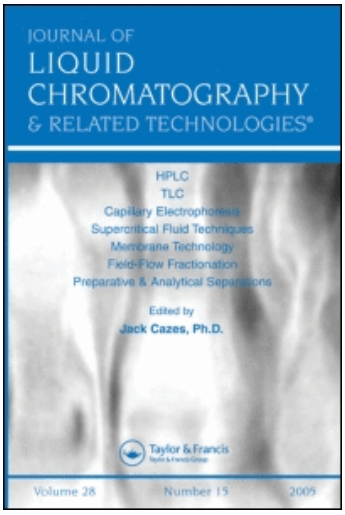

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A SIMPLE AND CONVENIENT METHOD FOR SIMULTANEOUS DETERMINATION OF FOUR MAJOR SPECIES OF ILLEGAL ADDITIVES IN SLIMMING HEALTH FOOD

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A SIMPLE AND CONVENIENT METHOD FOR SIMULTANEOUS DETERMINATION OF FOUR MAJOR SPECIES OF ILLEGAL ADDITIVES IN SLIMMING HEALTH FOOD

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□ *A simple and convenient method for the simultaneous determination of four major species of illegal additives (orlistat, cetilistat, sibutramine, and rimonabant) in health food has been developed. The analytes were separated and quantized by HPLC-PDA at 222 nm. Analytical separation was performed using a gradient elution with acetonitrile and 0.02 mol/L phosphoric acid aqueous solution on a C_{18} column. The recovery of the essay was in the range of 96.1 − 97.2%. The method was reproducible with intra- and inter–day variation of less than 1%. The limit of detection was 189 ng/mL and the calibration curves showed good linearity ($R^2 > 0.999$). In contrast to existing methods, the proposed method has obvious advantages of simplicity, rapidity, and good applicability, and it has been applied to analyze market products successfully.*

Keywords cetilistat, HPLC-PDA, orlistat, rimonabant, sibutramine, simultaneous determination

INTRODUCTION

Obesity is widely considered a major threat to health. In a WHO report from 2002, obesity ranked 5th among the risk factors conditioning health in developed countries, and in developing countries with low mortality rates. It is also expected to play a similar role during the next few decades in other developing countries, where high mortality rates are currently present. The impact of obesity on general health is nowadays also linked to its increasing prevalence worldwide.[1] Because of the prevalence of obesity, the market of health foods for weight reduction is enormous. However, it is helpful for health status of obesity, and can improve the effect of other prescription drugs. In general, synthetic drugs have obvious, fast

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effects, but a series of side effects would restrict its use. To achieve the obvious effects and enlarge the market share, many health foods were added to some kinds of contraband medicine. We aimed at four drugs (orlistat, cetilistat, sibutramine, and rimonabant), which have the most probability to be added from efficacy, price, and present situation of detection.[2–5] Orlistat is a novel, nonsystemically absorbed, anti-obesity agent, which selectively inhibits the absorption of approximately 30% of fatty components from the diet. The major adverse effects with orlistat are gastrointestinal, such as fatty and oily stool, faecal urgency, and oily spotting.[6,7] Cetilistat is a gastrointestinal lipase inhibitor that blocks fat digestion and absorption, leading to reduced energy intake, and thus weight loss. Adverse events were generally mild to moderate in intensity, occurring on only one occasion, such as flatus with discharge and oily spotting. Cetilistat is a newly developed anti-obesity medicine developed by Alizyme plc Company, which has successfully completed an extensive Phase I, Phase II, and Phase III clinical development programme in the West.[8,9] The State Food and Drug Administration (SFDA) of China has not approved that the medicine can be used in China and released the standard method to detect cetilistat. However, it is found out that the cetilistat is added to health food to improve the effect. To our knowledge, no methods for detecting cetilistat have been reported. Sibutramine is a tertiary amine, initially developed as an antidepressant, which inhibits serotonin, norepinephrine, and to a lesser extent, dopamine re-uptake, without presenting monoamine release activity. Sibutramine induces weight loss by reducing food intake, due to the hypophagic effect mediated by central b1, a1, and HT2A/2B/2C receptors. The most common side effects of sibutramine are generally minor, they include headaches, insomnia, anorexia, and dry mouth, changes in mental status (confusion, agitation, and restlessness), neuromuscular symptoms (shivering, ataxia, myoclonus, and hyperreflexia), and autonomic dysfunction (fever, diaphoresis, hypertension, and tachycardia).[10,11] Rimonabant is the first therapeutically relevant cannabinoid antagonist, and it is a selective antagonist of the cannabinoid CB1 receptor, licensed in Europe for treatment of obesity. Many effects have been described, such as attenuation of effects of smoked cannabis, hepatoprotective functions, modulation of cardiometabolic risk factors, anti-inflammatory, and anti-hyperalgesic properties, bronchospasm, and actions on neurotransmission. The most frequent adverse events leading to drug discontinuation in these trials were mood related disorders (depression, suicidal tendencies), which were the reason why the Food and Drug Administration did not yet approve rimonabant in the United States. Beside, many other adverse events have been described in data, such as nausea, dizziness, diarrhoea, and insomnia. Side effects leading to drug discontinuation occurred in 13 – 16% of patients taking the 20 mg dose. In

RIO-Europe, RIO-North America, and RIO-Lipids, drug discontinuation due to psychiatric disorders (mainly depression) occurred in 6 – 7% of rimonabant treated individuals.[12–15]

The above mentioned four synthetic drugs are prescription drugs and forbidden to be added into health foods. The structures of these drugs are illustrated in Figure 1.

Although many illegal dealers often add one or two of the four synthetic drugs into health food to pursue a remarkable profit, there were very few methods reported to determine them. The orlistat is generally determined by a HPLC-UV method,[16,17] the sibutramine can be detected by the LS/MS, or HPLC-UV method,[18] rimonabant is detected by the HPLC-MS method.[19–22] Maybe because cetilistat is a newly developed medicine, which has not been come into market, the detection method has not been described in data. Beside, these seldom reported methods are complicated, time consuming, and need different analytical instruments. It is not suitable for the undeveloped or developing areas to monitor the heath food. Also, it is not suitable for a quick assay in bulk. However, to our knowledge, there is a dearth of analytical methods reported in the literature to detect four of them at the same time.

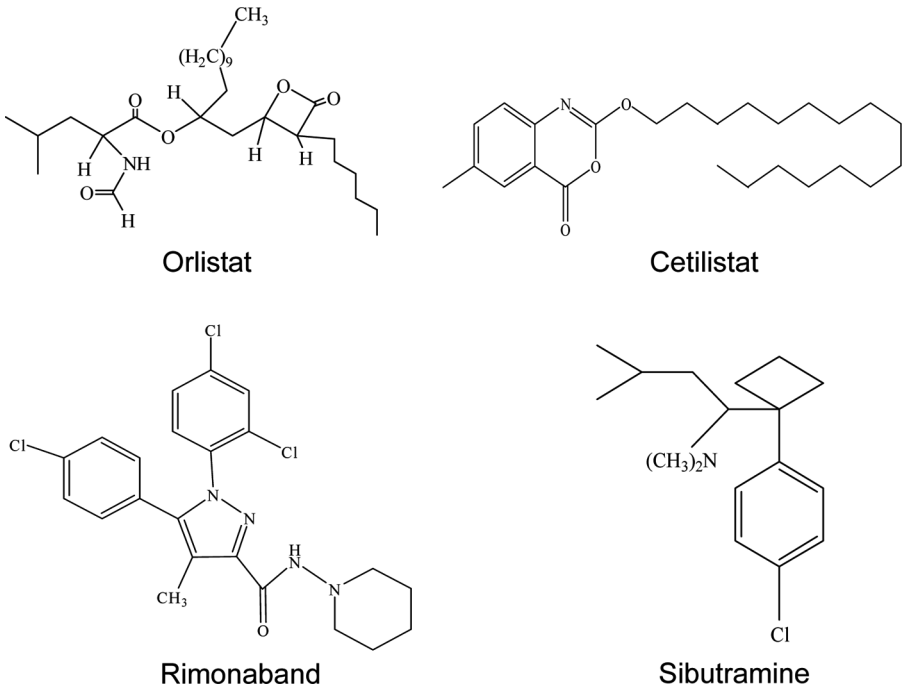

FIGURE 1 Structures of the studied compounds.

EXPERIMENTAL

Reagents

Methanol, acetonitrile were of HPLC grade. Ultrapure water obtained from Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. Analytical grade phosphoric acid and ammonium acetate (Yongda, Tianjin, China) was used for preparation of the mobile phase.

The standards for quantitative analysis were orlistat, cetilistat, sibutramine, and rimonabant purchased from DIO company (Kun Ming, China).

Instrumentation and Analytical Conditions

HPLC system 2695 series (Waters Technologies, USA) equipped with Empower 2.0 software (Waters Technologies, USA), comprised of a quaternary solvent delivery pump, an online vacuum degasser, an auto sampler, a thermostated compartment, and photodiode array (PDA) detector, were used for the chromatographic analysis. All separations were carried out on a Thermo C_{18} column (250 mm × 4.6 mm i.d. with 5.0 μm particle size).

Mobile phase A was acetonitrile and phase B was 0.02 mol/L phosphoric acid aqueous solution (pH 3.0). The linear gradient condition (from 38% to 45% A for 0–4 min and kept at 45% until 9 min, from 45% to 55% A for 9–13 min and kept at 55% until 15 min, from 55% to 62% A for 15–20 min and kept at 62% until 31 min, from 62% to 38% A for 31–32 min) was applied for the separation. The flow rate was 1.0 mL/min, column temperature was maintained at 30°C, effluent was monitored at 222 nm, and injection volume was 20 μL. The peak identification was based on the retention time and the PDA spectrum against the standard presented in the chromatogram.

Preparation of the Standard Solution

Quantification was based on the external standard method. The stock solutions of each analyte were prepared by dissolving them in methanol (1 mg/mL). The stock solutions were diluted and then mixed to produce the working solutions with concentrations of 2.0, 4.0, 10.0, 20.0, 100, and 200 μg/mL for each analyte. Both the stock and working solutions were stored at 4°C.

Sample Extraction Procedure

The sample powder (0.1 g) was extracted with 15% aqueous methanol (40.00 mL) for 15 min in the ultrasonic bath at 60°C. After cooling, it was diluted with methanol to 50 mL in a volumetric flask, and then sealed.

Subsequently, 1 mL of the solution was diluted with methanol to 10 mL in a volumetric flask. This diluted solution was filtered through a syringe filter (0.45 μm) before injection.

RESULTS AND DISCUSSION

Optimization of Wavelength

Orlistat, cetilistat, sibutramine and rimonabant was scanned by the PDA detector. As can be seen in Figure 2, the maximum absorbance values of the orlistat, cetilistat, sibutramine, and rimonabant are 219.2 nm, 221.5 nm, 222.7 nm, and 222 nm. Thus, in this study the detection wavelength was set at 222 nm.

Optimization of Mobile Phase

In this study, the composition of the mobile phase was optimized by adding different acids (ammonium acetate and phosphoric acids) to the aqueous phase. As a result, a mobile phase containing phosphoric acid was selected together with acetonitrile, which gave satisfactory resolution and a stable baseline. To improve separation selectivity and increase efficiency, different percentages of phosphoric acid were investigated. Finally, a mobile phase consisting of acetonitrile and 0.02 mol/L

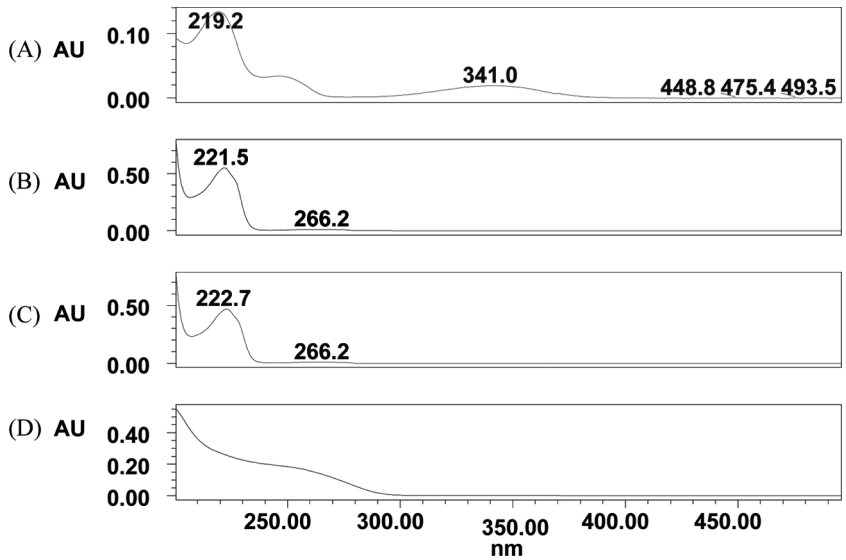

FIGURE 2 The ultraviolet absorption spectrum of orlistat, cetilistat, sibutramine, and rimonabant. (A): orlistat, (B): cetilistat, (C): sibutramine, (D): rimonabant.

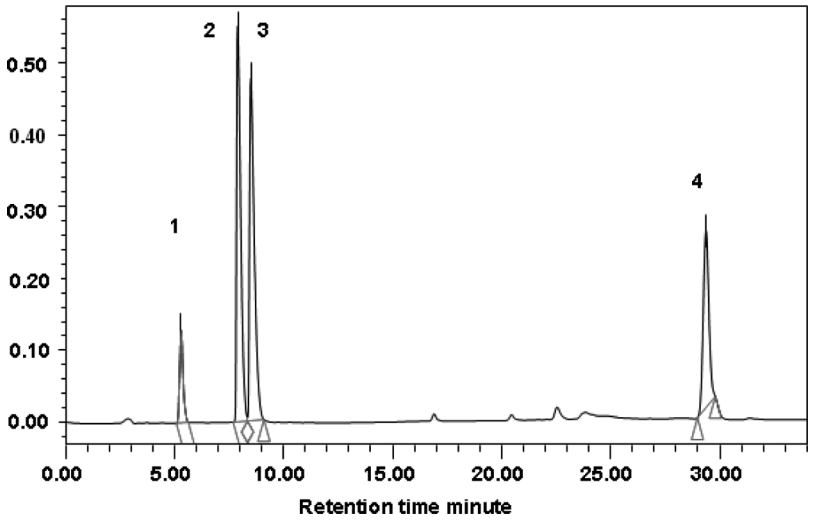

FIGURE 3 The HPLC chromatograms of 4 standards. 1: orlistat, 2: cetilistat, 3: sibutramine, 4: rimonabant.

phosphoric acid was chosen for the detection. Typical HPLC-PDA chromatograms of the standards of orlistat, cetilistat, sibutramine, and rimonabant are presented in Figure 3.

Preparation of the Standard Curve

The calibration curve of each analyte was found to be linear over the studied range of $2.0 - 200.0\,\mu g/mL$. The linear regression equations for compounds were expressed as $A = mC+c$, where C is the concentration, A is the peak area of the standards, and m and c are constants (Table 1). A good linearity (correlation coefficient >0.999) is shown for each calibration curve.

Precision

The instrument precision was examined by six replicated injections of $20.0\,\mu g/mL$ mixture of the standard solutions used above. The precision result of the solution was presented in Table 2.

TABLE 1 Linear Relationship Between Peak Area and Concentration

Components	Regression Equation	R^2	Linear Range(μg/mL)
Orlistat	$A = 0.970 \times 10^4 C - 9.04 \times 10^4$	$r^2 = 0.9998$	2.0∼200.0
Cetilistat	$A = 4.79 \times 10^4 C - 1.22 \times 10^4$	$r^2 = 0.9999$	2.0∼200.0
Sibutramine	$A = 4.67 \times 10^4 C - 4.12 \times 10^4$	$r^2 = 0.9998$	2.0∼200.0
Rimonabant	$A = 3.58 \times 10^4 C + 1.55 \times 10^4$	$r^2 = 0.9999$	2.0∼200.0

TABLE 2 Recoveries of Orlistat, Cetilistat, Sibutramine, and Rimonabant from the Samples (n = 6)

	Orlistat (μg/mL)	Cetilistat (μg/mL)	Sibutramine (μg/mL)	Rimonabant (μg/mL)
1	9.87	9.92	9.69	9.85
2	9.75	9.90	9.67	9.81
3	9.82	9.82	9.54	9.38
4	9.75	9.52	9.40	9.25
5	9.43	9.62	9.64	9.66
6	9.64	9.55	9.78	9.71
Quantity found (μg/mL)[a]	9.71	9.72	9.62	9.61
Quantity added (μg/mL)	10.0	10.0	10.0	10.0
Recovery (%)[b]	97.1	97.2	96.2	96.1
RSD (%)	1.62	1.85	1.38	2.52

[a]Data are means from six experiments (n = 6).
[b]Calculated as [(amount detected)/(amount added)] × 100.

Stability

For the stability test, the same sample solution was analyzed at 0 h, 2 h, 4 h, 12 h, 24 h, and 48 h at the room temperature. The RSD of contents of them ranged between 0.25% and 0.73%, which indicated that it was stable over 2 days under the experimental conditions.

Limit of Detection

Signals three times higher than the peak noise height were regarded as the limit of detection (LOD). Among them, the cetilistat has the highest LOD which is $\leq$189 ng/mL.

Recovery

Recovery was examined by adding 100.0 μg/mL mixture of standard solutions used above to 0.10 g samples that have been proven to not contain analytes. The samples were extracted and analyzed by using the method described above. Each sample was tested six times. Table 2 shows the recoveries of the components from the samples.

Application

Using the described method, orlistat, cetilistat, sibutramine, and rimonabant were analyzed in 50 market products from different companies in China. Results of the study showed that they did not contain these

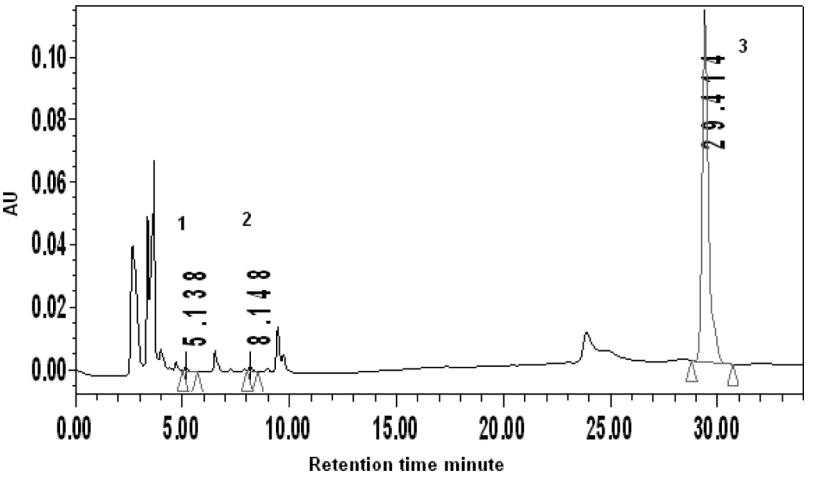

FIGURE 4 The HPLC chromatograms of the market product.

compounds. The results are in agreement with those obtained, when the official QC report was applied.

In general, it is not allowed that the related government department provides the sample to any institution. Although we spent a lot of time to file an application to the related department, we only got one sample containing rimonabant. We used the proposed method to analyze the sample. The representative HPLC-PDA chromatogram is presented as Figure 4 and the ultraviolet absorption spectrum are presented as Figure 5. The

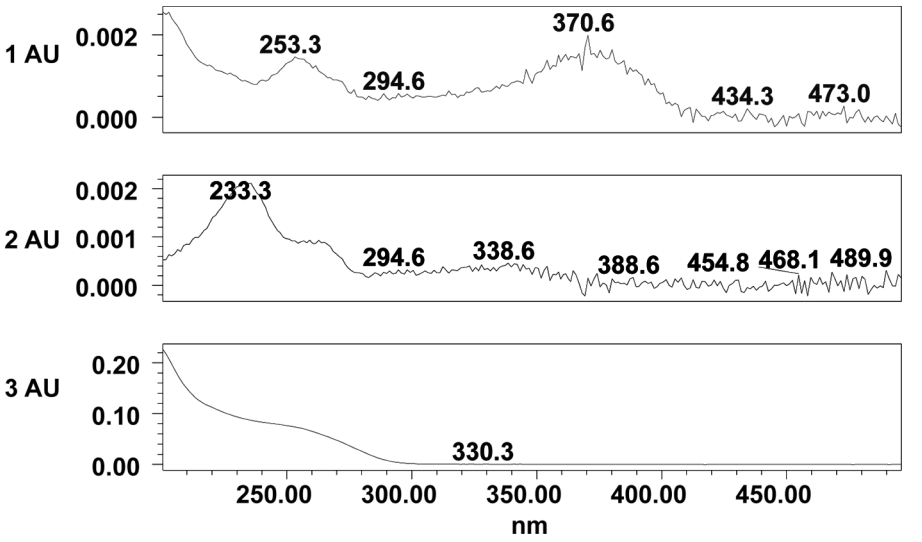

FIGURE 5 The ultraviolet absorption spectrum of three peaks of the analyte.

retention time of the Peak 1, Peak 2, and Peak 3 suggested the possibility of the presence of the orlistat, cetilistat, and rimonabant. However, the comparison of their ultraviolet absorption spectrum with the standard ultraviolet absorption spectrum showed that the Peak 1 and Peak 2 were not produced by orlistat and cetilistat and the peak 3 was from rimonabant. The results were identical with what was issued by the government.

CONCLUSION

Orlistat, cetilistat, sibutramine, and rimonabant are the most common illegal additives in health food for controlling body food. It is necessary to establish a convenient and reliable analytical method to detect them at the same time. However, there is a lack of reported analytical methods to detect these four components at the same time. Also, there is no method for simultaneously detecting two or three of them reported in the literature. It is the first developed method to detect all of them within one analyses.

In the present study, a sensitive and prompt analytical method for simultaneously detecting orlistat, cetilistat, sibutramine, and rimonabant in health food was developed. High linearity, repeatability, intra-day and inter-day assay precision, accuracy, and reliability were presented in the method validation procedure. From a practical point of view, the method described in this research allows a simple, rapid, and sensitive determination of of orlistat, cetilistat, sibutramine, and rimonabant in health food using HPLC-PDA at a single wavelength of 222 nm. It is promising to improve the quality controls of health food and other food supplementary.

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