Rapid Communication

Application of HRAM screening and LC–MS/MS confirmation of active pharmaceutical ingredient in “natural” herbal supplements

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Abstract

The growing market of herbal remedies worldwide could pose severe problems to consumers’ health due to the present possibility of potentially harmful, undeclared synthetic substances or analogues of prescription drugs. The present work shows a simple but effective approach to unequivocally identify synthetic anorectic compounds in allegedly ‘natural’ herbal extracts, by exploiting liquid chromatography/time-of-flight (Q-TOF LC/MS) technology coupled to liquid chromatography/triple quadrupole (LC–MS/MS) confirmation and quantitation. The procedure was applied to five tea herbal extracts and pills sold as coadjuvant for weight loss. The method exploited liquid–liquid sample extraction (LLE) and separation in a C18 (2.1 mm × 150 mm, 1.8 μm) column. QTOF acquisitions were carried out both in scan mode and all ion MS/MS mode and results were obtained after search against ad hoc prepared library. Sibutramine, 4-hydroxyamphetamine, caffeine and theophylline were preliminary identified samples. Confirmation and quantitation of the preliminary identified compounds were obtained in LC–MS/MS after preparation of appropriated standards. Sibutramine, caffeine and theophylline were finally confirmed and quantitate.

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1. Introduction

Adulteration of botanical food supplements with undeclared synthetic drugs to improve their effectiveness is becoming a widespread and mostly uncontrolled problem in many countries. Among them, slimming functional food, such as teas, coffee and ‘natural’ pills, are commercially readily available to a vast population, not always aware of the health risks [1–3]. At the moment, there are no established analytical protocols for the systematic detection of synthetic adulterants in these products, but a large body of literature is converging to the target screening approach, either by liquid chromatography or gas chromatography [4–7]. However, this approach may not be suitable due to the sheer number of chemicals. Instead, an accurate mass approach during first step extraction allows the discovering of a theoretically infinity number of chemicals and for what is generally called “retrospective analysis” in post-acquisition data mining for all samples. For this reason, high-resolution accurate-mass spectrometry (HRAM), enabling accurate-mass determination of ionic species (and metabolites), offers the potential to overcome the limitations of multi-target screening [8]. The concept of HRAM is not novel at all [9,10] and in recent years its use has become more widespread due to technological improvements [11].

The present work shows a simple but effective approach to detect stimulants and anorectic compounds in allegedly ‘natural’ herbal extracts (tea and pills) sold as coadjuvant of weight loss by exploiting Q-TOF LC/MS technology after simple sample preparation and the confirmation and quantitation of the identified compounds with LC–MS/MS. Furthermore, the presented approach highlights the importance of confirmation technique to overcome the huge number of possibilities after a screening performed in HRAM. In fact, the candidate molecules were confirmed in LC–MS/MS by using analytical standards and quantitative data were obtained. The overall procedure confirmed the presence of stimulants such as caffeine, as well as undeclared sibutramine, a synthetic anti-obesity drug, in two slimming products.

2. Materials and methods

2.1. Standards and solvents

Water, methanol and formic acid were of LC–MS grade, all from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). Sibutramine, 4-
hydroxyamphetamine, theophylline and caffeine reference material were from Cerilliant Corporation (Round Rock, Texas, USA).

2.2. Sample and standards preparation

Five samples of allegedly natural herbal supplements were acquired by both on-line shopping and regular stores on the period August 2015–February 2016. For analysis, six bags of each herbal tea (samples 1, 3, 4 and 5) were opened and homogenized, while soft-gel capsules (n. 6, sample 2) were squeezed in a tube and vortexed. Amounts of 400 mg for each sample were weighted and suspended in 16 mL of water under agitation. After centrifugation, 50 μL of sample solution was diluted in 150 μL of water and 5 μL directly injected into LC-QTOF. For screening, when the concentration was beyond the linear range of the instrument i.e. saturation of the detector, the sample solution was diluted to appropriate concentration. For confirmation and quantitation step, a second aliquot of 1 mL of each sample was liquid/liquid extracted using a simplified procedure exploiting diethyl ether extraction after basification of the solution with 10% aqueous NaOH (pH 9–10) [12] to remove any interferents (i.e. sugars, organic acids). The supernatant was dried and then reconstituted in 200 μL of water. In case analytes concentration exceeded the calibration range, samples were diluted in water. Stock standard solutions (10 μg/mL) were prepared by weighing and dissolving each chemical in methanol. Standard working solutions were prepared via dilution of the stock solutions in methanol. All standard solutions were stored at −20°C. Preliminary quantitation of each compound was performed by preparation of standard working solutions in methanol at five concentration levels. However, to overcome matrix effect in LC-MS/MS analyses and due to the lack of similar blank material for a calibration curve, the method of the standard addition was adopted for quantitation.

2.3. Chromatographic conditions

The separation of the analytes was carried on an Agilent 1290 LC system (Santa Clara, CA, US) equipped with a binary pump and a thermostatic auto-sampler. An Agilent ZORBAX Eclipse Plus C18 (RRHT 2.1 mm × 150 mm, 1.8 μm) was used by the gradient elution of 0.01% formic acid in water as mobile phase A and 0.01% formic acid in methanol as mobile phase B: 0–1 min, 5% B; 1–12 min, 5–95% B; 12–15 min, 95% B; 15.1–18.1 min, 5% B. The mobile phase was delivered at a flow rate of 0.2 mL/min and the injection volume was 5 μL. The autosampler tray temperature was set to 8°C, while the column temperature was 40°C.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Δppm</th>
<th>RT (min)</th>
<th>Score (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theophylline</td>
<td>C7H8N4O2</td>
<td>−4.5</td>
<td>5.7</td>
<td>93</td>
</tr>
<tr>
<td>4-hydroxyamphetamine/catheine</td>
<td>C9H13NO</td>
<td>4.1</td>
<td>6.3</td>
<td>98</td>
</tr>
<tr>
<td>Caffeine</td>
<td>C8H10N4O2</td>
<td>5.0</td>
<td>6.5</td>
<td>87</td>
</tr>
<tr>
<td>Sibutramine</td>
<td>C17H26ClN</td>
<td>1.1</td>
<td>9.9</td>
<td>97</td>
</tr>
</tbody>
</table>

2.4. Mass spectrometry conditions

Accurate mass and MS/MS spectral analyses were performed on a 6550 QTOF and 6460 Triple quadrupole spectrometers (Agilent technologies, Santa Clara, CA, US) respectively. The applied ESI ion source conditions both for QTOF and Triple quadrupole instruments were from an in-house validated method already published [13] and set as follows: drying gas 125°C, drying gas flow 20 L/min, capillary voltage 4000 V, nozzle voltage 300 V, nebulizer flow 20 L/min, sheath gas temperature and flow were 325°C and 10 L/min, respectively. Ion funnel high and low voltages were 150/100 V, respectively. The acquisition modes for HRAM were full scan MS, range 40–1000 m/z, rate 1 spectra/sec and all ion MS/MS 40–1000 m/z, rate 1 spectra/sec, with fixed collision energies (0 V, 10 V, 20 V and 40 V) which produced data-dependent MS accurate mass fragmentation spectra. For confirmation by LC–MS/MS, MRM transitions for each compound were selected from Personal Compound Databases and Libraries (PCDLs) from Agilent’s METLIN and Forensic Accurate Mass Library and acquired in dynamicMRM mode (sibutramine: m/z 280 → 125, CE 20 V; m/z 280 → 179, CE 10; m/z 280 → 139, CE 20 V; caffeine: m/z 195 → 138, CE 40 V, 195 → 110, CE 40 V, 195 → 123, CE 40 V; theophylline: m/z 181 → 124, CE 20 V, 181 → 110, CE 20 V). Data acquisition and processing were carried out using MassHunter software (Agilent Technologies, Santa Clara, CA, US) with Qual and Quan browsers.

3. Results and discussion

Under the described conditions, five samples commercialized for slimming purposes of tea and/or soft-gel capsules, all allegedly of natural plant origin, were screened for active pharmaceutical ingredients with anorectic and appetite suppressant properties. At this purpose, a specific compound library (PCDL) comprising stimulants and 80 amphetamines-like compounds with anorexigenic effects was prepared from wider Agilent’s METLIN and Forensic Accurate Mass Databases Libraries. In this library, all compounds were present with acquired experimental accurate mass fragments spectra at collision energies of 10, 20 and 40 V, i.e.
low, medium and high-collision energies. To optimize sensitivity
and mass accuracy of the HRAM screening method, the instrument
parameters was set as follow: ion funnel voltages were experi-
mentally optimized for amphetamine-like compounds; acquisition
rate was set at 1 spectra/sec, that means 5993 transient/spectrum,
very low acquisition rate to obtain well-defined peaks and good
quality spectra, tune parameters were optimized for wide dynamic
range to limit detector saturation (2 GHz, dynamic range). Under
these conditions, the screening method was evaluated in terms of
sensitivity and accuracy for the identified compounds in order to
set the applicability of the method for screening purposes. The
calculated limit of detection (LOD) for sibutramine, caffeine and
theophylline, defined as a signal to noise (S/N) of 10 and producing
a RDS below 20%, was 1 ppm, with mass accuracy better than
5 ppm [14]. Samples were acquired in HRAM scan mode and
reviewed with dedicated data mining algorithms. Database search
(with fixed Δppm of 5 and [M+H]+ adduct) was employed to screen
samples for the compounds present in the library. Blank injections
of solvent and water, after injection of positive samples, were
used to evaluate carry over and thus to eliminate false positive results
from the system. Basically, no carry over was observed. Results
with scores >85% and mass accuracy better than 5 ppm were
considered consistent and further investigated. Scores were
calculated by the software upon single scores from the mass
match, isotope abundance and isotope spacing match of the
obtained spectra against the spectra present in the library. Using
this approach, sibutramine, 4-hydroxyamphetamine/catheine, caf-
feine and theophylline were preliminary detected in samples with
mass accuracy better than 5 ppm for all compounds (Fig. 1, Table 1)
after the first step of the screening procedure.

To reinforce the screening approach, samples were acquired at
different collision energies (10, 20, 40V) in order to collect
fragments in accurate mass for each compound. Then, a dedicated
software provided to compare the obtained experimental frag-
ments to fragments present into the accurate mass library. Sibutramine, caffeine and theophylline were still confirmed as
possible candidates also by the verified presence of specific
fragments with mass accuracies better than 5 ppm between the
target and measured masses. The acquisition of fragments in
accurate mass for each candidate and their comparison to a specific
library containing spectra of reference standard helped to reduce
the number of total presumptive candidates from 4 to 3, removing
both the two isomers 4-hydroxyamphetamine or cathine from the
candidate list. This indicates that a screening procedure relying
both on accurate mass information, comprehensive of isotopic
pattern evaluation (abundance, spacing) and accurate mass
fragments collection, instead of only accurate mass information,
as applied in most laboratories for rapidity, could better point out
which data to investigate further. It must be emphasized that the
number of candidates identified by LC-QTOF could have been much
numerous if different criteria for data mining were adopted. The
potentiality of unknown screening performed with accurate mass
technology is in fact, ideally infinity. On this ground, for
confirmation of all possible candidates, it is still recommendable
to use the relative reference material, to be analysed with the same
or a different technique. In this case LC–MS/MS was used for
confirmation of each analyte and quantitation by the standard
addition method was chosen to limit matrix effect and to overcome
the lack of blank matrices. In fact, one of the usually adopted
methods to solve these problems is the method of standard
additions, that means spiking known quantities of the analyte to
the solution of interest and measuring the solution’s analytical
signals in response to each addition. After a preliminary evaluation
of method linearity in the range 0.05–5 μg/mL for each analyte,
triplicate prepared samples were properly diluted to fit the
calibration range and preliminary quantitate. The size of each
addition was set at 2–3 times that of the calculated concentration
in the sample.

Sibutramine, was finally confirmed at concentrations of 15 and
26 μg/mg in two samples, consisting in 3.6 and 6.2 mg of
sibutramine for each unit (pill or bag); caffeine was confirmed
at 0.1 and 2.0% in weight, for a total amount of 5 and 100 mg/bags in
two samples (one of these also containing sibutramine) and
theophylline was present at 0.004 and 0.006% in weight, for a total
amount of 0.2 and 0.3 mg/bags (Table 2). These data were found
consistent to other similar studies, in which undeclared synthetic
drugs were confirmed in herbal weight loss products present on
the market. The most commonly undeclared ingredients, which are
illegally added, include sibutramine, phenolphthalein, bume-
tanide and phenytin. [17–20]. These adulterants are not
mentioned on labels and therefore consumers are kept unaware
of their presence and thus their side effects. In particular,
sibutramine is known to be associated with psychosis and mood
changes, cardiovascular problems and heart failure. For this
reason, it has been withdrawn from the market in the USA, European
Union, Australia, Canada and some Asian countries. In those
countries where sibutramine is still available as a pharmaceutical
drug, patients are regularly monitored. Contrary to sibutramine,
caffeine and theophylline belongs to the natural stimulant class of
substances, and their presence in this study is congruent with the
herbal or natural origin of the starting material. The caffeine
content, varying from 5 to 100 mg per pack, were compared to the
caffeine content in relation to a ‘generic’ cup of coffee (typically
80–150 mg for a French press or plung cup of coffee). Although
one pack contained what could be considered a negligible amount,
the second pack contained 100 mg of caffeine, which could
consistently increase the consumer’s total daily caffeine intake,
leading to caffeine adverse effects. Theophylline content in the
samples was below the regular quantitative in tea [15] but in line
with the results reported in similar studies on slimming material
[16].

4. Conclusions

In this paper, the applicability of accurate mass measurements
(Q-TOF LC/MS) screening and LC–MS/MS confirmation in
the investigation of undeclared active compounds in commercial
foodstuffs was demonstrated in an easy and rapid method set-up.