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Hydrophobic solvent induced phase transition extraction to extract drugs from plasma for high performance liquid chromatography–mass spectrometric analysis

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ABSTRACT

Novel sample preparation approaches for HPLC bioanalysis based on the phenomenon that acetonitrile can be separated from water by adding salts or cooling at subzero temperatures have been reported. These two methods are superior to conventional liquid–liquid extraction since the separated acetonitrile phase can be directly injected to the RP–LC system. However, the salting-out method suffers from a potential problem that the remained salt in the acetonitrile phase may harm the MS detector, while the subzero-temperature method is troublesome to operate. Here, we have reported a similar phase separation phenomenon that the acetonitrile aqueous mixture can be separated by adding a hydrophobic solvent; and capitalising on this phase transition phenomenon, we have proposed an alternative approach, named solvent induced phase transition extraction (SIPTE), to extract drug from plasma for HPLC–MS analysis. The proposed SIPTE method is much simpler and avoids contaminating the MS detector. Three structurally diverse drugs were selected as test compounds to design the SIPTE method and to validate the efficiency of this method. The four goals of plasma sample pretreatment for HPLC–MS analysis, i.e. removal of proteins, removal of other low-molecular interferences, preconcentration of the analytes of interest, and matching the sample solvent with the HPLC–MS system, can be rapidly performed in a very simple step by using the SIPTE method.

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1. Introduction

Plasma is the most important type of biological sample for the evaluation of drugs. The in vivo pharmacokinetic properties of a drug, including absorption, distribution, metabolism and excretion (ADME), which usually involve quantitative measurement of drugs in plasma, are of paramount importance for drug discovery and development in the pharmaceutical industry [1]. The matrix of plasma is extremely complex as it contains high concentrations of proteins, low-molecular weight compounds and inorganic salts. Therefore, it has been a great challenge to quantify compounds of interest in plasma sensitively, accurately and fast. In general, a chromatographic method for the plasma analysis includes different steps, i.e. sample pretreatment, separation of analyte from endogenous components and analyte detection. In recent years, considerable progress in high performance liquid chromatography–mass spectrometry (LC–MS) instrumentation and techniques has been achieved. The existing technologies can provide robust and rapid chromatographic separation and selective and sensitive MS detection of target compounds in plasma [2,3], thus leaving the sample pretreatment as the rate-limiting step in many cases.

Sample pretreatment is an important step as it will clean the complex matrix. In the past few years, a number of new and effective liquid sample preparation methods have been developed, such as liquid phase microextraction [4–6], membrane extraction [7–9], and hollow fibre membrane extraction [10,11], etc; however, they are usually too complex and not rapid enough to be adopted as a common plasma sample pretreatment method in an in vivo pharmacokinetic study. Traditional protein precipitation (PPT), liquid–liquid extraction (LLE) and solid phase extraction (SPE) are still the three most widely used sample preparation techniques for determination of analytes in blood or plasma [2,3,12]. PPT is simple and fast, but the obtained extract still contains a significant amount of impurities which could result in relatively high background in the chromatogram, ion suppression of target analytes

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and column deterioration. LLE provides much cleaner extracts than does PPT, but it is a laborious and relatively slow process since the hydrophobic organic solvent must be evaporated and the dried extract should be redissolved in a hydrophilic solvent (i.e. methanol, acetonitrile or the mobile phase) prior to injection for RP–LC separation. SPE integrates clean-up and preconcentration into one step with high selectivity, but it suffers from relatively complicated procedures, sometimes poor reproducibility and high cost. Therefore, an ideal sample preparation method having the abilities to effectively remove the interferences and automatically enrich the analytes but still be simple and economical is desiderated.

Acetonitrile is hydrophilic and miscible with water, thus LLE based on acetonitrile in common condition is hard to be achieved. However, Rustum in 1989 [13] and Yoshida et al. in 2004 [14] have reported the phase transition phenomenon that acetonitrile can be separated from water by adding salts; and based on these, novel sample preparation methods used for HPLC–UV analysis of drugs in serum were developed. Recently, the feasibility of applying this salting-out method for high throughput HPLC–MS bioanalysis [15,16] or CE analysis [17] has been also demonstrated by other analysts. This approach is essentially an LLE extraction, but it is a new extraction concept and has the following advantages over conventional LLE: first, the homogeneous state of the mixture in the mixing stage results in a substantially superior contact between the solvents, thus avoiding the need of intense agitation and providing higher extractability and reproducibility; second, as the extract solution (acetonitrile) is hydrophilic, higher partition coefficient for analytes especially for the hydrophilic ones will be obtained; third, the recovered acetonitrile solution can be directly injected into the RP–LC system, which greatly simplifies the preparation procedure. However, this method suffers from a severe problem that the recovered acetonitrile phase will contain high concentration of salts [14] which is harmful to the MS detector. This is the key reason that this simple and effective salting-out method has not gained much attention in practical bioanalysis using LC–MS. Alternatively, Yoshida and Akane reported that phase separation of the acetonitrile aqueous mixture can be obtained by cooling at subzero temperatures (approximately −20 °C) as well [18,14], and corresponding serum sample preparation method has been proposed. This method can be compatible with the MS detector, but transferring the extractant in this method is very troublesome to operate since the clear separated system will soon become cloudy when the sample is not refrigerated.

Besides adding salts and cooling the sample at subzero temperatures, we further observed that similar phase separation can be obtained when a hydrophobic solvent such as chloroform is added to the acetonitrile aqueous mixture. Thus, capitalising on this phase transition phenomenon, an alternative sample preparation approach for HPLC–MS analysis of drugs in plasma is proposed in the current study. Plasma is first mixed with acetonitrile; then a drop of chloroform (or another non-oxygenated hydrophobic solvent) is added as the modifier to induce phase separation of the mixture. The separated organic phase with the analytes extracted can be directly subjected to the HPLC–MS system. Compared to the previous salting-out and subzero-temperature methods, this method is much simpler and avoids contaminating the MS detector. In two literatures by Ullmann et al. and Gupta et al., the concept of phase transition extraction (PTE) has been well defined, which is based on the fact that the mixed solution is initially homogeneous but is separated into two phases by varying experimental condition(s), e.g. cooling the sample or adding modifier(s) [19,20]. The method proposed here and both the previous salting-out and subzero-temperature methods all are PTE processes, and we further denoted the proposed method here as solvent induced phase transition extraction (SIPTE).

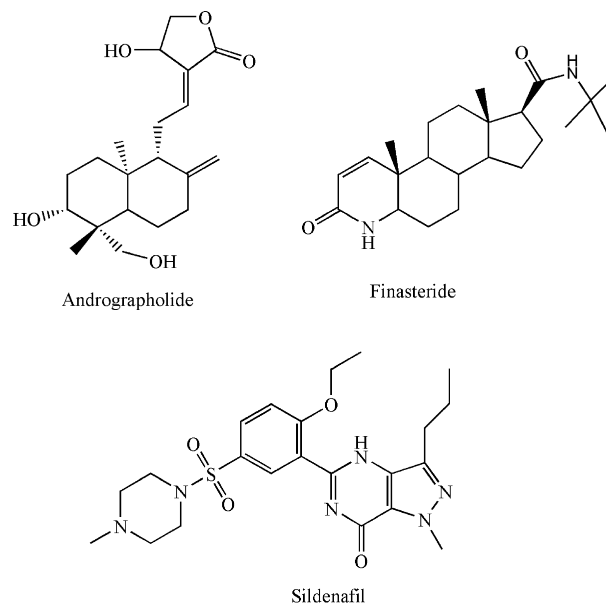

Fig. 1. Structures of the three test drugs.

In this study, three structurally diverse drugs, i.e. andrographolide, sildenafil and finasteride (see Fig. 1) spiked in blank plasma were used as test compounds to design the SIPTE method and to validate the efficiency of this method. The influences of different modifiers, the added amount of acetonitrile and modifier on the extraction efficiency were investigated. The conventional LLE and PPT methods were also used to treat the spiked plasma samples under study, and the results obtained were compared with those provided by the proposed method in terms of matrix effect and final assay sensitivity.

2. Experimental

2.1. Materials

Andrographolide, sildenafil, finasteride and fenfluramine (internal standard (I.S.) for sildenafil used in the method characterisation study) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Baicalein (I.S. for andrographolide used in the characterisation study) and rutaecarpine (I.S. for finasteride used in the characterisation study) were purchased from Shanghai Usea Biotech Company (Shanghai, China). HPLC-grade acetonitrile was purchased from Tedia Company (Fairfield, OH, USA). Ultrapure water was prepared by a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA). Other common reagents including dichloromethane, chloroform, toluene, 1,2-dichloroethane, 1,2-dibromoethane, fluorobenzene, ethyl acetate, ethyl ether, n-hexanol and ammonium formate were all analysis-grade. Blank human plasma was obtained from volunteers and was stored below −40 °C until used for analysis.

2.2. Liquid chromatography

In this SIPTE study, the sample solution is acetonitrile containing chloroform, thus requiring strict demand for the separation column. Indeed, when using the commonly used C_{18} or C_8 column of which the carbon loadings is low (lower than 12%), very broad peaks and shortened retention times were obtained for all test compounds. However, we found that a 5 μm ultimate XB C_{18} column (250 mm × 4.6 mm) of which the carbon loading is 17% (Welch Materials, Ellicott, USA) was insensitive to the presence

of chloroform and other hydrophobic solvents, giving good peak shapes for all test drugs in all SIPTE experiments. Thus this high carbon loading C_{18} column was selected to match with the proposed SIPTE method in this study. Chromatographic separation was performed based on a Waters Alliance 2695 (Milford, MA, USA) HPLC system. For analysis of andrographolide, isocratic elution using acetonitrile–water (55:45, v:v) was performed; for sildenafil, acetonitrile–water containing 20 mM of ammonium formate (70:30, v:v) was used as the mobile phase; for finasteride, isocratic mobile phase of acetonitrile–water containing 20 mM of ammonium formate (78:22, v:v) was used. For all analyses, the flow rate was set at 1 ml/min and the injection volume was 10 μl.

2.3. Mass spectrometry

MS detection was performed on a Micromass ZQ 2000 mass spectrometer with an electrospray ionisation interface (ESI) (Manchester, UK). The outlet of the column was split, and only 0.2 ml/min portion of the column effluent was delivered into ESI source. For respective assays of the three test drugs, the MS parameters were set as follows: for andrographolide, mass spectrometer was operated in negative ion mode, selective ion monitors (SIM) were set at m/z 331 ($[M-H_2O-H]^-$) for andrographolide and at m/z 269 ($[M-H]^-$) for baicalein (I.S.), capillary voltage, 3.5 kV, cone voltage, 35 V, source temperature, 110 °C, desolvation temperature, 300 °C, desolvation gas flow rate, 300 l/h, cone gas flow rate, 40 l/h; for sildenafil, positive ion mode was performed, selective ion monitors (SIM) were set at m/z 475 ($[M+H]^+$) and at m/z 232 ($[M+H]^+$) for sildenafil and fenfluramine (I.S.) respectively, capillary voltage, 4.5 kV, cone voltage, 45 V, source temperature, 105 °C, desolvation temperature, 350 °C, desolvation gas flow rate, 300 l/h, cone gas flow rate, 40 l/h; for finasteride, mass spectrometer was also operated in positive ion mode, selective ion monitors (SIM) were set at m/z 373 ($[M+H]^+$) for finasteride and at m/z 288 ($[M+H]^+$) for rutaecarpine (I.S.), capillary voltage, 4.0 kV, cone voltage, 40 V, source temperature, 110 °C, desolvation temperature, 400 °C, desolvation gas flow rate, 300 l/h, cone gas flow rate, 30 l/h.

2.4. Optimised solvent induced phase transition extraction (SIPTE) method

1 ml of spiked plasma was first mixed well with 0.7 ml of acetonitrile, and then 0.07 ml of chloroform was added. After mild mixing and centrifugation, clear phase separation of the mixed solution was obtained. 0.37 ml of the organic phase (the upper phase) was obtained and 10 μl of it was directly injected to the HPLC–MS system.

2.5. Recovery rate calculation

In the study of investigating the SIPTE conditions, extraction efficiency was evaluated in terms of response (peak area) and recovery rate. SIPTE experiments of respective spiked plasma (i.e. plasma containing 2 μg/ml of andrographolide, 100 ng/ml of sildenafil or 100 ng/ml of finasteride) were performed under several series of different conditions. After phase separation, the organic phase was directly analysed by HPLC–MS. In addition, several blank extract solutions were prepared from blank plasma by using the optimised SIPTE conditions described above (0.37 ml extract solution (the organic phase) was obtained). These blank extract solutions were used to prepare respective post-extraction spiked samples in which the total amount of a corresponding drug is equal to that in 1 ml corresponding spiked plasma investigated. These samples were used as the contrast sample and were assayed after every analysis of the investigated sample. Each experiment was performed in triplicate. Recovery rate was calculated by comparing the total response detected in the organic phase for the investigated sample (the mean detected peak area multiplying the mean measured volume, $A_{org}V_{org}$) with that for the contrast sample ($0.37A_{con}$): recovery rate (%) = $[A_{org}V_{org}/0.37A_{con}] \times 100$.

2.6. Protein precipitation (PPT) method

For comparison purpose, conventional PPT method was used to prepare the spiked plasma under study. Respective spiked plasma samples of the three test drugs was treated by the same PPT method: 1 ml of plasma was mixed with 3 ml of acetonitrile. After vortex-mixing and centrifugation, 10 μl of the supernatant was injected to the HPLC system.

2.7. Liquid–liquid extraction (LLE) method

For comparison purpose, the conventional LLE method was also performed for the spiked plasma under study. To extract andrographolide from plasma, the adopted LLE method is similar to the previous method reported by Gu et al. [21]. 1 ml of plasma was mixed with 4 ml of ethyl acetate, and the mixture was vortex-mixed for 4 min. Following centrifugation, the organic phase was recovered and was dried under a nitrogen stream at 38 °C. The residue was dissolved in 0.37 ml of mobile phase, and after centrifugation 10 μl of this solution was injected to the HPLC–MS system. This method resulted in 60% of andrographolide recovered from plasma.

To extract sildenafil from plasma, the adopted LLE method is similar to the study by Lee et al. [22]. 1 ml of plasma was mixed with 1 ml of 50 mM NaOH solution, and 7 ml of ethyl acetate was added to extract. After mixing (4 min) and centrifugation, the recovered organic layer was taken and dried under a nitrogen stream at 38 °C. The residue was dissolved in 0.37 ml of mobile phase, and after centrifugation 10 μl of this solution was injected to the HPLC–MS system. This method resulted in almost 100% of sildenafil extracted from plasma.

The LLE method adopted for finasteride is the same as the method for sildenafil described above as this method also resulted in almost 100% of finasteride extracted from plasma.

2.8. Matrix effect

Matrix effects of the proposed SIPTE method and the two compared methods (LLE and PPT) for the three test drugs were investigated, respectively. Respective blank extract solutions were prepared from blank plasma processed by different methods described above. These extract solution were used to prepare post-extraction spiked samples: 10 μl of each working solution with an appropriate concentration was mixed with 190 μl of each extract solution to result in spiked extract samples containing 1 μg/ml of andrographolide, 100 ng/ml of sildenafil and 100 ng/ml of finasteride (n = 3 for each), respectively. After mixing and centrifugation, these samples were injected to HPLC–MS. Furthermore, a set of neat standard solutions of 1 μg/ml of andrographolide, 100 ng/ml of sildenafil and 100 ng/ml of finasteride (n = 3 for each) were prepared by diluting corresponding working solutions using corresponding mobile phase and were directly injected into the HPLC–MS system. Matrix effect was expressed as the ratio of the mean peak area of each analyte detected in the spiked extract solution to that detected in the neat solution.

2.9. Method characterisation

To obtain satisfactory characterisation results, three internal standards corresponding to the three test drugs were used (fenfluramine for sildenafil, rutaecarpine for finasteride and baicalein for

andrographolide). Calibration samples of the three test drugs were prepared by adding corresponding standard solutions and internal standard solutions into blank plasma to construct concentrations of 40, 100, 250, 500, 1000 and 2000 ng/ml for andrographolide with baicalein (I.S.) concentration at 200 ng/ml, 2, 10, 50, 100, 333, 667 and 1000 ng/ml for sildenafil with fenfluramine (I.S.) concentration at 50 ng/ml, and 0.5, 10, 50, 100, 333, 667 and 1000 ng/ml for finasteride with rutaecarpine (I.S.) concentration at 50 ng/ml. These calibration standard samples were prepared in triplicate and the standard curves were obtained by least-square linear regression of the mean peak area ratios of analyte to corresponding internal standard versus the concentrations of calibration samples. Furthermore, the quality control (QC) samples at three concentration levels (100, 500 and 2000 ng/ml for andrographolide with baicalein concentration at 200, 10, 100 and 1000 ng/ml for both sildenafil and finasteride with their internal standard concentrations all at 50 ng/ml) in six replicates on the same day and on three successive days were prepared in the same manner to verify the intra- and inter-day precision and accuracy for the proposed SIPTE method. The accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of determined concentration (C_{det}) as follows: accuracy (bias, %) = $[(C_{nom} - C_{det})/C_{nom}] \times 100$. The precision (relative standard deviation, RSD) was calculated from the standard deviation and observed concentration as follows: precision (RSD, %) = [standard deviation (SD)/C_{det}] $\times$ 100.

3. Results and discussion

Acetonitrile is hydrophilic and miscible with water. However, Rustum [13] and Yoshida et al. [14] reported that phase separation of acetonitrile aqueous solution can be obtained by adding a salt. The added strong polar salt may mainly dissolve with water, thus leading to enlarging the polarity difference between water and acetonitrile and consequently resulting in phase separation of this system. Likewise, we found that acetonitrile aqueous solution can be separated by adding a hydrophobic solvent such as chloroform as well. The mechanism of this phenomenon may be a reversed process to the salting-out method: the added chloroform mainly dissolves with acetonitrile and thus results in a decrease of the polarity of acetonitrile, which also leads to enlarging the polarity difference between water and acetonitrile and consequently induces phase separation of acetonitrile aqueous solution. In the present text, this hydrophobic solvent induced phase separation phenomenon was used to develop a novel plasma sample preparation method for HPLC–MS analysis and we named it as solvent induced phase transition extraction (SIPTE).

3.1. *Investigation of SIPTE conditions*

3.1.1. *Effect of using different modifiers*

Besides chloroform, we found that almost all organic solvents which are hydrophobic but still are miscible with acetonitrile can be used as modifiers to result in phase separation of acetonitrile aqueous mixture. Thus, the extraction efficiencies of different SIPTE modes using different modifiers, including six commonly used non-oxygenated organic solvents, i.e. dichloromethane, chloroform, 1,2-didichloroethane, 1,2-dibromoethane, toluene, fluorobenzene, and four oxygenated organic solvents, i.e. ethyl acetate, ethyl ether, n-hexanol and n-octanol, were evaluated here. For this investigation, 0.3 ml of each modifier was added to 2 ml of each spiked plasma–acetonitrile mixture (1:1, v:v). As indicated in Table 1, the results obtained by using the six non-oxygenated modifiers are very similar. High recoveries for all test compounds were obtained in all these cases (higher than 85% for andrographolide while nearly 100% for sildenafil and finasteride). However, when using oxygenated solvents as the modifier, the results are bad since much lower extraction recoveries were obtained (lower than 56, 86 and 85% for andrographolide, sildenafil and finasteride respectively). Furthermore, we found that at least 0.3 ml of a oxygenated modifier should be added to separate 2 ml of the plasma–acetonitrile mixture (1:1, v:v); in contrast, 0.05 ml of a non-oxygenated one was enough to do that. So, all hydrophobic non-oxygenated solvents can be used as proper modifiers for the SIPTE method, whereas the oxygenated solvents are inefficient ones.

Table 1
Recovery rates (%) of the three test drugs in spiked plasma extracted by SIPTE using different modifiers.[a]

	Andrographolide	Sildenafil	Finasteride
Non-oxygenated solvents			
Dichloromethane	87	99	97
Chloroform	86	99	96
1,2-Dichloroethane	89	99	96
1,2-Dibromoethane	90	99	96
Toluene	85	99	97
Fluorobenzene	86	99	98
Oxygenated solvents			
Ethyl acetate	45	86	81
Ethyl ether	55	83	76
n-Hexanol	56	72	85
n-Octanol	55	67	84

[a] Spiked concentrations, 2 μg/ml of andrographolide, 100 ng/ml of sildenafil and finasteride.

3.1.2. *Effect of added amount of acetonitrile and modifier*

Acetonitrile is an efficient deproteinisation reagent for preparation of biological samples in conventional method. In general, at least equal volume of acetonitrile to that of plasma should be added to remove most of plasma proteins prior to HPLC analysis since the unprecipitated proteins may rapidly clog the chromatographic column. However, this issue is avoided in the current study as the unprecipitated proteins will dissolve in the aqueous phase which is not the injection solution. Contrarily, the use of less acetonitrile may result in smaller volume of the recovered organic phase and thus enhance the concentration ratio of analytes. Hence, the effect of the added amount of acetonitrile on the extraction behaviors was investigated. As shown in Fig. 2, the recovered volume of the organic phase was decreased linearly when smaller amount of acetonitrile was used. As a result, as shown in Fig. 3 the response intensity (peak area detected) of each test compound was dramatically increased. Interestingly, the recovery rate of each test drug only varies slightly, high recovery rates, 70, 93 and 87% for andrographolide, sildenafil and finasteride, respectively, were still obtained when only 0.5 ml of acetonitrile was used (Fig. 3). This indicates that, when using the SIPTE method, the assay sensitivity can be greatly enhanced without remarkably sacrificing the recovery rate only by using less acetonitrile.

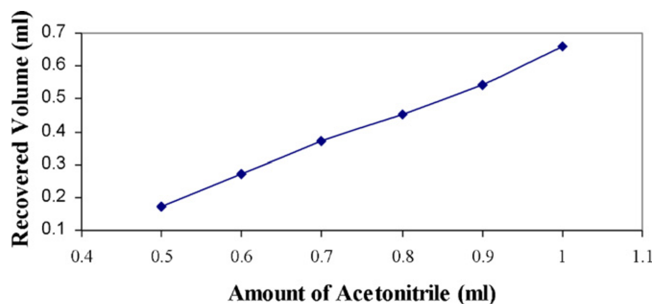

Fig. 2. Effect of added amount of acetonitrile on recovered volume of the organic phase. The ratio of the amount of modifier (chloroform) added subsequently to that of acetonitrile fixed at 0.1.

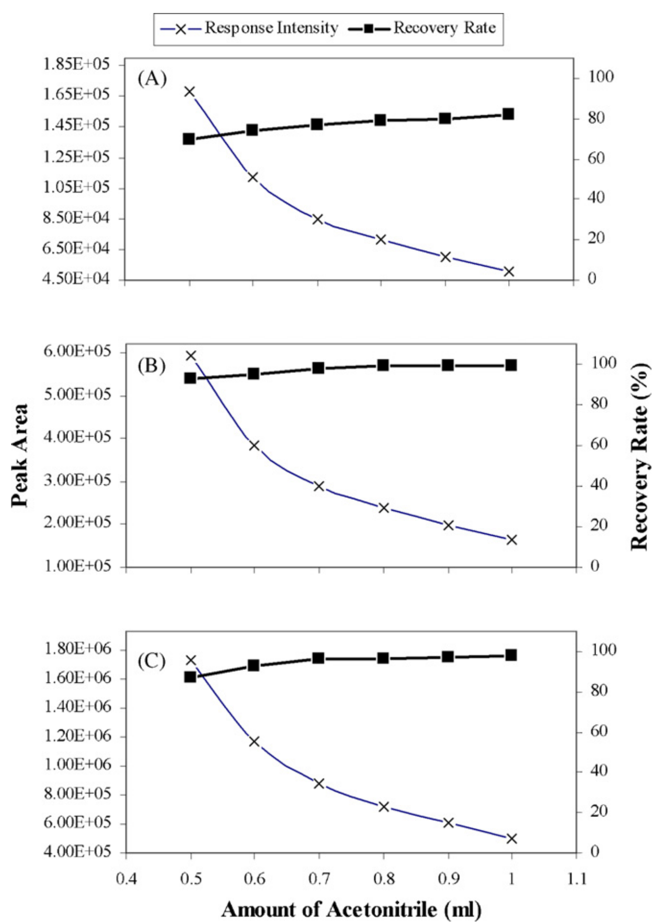

Fig. 3. Variations in response intensities (peak area detected) and recovery rates of andrographolide (A), sildenafil (B) and finasteride (C) as a function of added amount of acetonitrile. The ratio of the amount of modifier (chloroform) added subsequently to that of acetonitrile fixed at 0.1; spiked concentrations, 2 μg/ml for andrographolide, 100 ng/ml for sildenafil and finasteride.

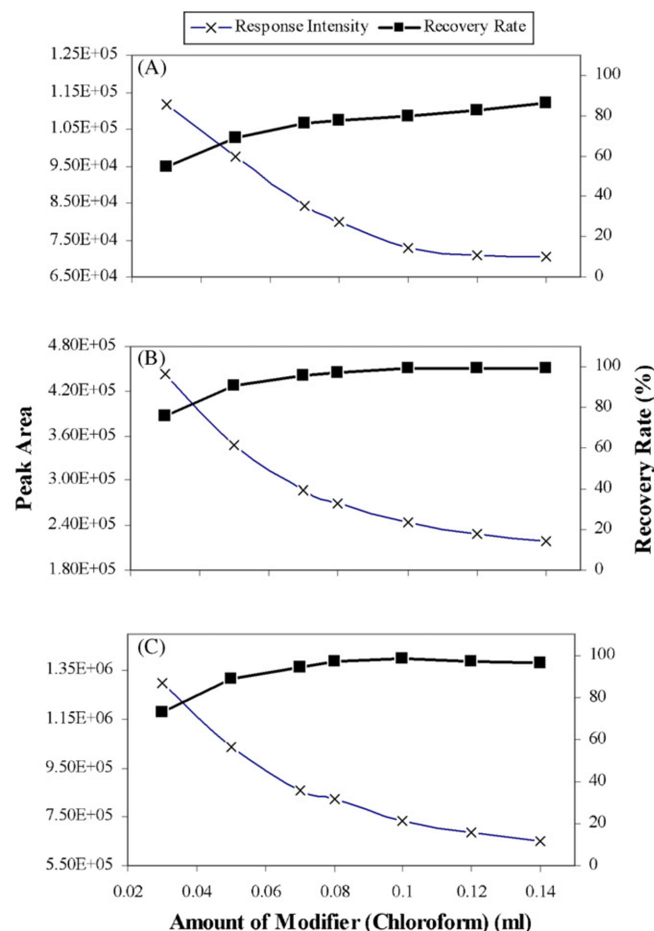

Fig. 5. Variations in response intensities (peak area detected) and recovery rates of andrographolide (A), sildenafil (B) and finasteride (C) as a function of added amount of modifier. The added amount of acetonitrile fixed at 0.7 ml; spiked concentrations, 2 μg/ml for andrographolide, 100 ng/ml for sildenafil and finasteride.

Similar investigation for the added amount of modifier (using chloroform as an example) was also carried out. As expected, the use of smaller amount of modifier resulted in smaller volume of the recovered organic phase (Fig. 4), and thus increased the response intensity of each compound (Fig. 5). However, when too small amount of modifier was used, the recovery rate was decreased remarkably, only 54, 76, and 73% of andrographolide, sildenafil and finasteride, respectively, were recovered when the least amount of modifier (0.03 ml) was used (Fig. 5). Therefore, using less amount of modifier also greatly results in enhancement of the assay sensitivity but decreases the recovery rate.

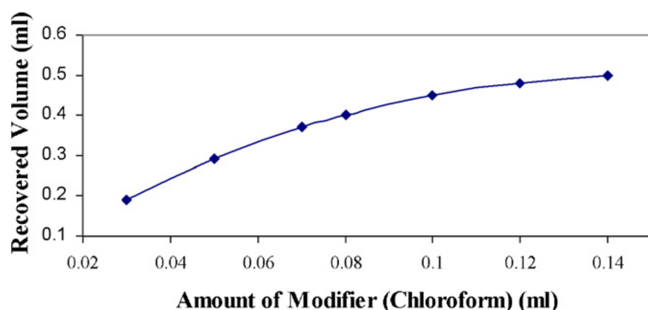

Fig. 4. Effect of added amount of modifier on recovered volume of the organic phase. The added amount of acetonitrile fixed at 0.7 ml.

Additionally, it is necessary to work under such conditions that the volume of the organic phase is easy to handle (that is, at least 0.3–0.4 ml). Thus, the optimised added amounts of acetonitrile and modifier (chloroform) used in the following studies were set at 0.7 and 0.07 ml (to 1 ml of plasma), respectively (0.37 ml of the organic phase was recovered in this case). Using these conditions, the recovery rates for andrographolide, sildenafil and finasteride were 77, 98 and 96%, respectively.

3.2. Comparison with conventional methods

The application of the proposed SIPTE method to, respectively, extract the three test drugs from plasma for HPLC–MS analysis was

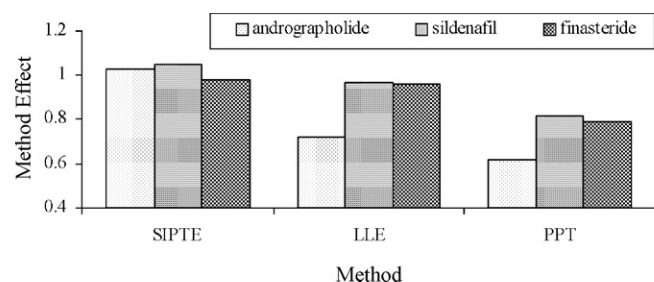

Fig. 6. Matrix effect of the three test drugs in the extract from blank plasma processed by SIPTE, LLE and PPT.

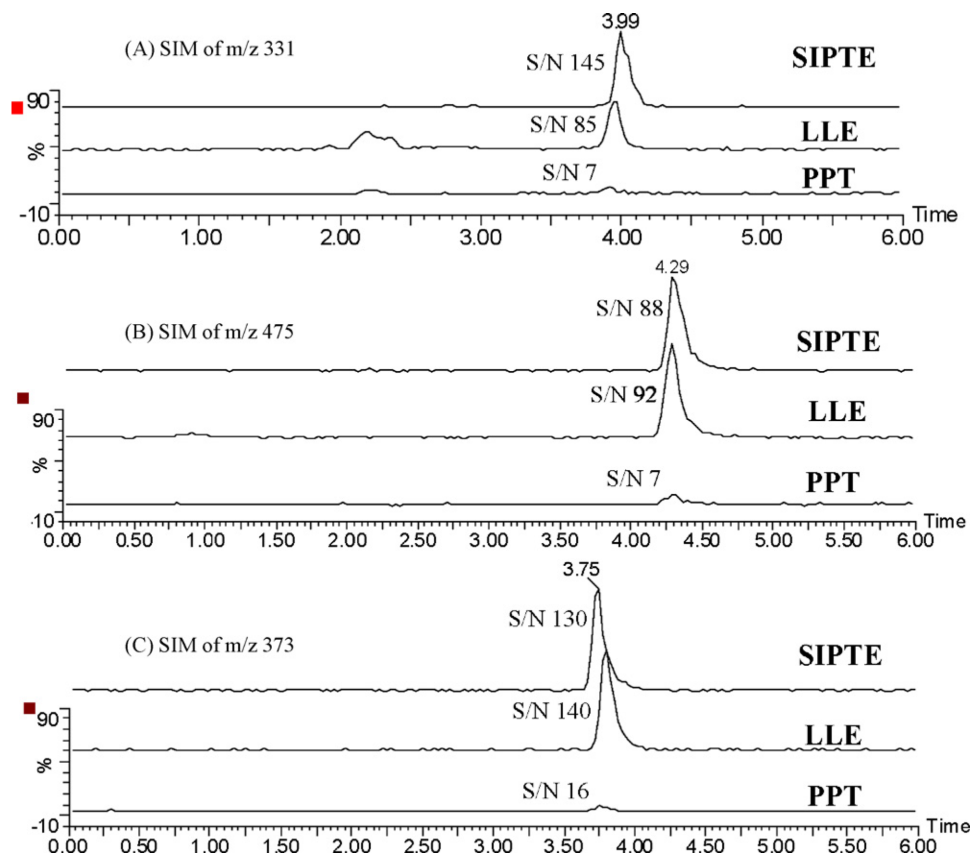

Fig. 7. SIM chromatograms of andrographolide (A), sildenafil (B) and finasteride (C) obtained from HPLC-MS analysis of the same spiked samples but processed by different sample preparation methods. Spiked concentrations, 300 ng/ml of andrographolide, 15 ng/ml of sildenafil and 5 ng/ml for finasteride.

compared with the conventional PPT and LLE methods. First, the matrix effect of each extraction method for each test drug, which reflects the presence or absence of MS signal suppression of target analyte caused by impurities from the extract, was evaluated. In this work, matrix effect was measured as the ratio of the analyte peak area in matrix over that in neat solution (mobile phase). As shown in Fig. 6, matrix effect values for the three test compounds using SIPTE were slightly higher than or close to 1, meaning no (or low) matrix effect. However, matrix effects were clearly observed for the three drugs when PPT was used. Only 59–75% of the signals were left compared with that in neat solution. Matrix effect for andrographolide was also observed when conventional LLE was used. These experimental results indicate that the simple SIPTE method is superior to conventional ones in sample clean-up.

Now the properties of high recovery rate, high preconcentration ratio and no matrix effect have been demonstrated for the SIPTE method, foreshowing the SIPTE method will be characterised with high assay sensitivity. As shown in Fig. 7, SIM chromatograms for each test compound from the same spiked plasma but prepared by different extraction methods were compared. Both signal intensities and signal to noise ratios for the three test drugs using SIPTE are all more than ten times higher than that using PPT, and are equal to or higher than (in the case of andrographolide) that using LLE. Thus, the extraordinary advantage of enhancing assay sensitivity for the proposed SIPTE method is clearly confirmed. The proposed SIPTE method is as simple as the PPT method but provides much higher assay sensitivity due to the functions of automatically preconcentrating the analytes and eliminating the matrix effect. The LLE method can provide similar assay sensitivity for sildenafil and finasteride to the SIPTE method, but this method is much more labor-consuming because of the additional needs of adjusting the pH value, drying the recovered organic solvents and redissolving the extract with mobile phase. The lower signal intensity observed for andrographolide using LLE should be due to the presence of matrix effect and the poor extraction recovery (the recovery rate was only 60% in this case).

3.3. Method characterisation

When 0.7 ml of acetonitrile and 0.07 ml of chloroform were added to 1 ml of plasma, the assay limits of the SIPTE method for andrographolide, sildenafil and finasteride were 40, 2 and 0.5 ng/ml (S/N 10:1; RSD 11.5, 8.5 and 13.4%, respectively), respec-

Table 2
Intra- and inter-precision and accuracy of the SIPTE method for determination of the three test drugs in plasma.

Nominal (ng/ml)	Intra-day		Inter-day	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Andrographolide				
100	4.1	2.0	1.2	−1.8
500	2.2	3.0	2.1	−0.3
2000	2.5	−0.2	5.2	−1.4
Sildenafil				
10	2.8	−1.7	3.4	−5.1
100	3.9	3.5	3.2	0.4
1000	2.7	2.7	1.2	0.8
Finasteride				
10	6.3	−3.0	1.1	−0.9
100	0.3	−0.2	2.6	−0.5
1000	3.7	−0.9	3.5	3.7

tively. Linear calibration curves in the range of 40–2000 ng/ml for andrographolide, 2–1000 ng/ml for sildenafil and 0.5–1000 ng/ml for finasteride spiked in blank plasma were obtained with correlation coefficients (r^2) of 0.9998, 0.9995 and 0.9994, respectively. The intra- and inter-day precisions and accuracies of the SIPTE method were also evaluated. As shown in Table 2, excellent results were obtained for both inter- and intra-day precisions (<6.3%) and accuracies (ranged from −5.1 to 3.7%), reflecting the robustness of the method.

4. Conclusion

The invention and characterisation of the novel SIPTE method for plasma sample preparation prior to HPLC–MS analysis have been reported here. This approach is obviously superior to previous ones in the follow aspects: (1) compared with the previous salting-out and subzero-temperature methods, the proposed SIPTE method is much simpler and can be compatible with the MS detector; (2) compared with the simple PPT method, though the two methods are all simple, the SIPTE method provides ten-fold higher sensitivity; (3) compared with the LLE method, the SIPTE method is much simpler and gives higher sensitivity for weak lipophilic analytes; (4) as the conditions for this method are very mild, degradation of thermal labile analytes will be avoided. In a word, the proposed SIPTE method is superior to conventional ones with much simpler operation and much higher efficiency. For analysis of hydrophobic drugs, the conventionally used LLE and PPT methods can be fully replaced by the SIPTE method. As this method is essentially a phase separation process of the acetonitrile aqueous system, this technique should have the potential for bioanalysis of other matrix such as urine and saliva.

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