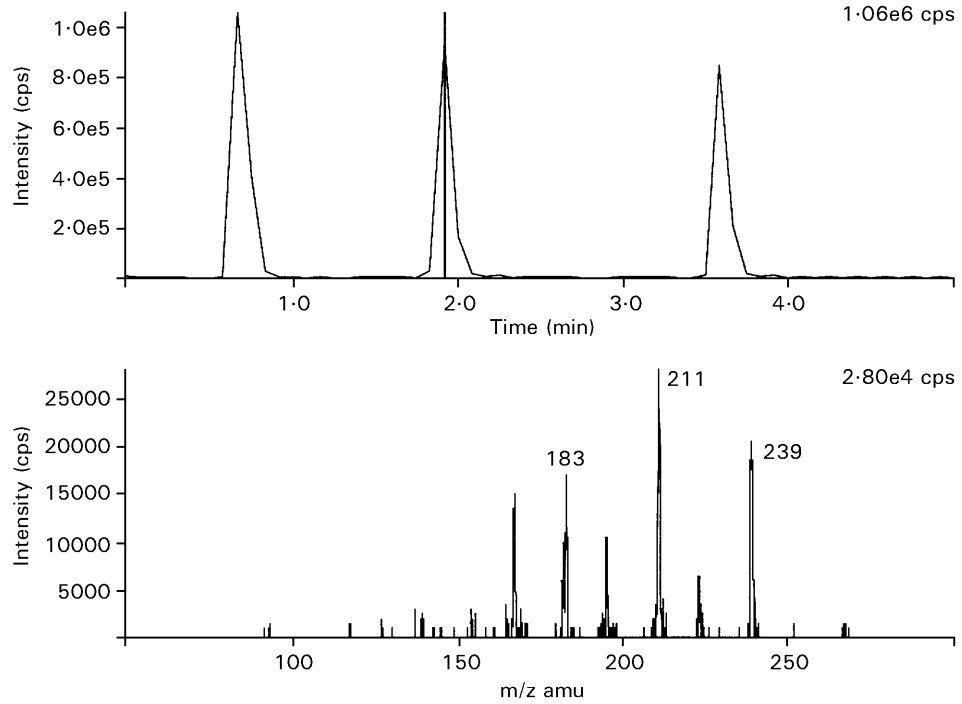

Figure 3. Product ion mass spectrum of aloe-emodin with deprotonated molecule as precursor ion.

those of known standards. The product ion spectra of the compounds are shown in Figures 2–6.

The chromatographic conditions (column packing, mobile phase composition and LC pump flow rate) were chosen to keep analytical run-time to less than 35 min. A typical total ion chromatogram of an extract in negative multiple reaction monitoring mode is shown in Figure 7.

The advantage of multiple reaction monitoring in LC/MS/MS analysis is that it can provide at least two pieces of important information; firstly, the chemical fingerprints for all compounds detected

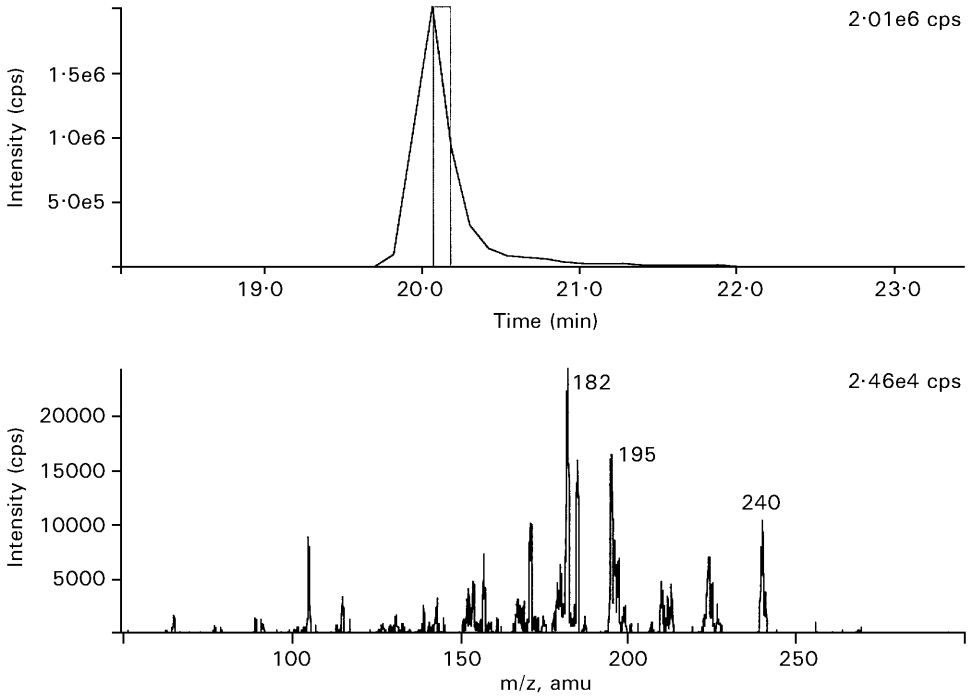

Figure 4. Product ion mass spectrum of emodin with deprotonated molecule as precursor ion.

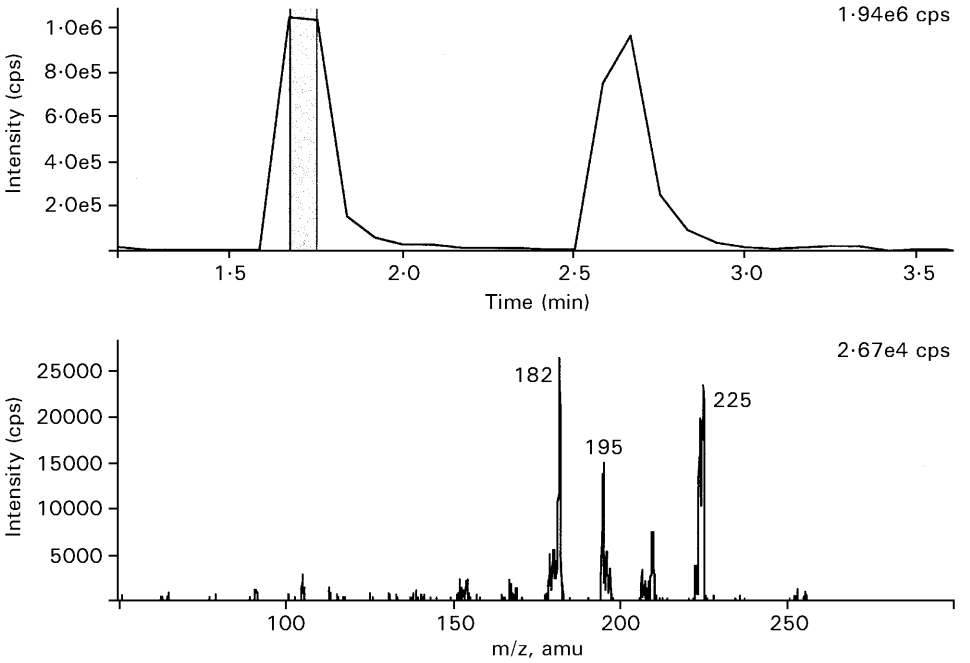

Figure 5. Product ion mass spectrum of chrysophanol with deprotonated molecule as precursor ion.

from the precursor/product ion combination used in the multiple reaction monitoring; and secondly, the concentrations of all compounds detected from each calculated peak area. For example, the multiple reaction monitoring for emodin (at 20·00 min) can only be observed when the precursor ion (m/z 269·0 M-H) can be observed, and the fragment ions (m/z 239, 195, 182) which are generated from this precursor ion are also observed. It is this unique combination of precursor/product ion that gives such high specificity and sensitivity (Wang et al 1998).

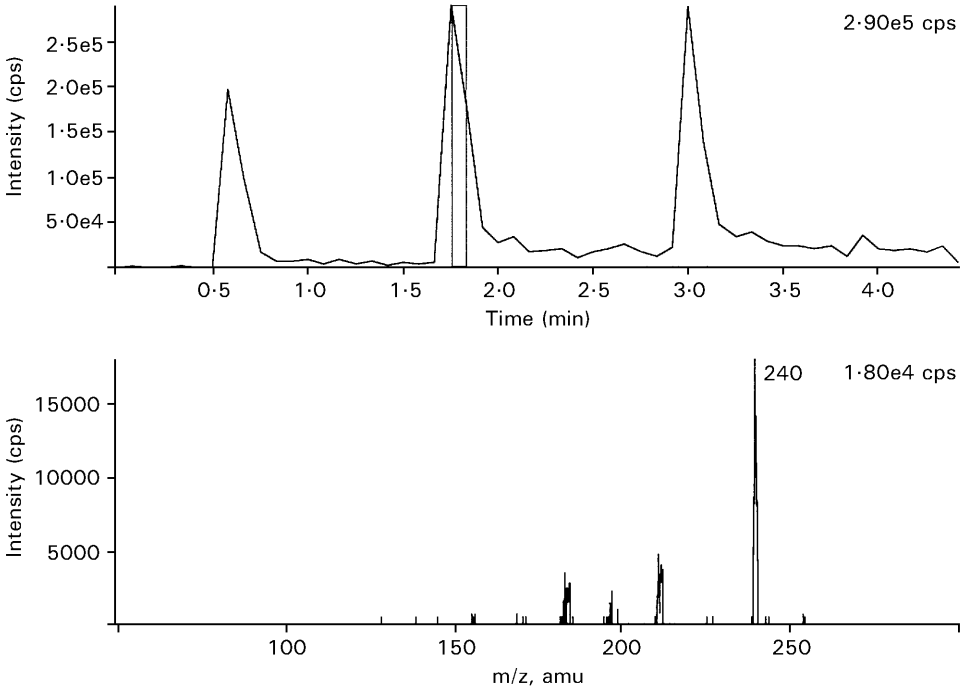

Figure 6. Product ion mass spectrum of physcion with deprotonated molecule as precursor ion.

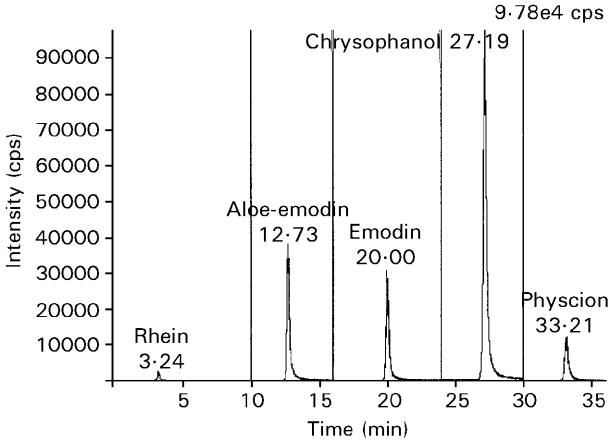

Figure 7. A typical reconstructed ion chromatogram of Rhubarb LC/MS/MS multiple reaction monitoring.

Quantitative analysis

Standard quantities of each analyte were processed along with unknown samples on each analysis. The four non-zero point calibration curves of five standards showed good linearity in a broad concentration range from 110 ng mL^{-1} to 11 μg mL^{-1} (Table 3). The concentrations of rhein, emodin, aloe-emodin, physcion and chrysophanol in the sample were estimated to be 0·16, 0·16, 0·59, 0·53 and 0·34% (w/w), respectively. These concentrations were determined by interpolation from the corresponding standard calibration curves.

Table 3. The calibration curves of standards.

Analyte	Slope	Y-intercept	Correlation coefficient
Rhein	475·3	−8865·0	0·9967
Aloe-emodin	3714·0	−12140·0	0·9999
Emodin	11950·0	−79750·0	0·9996
Chrysophanol	16070·0	−99320·0	0·9997
Physcion	7075·0	−14670·0	0·9997

Conclusion

A total of five compounds (rhein, emodin, aloe-emodin, physcion and chrysophanol) in Rhubarb have been tentatively identified by the described LC/MS/MS method. The mass spectra and retention times of these compounds were comparable with the respective authentic standards. Unique product ions of the extracts were selected for screening in the multiple reaction monitoring mode. Owing to the greatly enhanced selectivity, the interference from the sample matrix was greatly reduced. This resulted in higher sensitivity and was less affected by isobaric interference. A lesser demand on the chromatographic separation resulted. It was also suitable for monitoring the components of Rhubarb in a complex mixture such as that in multi-herb preparations. Therefore, a more drastic gradient profile could be used and hence lead to shorter QA/QC analysis times of traditional Chinese herbal medicines.

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