Use of Cloud Point Extraction with Derivatizing Reagent for the Extraction and Determination of Isoniazid

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Abstract

A simple and sensitive cloud point extraction high-performance liquid chromatography method is proposed for the determination of isoniazid in blood. The procedure is based on the product of the reaction of isoniazid with benzaldehyde. It can be validated that there is a linear relationship between the signal of isonicotinyl hydrazone and the concentration of isoniazid. A cloud point extraction system of nonionic surfactant Triton X-100 is applied for preconcentration of isonicotinyl hydrazone. Then the analytes in surfactant-rich phase are detected with HPLC-UV system. calibration graph was obtained in the range of 2.0×10^{-3} – 0.5 mg/L, the detection limit was 5.0×10^{-4} mg/L. Method validation is performed on serum samples spiked at two levels, the recoveries ranging from 82.17–83.81%, with relative standard deviations from 2.45% to 3.89%.

Introduction

Because isoniazid, ethambutol, rifampicin, and pyrazinamide are the most effective antitubercular compounds, they are usually administered in combination. The World Health Organization recommend a 6 month regimen comprising previously mentioned drugs, which are given together for the first 2 months, followed by isoniazid and rifampicin therapy for the next 4 months (1). Isoniazid is efficient in the treatment of tuberculosis. On the other hand poisoning accidents, even death, have sometimes happened because of overdosage with isoniazid (2). For this reason, the determination of isoniazid level in human body fluid and pharmaceutical formulations is vital. Although many methods have been reported for the determination of isoniazid (3–7), these methods have involved a extraction technique which suffer from some disadvantages such as a great expense and toxicity of solvents used, so the techniques should be changed in order to reduce their disadvantages.

The cloud point of aqueous solutions of the surfactant micellar systems is a temperature at which the solution becomes turbid before separating into two phases (i.e., a surfactant-rich phase and an aqueous phase). The surfactant-rich phase is able to extract and pre-concentrate analytes. It should be noted that cloud point extraction (CPE) has been applied in many studies concerning the extraction and pre-concentration of analytes from water samples (8,9), soil (10), human serum (11,12), and plants (13–16). The CPE technique can achieve results similar to those obtained by traditional extraction techniques. But, hydrophilic isoniazid can not be satisfyingly extracted with CPE method. The problem can be overcome by derivative reaction of isoniazid.

The derivatization of isoniazid with an aldehyde has been applied for the quantification of isoniazid (17–20). The method was improved by modification and standardization to allow quantitation of isoniazid in biological fluid samples.

In the work, benzaldehyde was added to the samples as a pre-column derivatizing reagent. Isoniazid reacted with benzaldehyde to form isonicotinyl hydrazone, subsequently, the isonicotinyl hydrazone was extracted into a surfactant-rich phase of Triton X-100 aqueous solution by a cloud point procedure. Compared with the absorbance maximum of isoniazid (λ = 269 nm), the wavelength position of isonicotinyl hydrazone is changed to 305 nm. In this case, the background absorbance of Triton X-100 did not overlap with the peak of isonicotinyl hydrazone. Therefore, the surfactant-rich phase was directly injected into the high-performance liquid chromatography (HPLC) system for analysis. Until now, literature concerning this method has not been reported.

The method has major advantages. For instance, the high background absorbance shown by many surfactants in UV region may be eliminated, due to the fact that the surfactants do not absorb at the working wavelength. Also, the extraction efficiency for isonicotinyl hydrazone is raised because the polar character of isonicotinyl hydrazone molecules is lower than that of isoniazid. In addition, the determination sensitivity of isonicotinyl hydrazone is higher because it has a higher molar absorptivity as compared with isoniazid.

Experimental

Reagents and solutions

Isoniazid and non-ionic surfactant Tween 20 (Polyethylene glycol sorbitan monolaurate) were obtained from Sinopharm Chemical Reagent Co. Ltd., (Shanghai, China). Benzaldehyde,

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trichloroacetic acid, non-ionic surfactant Triton X-100 (Isooctyl phenoxy polyethoxy ethanol) and acetonitrile were obtained from LingFeng Reagent Co. Ltd., (Shanghai, China). NaCl, Na_2SO_4, Na_2CO_3, and NaOH were purchased from Nanjing Reagent plant (Jiangsu, China). Acetonitrile is of HPLC-grade, and all other reagents are of analytical reagent grade. Stock solution containing 1.00 g/L of isoniazid was prepared in purified water. A stock solution containing 1.0 g/L of benzaldehyde was prepared with 0.1 mol/L of trichloroacetic acid and 0.2 mol/L of trichloroacetic acid were prepared, the concentration of surfactant aqueous solutions was 300 g/L. Redistilled water was used in all the experiments.

Apparatus

All analysis was performed on a Waters HPLC (Waters, Milford, MA) comprised of a solvent delivery pump (Model 515), an injector with a 20 µL loop, a UV–vis detector (Model 2487) and a Lichrospher C18 column (250 mm × 4.6 mm, particle 5 µm) (Hanbon Reagent Company, Jiangsu, China). A centrifuge from Changsha Pingfan Instrument and Meter Co., Ltd (Hunan, China) was used to accelerate phase separation. A thermostat from Tongzhou Instrument Plant (Jiangsu, China) was applied to maintain the desired temperature within ± 1.0°C. All solutions were degassed by ultrasonication (Kunshan Ultrasonic Instrument Plant, Jiangsu, China).

Derivatization experiments

One milliliter of 0.1 mg/L isoniazid solution was placed in 10.0 mL volumetric flask, followed by addition of 0.5 mL of a solution containing 1.0 g/L of benzaldehyde and 0.5 mL of a solution containing 0.2 mol/L trichloroacetic acid. Finally, the mixture was diluted to 5.0 mL with distilled water.

CPE procedure

5.0 mL solution, prepared as described in the prior section, were placed in 10 mL centrifugal vial, followed by adding 3.0 mL of 30% (w/v) of Triton X-100 solution, 0.15 mL of 1.0 mol/L NaOH and 2.0 g of sodium chloride. The mixture was diluted to the mark with distilled water, and then the vial was left in the water bath at 70°C for 20 min, and surfactant-rich phase and aqueous phase were separated by centrifugation for 5 min at 4000 rpm. The aqueous phase was sucked out by the aid of a syringe and surfactant rich phase was left in the vial. Its volume was a little smaller than 0.5 mL, a preconcentration factor of 10 times could be achieved, but in order to reduce the viscosity, the surfactant-rich phase was diluted to 0.6 mL with mobile phase. Therefore, a preconcentration of 8.3 times was finally obtained. Surfactant-rich phase solution, 20 µL, was injected into the LC system.

Determination of isonicotinyl hydrazone from spiked blood samples

The isoniazid-free blood samples spiked with different concentrations of isoniazid were used to test the applicability of the method. The blood samples were kept at room temperature for approximately 1–2 h to coagulate. The serum was isolated by centrifuged for 10 min at 12000 rpm. The serum (0.5 mL) was mixed with trichloroacetic acid (0.5 mL, 0.2 mol/L) by sonicating for 10 min. After the mixture was centrifugalized for 5 min at 12000 rpm, the supernatant solution was placed in 10 mL centrifugal vial, followed by addition of 0.5 mL of a solution containing 1.0 g/L of benzaldehyde, and then the solution was diluted to the 5.0 mL with distilled water. Then 3.0 mL of 30% (w/v) of Triton X-100 solution, 0.15 mL of 1.0 mol/L NaOH solution, and 2.0 g of NaCl were added, and diluted to 10 mL with doubly distilled water. The mixture solution was left in the water bath at 70°C for 20 min. After centrifugation for 5 min at 4000 rpm, the surfactant-rich phase was diluted to 0.6 mL with mobile phase for the determination.

LC analysis

The HPLC mobile phase was a mixture of acetonitrile–water (70:30, v/v). The flow rate was 0.6 mL/min and wavelength at 305 nm was selected to detect the compounds. 20 µL of the surfactant-rich phase were injected into HPLC system. According to the linear relationship between the signal of isonicotinyl hydrazone and the concentration of isoniazid, the amount of isoniazid was obtained.

Results and Discussion

Relationship of isonicotinyl hydrazone and isoniazid

The absorption spectra of analytes are showed in Figure 1. Isonicotinyl hydrazone shows a maximum of absorption located at 305 nm. The wavelength positions of absorption maximum of isoniazid and benzaldehyde are located at 269 nm and 248 nm, respectively.

The reaction of isoniazid with benzaldehyde was investigated in the presence of trichloroacetic acid at pH 1.7~4.0. The results show the absorbance of reaction product (isonicotinyl hydrazone) increased with pH, reached a maximum at pH = 2 and then gradually decreased.

The effect of concentration of benzaldehyde was investigated at pH = 2. The results show that the absorbance of isonicotinyl hydrazone increased with concentration of benzaldehyde. The

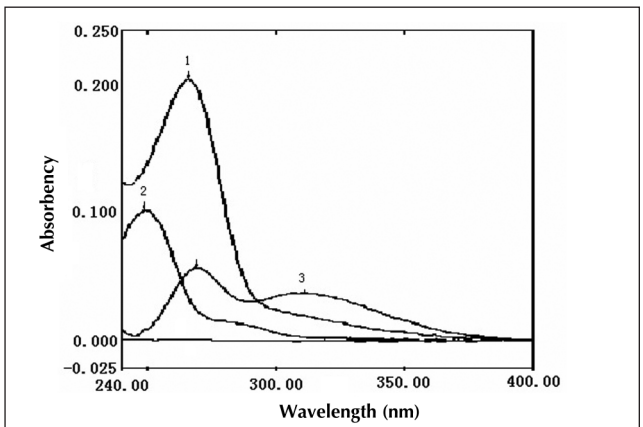

Figure 1. UV absorption curve: (A) 10.0 mg/L isoniazid + 0.02 mol/L trichloroacetic acid, (B) 1.0 mg/L benzaldehyde + 0.02 mol/L trichloroacetic acid, and (C) 10.0 mg/L benzaldehyde + 1.0 mg/L isoniazid + 0.02 mol/L trichloroacetic acid.

absorbance was increased up to 0.1 g/L. From which point onwards, the absorbance remained constant. Under previously mentioned optimum conditions, it was proven there was a linear relationship between the absorbance of isonicotinyl hydrazone and isoniazid in specified concentration range (Figure 2).

CPE procedure

Effect of surfactant concentration on extraction efficiency

The surfactant concentration is an important parameter in CPE procedure because the concentration should be sufficient for the quantitative extraction of the analytes. Triton X-100 and Tween 20 were used as extractant reagents. The effect of concentration of two surfactants on the extraction efficiencies of isonicotinyl hydrazone was studied with NaCl of 20% (w/v), pH = 7 and different concentrations of Triton X-100 or Tween 20 at 70°C. It can be found that the extraction efficiency of isonicotinyl hydrazone increased with concentration of the two surfactants. At Triton X-100 concentration of 9% (w/v), isonicotinyl hydrazone extraction efficiency was ~87%, which was twice as high as that with 9% (w/v) concentration of Tween 20. Beyond 9%, the efficiency remained constant. However, the increase of surfactant concentration would increase volume of surfactant-rich phase, which can induce the decrease of the pre-concentration factor. In order to obtain a good extraction efficiency and high pre-concentration factor, 9% (w/v) of Triton X-100 was chosen as extractant reagent.

Effect of pH on extraction efficiency

The phase behavior was also investigated as a function 9% (w/v) of Triton X-100 at 70°C. The results showed that the recovery of isonicotinyl hydrazone increased over the pH range 3–6. The recovery remained relatively constant when the pH of the solutions increases from 6 to 8. Beyond pH = 8, for isonicotinyl hydrazone was decomposed which leaded to a decrease in recovery. According to the results, pH value was controlled at 7 in the following experiments.

Effect of salt on extraction efficiency

The influence of salt on the recovery of analyte was studied at 70°C as the conditions of the previous sections. The salt concentration had a significant impact on the recovery of isonicotinyl hydrazone. The extraction efficiencies varied from 5% to 88% when concentration of salt increased from 5% to 25%. Among aforementioned three CPE systems, the extraction efficiency of CPE-NaCl system is the highest. Addition of salt accelerated phase separation by enhancing the micellar concentration in the surfactant rich phase, and high concentration of salt induced a fall of temperature of cloud point in CPE procedure. The volume of surfactant rich phase can be decreased by adding salt, which can allow increasing pre-concentration factor, but the phase will become too sticky to be dealt with. Taking the previous data into account, 20% (w/v) NaCl was chosen for cloud point extraction.

Effect of temperature on extraction efficiency

In order to evaluate the effect of temperature on the extraction process, a series of solutions containing Triton X-100 (9%, w/v) and NaCl (20%, w/v) were tested. Pre-concentration factor calculated as the ratio of the volume of surfactant-rich phase (Vu) to the total volume of the solution (VL) used for CPE. Regarding VL/Vu, the general tendency expected would be a decrease of volume of rich phase. As a universal observation, elevated temperature leads to dehydration of the micelle and increase of the phase-volume ratio, therefore, the pre-concentration efficiency can be enhanced. The extraction recovery of isonicotinyl hydrazone increased from 57% to 87% when temperature was elevated from 50°C to 70°C, and the recovery remained constant when temperature is over 70°C. The results obtained indicated that the cloud point process should be studied at 70°C.

The thermal stability of isonicotinyl hydrazone was also studied. The results showed the ultraviolet absorbance of reaction product (isonicotinyl hydrazone) has no significantly changes with temperature increased from 50°C to 70°C.

Effect of equilibration time on extraction efficiency

In order to evaluate the effect of equilibration time on CPE process, a series of solutions was tested under the previously described conditions. The recovery of the analyte increased from 50% to 87% when equilibration time increased from 15 to 20 min. Beyond 20 min, the extraction efficiency became constant. Therefore, equilibration time of 20 min was selected.

Interference studies

Isoniazid, pyrazinamide, rifampicin, and ethambutol are usually administered in combination. Therefore, the effect of other drugs on the determination of isoniazid should be studied. The detection wavelength of ethambutol was 258 nm, and it would not interfere with the detection of isonicotinyl hydrazone. In this work, isonicotinyl hydrazone that was derived from isoniazid was determined. Isonicotinyl hydrazone and pyrazinamide showed very similar spectral characteristics, with absorption maxima at 305 nm for isonicotinyl hydrazone, and at 265 nm and 315 nm for pyrazinamide. However, at 315 nm, the absorption of pyrazinamide was poor, and the effect of pyrazinamide was negligible at the 50 mg/L level. Rifampicin exhibited two absorption maxima at 340 nm and 474 nm. However, the polarities of isonicotinyl hydrazone and rifamipcin showed significance difference. The two components could be easy separated by the LC system.

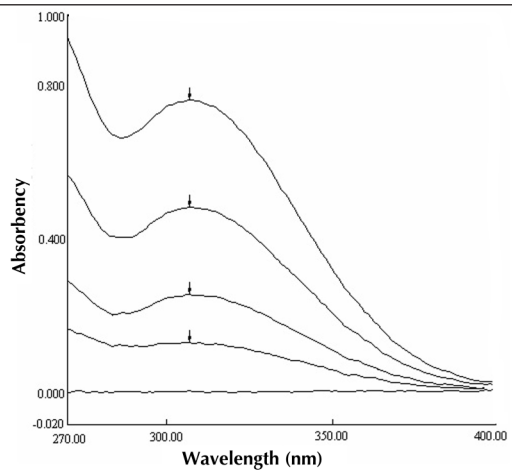

Figure 2. UV absorption curve of isonicotinyl hydrozone in different concentration. conditions: 0.1 g/L benzaldehyde + isoniazid, pH = 2.0.

HPLC characteristic of the components

In order to set up CPE procedure, one of the important problems to study was background absorbance of Triton X-100. The maximum absorption wavelength of isoniazid and isonicotinyl hydrazone were at 269 nm and 305 nm, respectively. Figures 3A and 3C show the chromatograms of aqueous solutions of isoniazid and Triton X-100 at 269 nm. It was found that background absorbance of Triton X-100 can mask chromatographic peak of isoniazid when the working wavelength was at 269 nm.

After isoniazid reacted with benzaldehyde, isonicotinyl hydrazone was formed, a mixture containing Triton X-100, isonicotinyl hydrazone, and benzaldehyde were detected at 305 nm. The components were separated within 7 min, the retention times of Triton X-100, isonicotinyl dydrazone and benzaldehyde were 3.153, 4.245, and 6.245 min, respectively. The target compound was detected at 305 nm, therefore, the background absorbance of Triton X-100 should not interfere with the detection of isonizaid. Figure 3B showed the chromatogram of reacted solution. It could be observed that the intensity of isonicotinyl dydrazone was much higher than that of isoniazid.

Method calibration

The standard solutions containing different concentrations of isoniazid were described previously, and 20 µL of the mixture solution was injected into the chromatographic system as previously described in the chromatographic conditions. It can be proven that there was a linear relationship between the peak areas of isonicotinyl hydrazone and the concentration of isoniazid in a range of 2.0×10^{-3} – 0.5 mg/L. The limit of detection was calculated for isoniazid using a signal/noise ratio (S/N = 3), the result obtained was 5.0×10^{-4} mg/L.

In order to test the reliability of the CPE procedure for the separation and preconcentration of isoniazid, a standard solution at 0.01 mg/L of isoniazid was treated and determined as in the CPE procedure and the LC analysis sections, and 5 replicates were analyzed. Average recovery and relative standard deviation (RSD) were 89.42% and 3.28%, respectively.

Determination of isoniazid in pill and blood sample

The method was applied to the determination of isoniazid in a pill containing 50 mg isoniazid. The pill was pulverized and dissolved by 800 mL purified water. The sample was kept in ultrasonication for 10 min and was finally diluted to 1000 mL with purified water. The sample solution was filtered through 0.45-µm membrane. Aliquots of 1.0 mL of filter liquid were treated as in the CPE conditions section and diluted to 10 mL with solution of NaH_2PO_4 (pH = 6.8), respectively. The former was analysed as in the Liquid chromatographic analysis section and the determination of the latter was based on the parameters specified by USP (21) for gradient HPLC analysis of anti-tuberculosis drugs. The results were shown in Table I. There were no significant differences between two methods.

Isoniazid was spiked into the isoniazid-free blood samples and then were treated as previously described. The chromatogram of one spiked serum sample showed that the peak of isonicotinyl hydrazone was separated from those scattered peaks (Figure 4). The accuracy and precision of the method were determined using five different replicate samples containing 0.02 and 0.1 mg/L of isoniazid, respectively. The values of recovery and relative standard deviation listed in Table II, it was found that the

Table I. Determination of Isoniazid in a Pill*

Methods	Amount (mg)	Found (mg)	Average recovery (%)	RSD‡ (%), *n* = 5
CPE–HPLC	50	45.2	90.4	3.26
USP†	50	46.9	93.8	2.48

* 50 mg per pill
† United States Pharmacopeial. Expert Committee: (PA7) Pharmaceutical Analysis 7. USP28–NF23, Page 1732.
‡ Relative standard deviation, *n* = 5.

Table II. Recovery, Repeatability, and RSD* of Serum with Isoniazid

Methods	Added (mg/L)	Found (mg/L)	RSD† (%) *n* = 5	Recovery (%)
CPE–HPLC	0.02	0.016	2.45	82.17
	0.1	0.084	3.89	83.81
Reference	0.02	–	–	–
method†	0.1	0.098	4.02	98.41

* Relative standard deviation, *n* = 5.
† Nouredine Sadeg, Nicole Pertat, Helene Dutertre, and Michel Dumontet. *J. Chromatogr. B* **675:** 113 (1996).

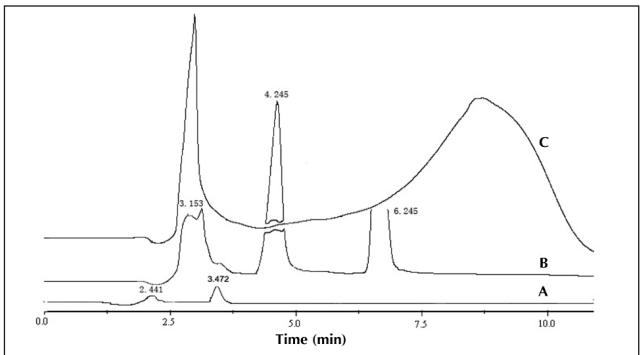

Figure 3. HPLC chromatograms: (A) 0.1 mg/L isoniazid solution, λ = 269 nm. (B) Mixture solution of Triton X-100, isoniazid and benzaldehyde after isoniazid derivatization. λ = 305 nm. Isoniazid 0.1 mg/L, Triton X-100 9% (w/v) and benzaldehyde 0.1 g/L. (C) 9% (w/v) of Triton X-100 solution, λ = 269 nm.

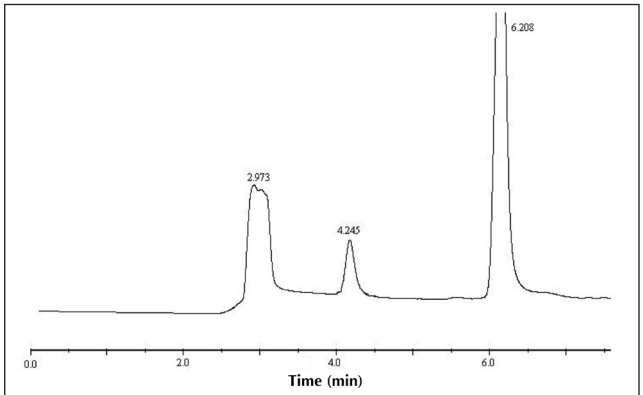

Figure 4. HPLC chromatograms of 0.02 mg/L isoniazid in blood sample after CPE. CPE conditions: 70°C, 20 min, pH 7.0, Triton X-100 9% (w/v), NaCl 20% (w/v). λ = 305 nm.

recovery and repeatability varied from 82.17% to 83.81% and from 2.45% to 3.89%, respectively.

In order to estimate the HPLC method for determination of isoniazid involving CPE, the determination results in blood samples were compared with these obtained by the method of Nouredine Sadeg et al. (19) (Table II). The results shown there were differences between two methods, but LOD of the method was lower than that of Nouredine Sadeg et al.

Conclusions

In order to overcome the problems arising from the use of organic solvents in an extraction procedure, CPE was introduced as a promising separation and extraction technique. However, for a polar compound, isoniazid, extraction efficiency was notably poor and the compound can not be determinated by HPLC–UV method for the absorption of surfactant in UV region. In this work, a simple way to overcome the drawback, which is based on the formation of colored products derived from isoniazid and benzaldelyde, is proposed.

The follow conclusions can be obtained from the present work: (*i*) Compared with the absorbance maximum of isoniazid, the wavelength position of isonicotinyl hydrazone is 305 nm. In this case, the background absorbance of Triton X-100 does not overlap with the peak of targets. Therefore, the surfactant-rich phase was directly analyzed with HPLC system in visible region. (*ii*) Isonicotinyl hydrazone is determined by UV–vis detector, the absorptive signals of isonicotinyl hydrazone is much higher than that of isoniazid and higher concentration factor can be obtained by CPE. Therefore, the sensibility of the new method is much higher than that of determinating isoniazid directly. (*iii*) Organic solvent was not used in procedures of sample treatment and CPE, the analysis method is friendly to both environment and operators.

References

1. P. Nicholas. http://www.mssm.edu/pharmacology/Pharm Course/Syllabus 1998/78/78.htm
2. S.Z. Yao, W.F. Li, X. L. Su, X.B. Zuo, and W.Z. Wei. A sensitive and specific method for isoniazid determination based on selective adsorption using an isoniazid ion-selective piezoelectric sensor. *Talanta* **50:** 469–480 (1999).
3. A. Safavi, M.A. Karimi, and M.R.H. Nezhad. Flow injection determination of isoniazid using Nbromosuccinimide- and *N*-chlorosuccinimide-luminolchemiluminescence systems. *J. Pharm. Bio. Anal.* **30:** 1499–1506 (2003).
4. A.N. Wang, W. Zhang, J. Sun, J.F. Liu, Y.S. Sang, S. Gao, and Z.G. He. HPLC–MS analysis of isoniazid in dog plasma. *Chromatographia* **66:** 741–745 (2007).
5. M.R. Majidi, A. Jouyban, and K. Asadpour-Zeynali, Voltammetric behavior and determination of isoniazidin pharmaceuticals by using overoxidized polypyrrole glassycarbon modified electrode. *J. Electroanal. Chem*. **589:** 32–37 (2006).
6. K. Kitamura, M. Hatta, S. Fukuyama, and K. Hozumi. Determination of isoniazid in tablets by second-derivative ultraviolet spectrophotometry of scraped-spot solutions from thin-layer chromatography. *Anal. Chim. Acta*. **201:** 357–361 (1987).
7. S. Guermouche and M.H. Guermouche. Solid-phase extraction and HPTLC determination of isoniazid and acetylisoniazid in serum comparison with HPLC. *J. Chromatogr. Sci.* **42:** 250–253 (2004).
8. Q. Fang, H.W. Yeung, H.W. Leung, C.W. Huie, Micelle-mediated extraction and preconcentration of ginsenosides from Chinese herbal medi. *J. Chromatogr., A* **904:** 47–55 (2000).
9. L. Wang, Y.Q. Cai, B. He, C.G. Yuan, D.Z. Shen, J. Shao, and G.B. Jiang. Determination of estrogens in water by HPLC–UV using cloud point extraction. *Talanta* **70:** 47–51 (2006).
10. F. Merino, S. Rubio, and D. Pérez-Bendito. A cid-induced cloud point extraction and preconcentration of polycyclic aromatic hydrocarbons from environmental solid samples. *J. Chromatogr., A* **962:** 1–8 (2002).
11. S.R. Sirimanne, J.R. Barr, D.G. Patterson. Quantification of polycyclic aromatic hydrocarbons and polychlorinated dibenzo-p-dioxins in human serum by combined micelle-mediated extraction (cloud-point extraction) and HPLC. *Anal. Chem*. **68:** 1556–1560 (1996).
12. S.R. Sirimanne, D.G. Patterson, L. Ma, and J.B. Justice. Application of cloud-point extraction–reversed-phase high-performance liquid chromatography A preliminary study of the extraction and quantification of vitamins A and E in human serum and whole blood. *J. Chromatogr. B* **716:** 129–137 (1998).
13. Z.H. Shi, J.T. He, and W.B. Chang. Micelle-mediated extraction of tanshinones from Salvia miltiorrhiza bunge with analysis by high-performance liquid chromatography. *Talanta* 64: 401–407 (2004).
14. Q. Fang, H.W. Yeung, H.W. Leung, and C.W. Huie. Micelle-mediated extraction and preconcentration of ginsenosides from Chinese herbal medicine. *J. Chromatogr., A* **904:** 47–55 (2000).
15. W. Liu, W. J. Zhao, J.B. Chen, and M.M. Yang. A cloud point extraction approach using Triton X-100 for the separation and preconcentration of Sudan dyes in chilli powder. *Anal. Chim. Acta.* **605:** 41–45 (2007).
16. H.Z. Zhu, W. Liu, J.W. Mao, and M.M. Yang. Cloud point extraction and determination of trace trichlorfon by high performance liquid chromatography with ultraviolet-detection based on its catalytic effect on benzidine oxidizing. *Anal. Chim. Acta.* **614:** 58–62 (2008).
17. H.I. Seifart, W.L. Gent, D.P. Parkin, P.P. van Jaarsveld, and P.R. Donaid. High-performance liquid chromatographic determination of isoniazid, acetylisoniazid and hydrazine in biological fluids. *J. Chromatogr., B*. **674:** 269–275 (1995).
18. M.Y. Khuhawar and F.M.A. Rind, Liquid chromatographic determination of isoniazid, pyrazinamide and rifampicin from pharmaceutical preparations and blood. *J. Chromatogr., B* **766:** 357–363 (2002).
19. N. Sadeg, N. Pertat, H. Dutertre, and M.l Dumontet. Rapid, specific and sensitive method for isoniazid determination in serum. *J. Chromatogr., B* **675:** 113–117 (1996).
20. Z.M. Zhou, D.Y. Zhao, J. Wang, W.J. Zhao, M.M. Yang. Study of cloud point extraction and high-performance liquid chromatographic determination of isoniazid based on the formation of isonicotinylhydrazone. *J Chromatogr., A* **1216:** 30–35 (2009).
21. The United States Pharmacopeial (USP). Expert Committee: (PA7) Pharmaceutical Analysis 7. USP28–NF23, pp. 1732.

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