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Excessive amounts of antifungal agent and preservatives were illegally added in an adulterated herbal product for kids

Daopeng TAN[1#], Jinguo CUI[2#], Xiangqian SHE[3*]

Abstract

Illegally manufactured and counterfeit herbal medicines are growing a worldwide issue. And internet sales have simplified the distribution and payment of these counterfeit medicines. In 2019, a paper in The Lancet revealed the tip of the iceberg of counterfeit Chinese herbal medicines in Yongfeng County, Jiangxi Province, China. After 2 years, some so-called Chinese herbal poultices are still being manufactured in these districts. Here we report on a so-called herbal kids creams, which was advertised for treatment of childhood eczema. By means of NMR and HPLC-PDA, we found overdosed antimicrobial agents in the creams, including Terbinafine hydrochloride, Methylparaben, Propylparaben. Hence, compared to glucocorticoids, which are of high concern, the illegal addition of non-hormonal drugs in products for children requires particular attention.

Keywords: illegal addition; adulterated herbal products; HPLC-PDA; terbinafine hydrochloride; methylparaben; propylparaben.

Practical Application: This analytical method can be used for the regulation of illegal additions in antimicrobial preparations.

1 Introduction

Nowadays, herbal products including herbal medicines, dietary supplements and cosmetics are extensively used to improve or maintain health since they were believed as safe and free of side effects (Guo et al, 2010; Nili-Ahmadabadi et al., 2019; Aghababaei et al., 2018; Firozian et al., 2021). Among them, natural dermatological external medicines are the most popular ones. Visiting the internet, such as "Taobao" in China, a huge selection of herbal products against skin problems were provided. For many Chinese patent medicines, the pharmacological activity was well-described. However, a lot of so-called Chinese herbal medicines fashionable in internet, illegal addition of chemical defined drugs was more popular, but the chemical ingredients were not labeled (Mose & Bygum, 2019). According to the literature (Fiori & Andrisano, 2014; Luo et al, 2017; Gaudiano et al., 2010), glucocorticoids such as betamethasone and clobetasol propionate were once the main illegal ingredients added in these herbal products.

Identification of the chemical ingredients of the herbal medicines is still a challenge, despite many combination of new techniques such as high performance liquid chromatography coupled with mass or NMR spectrometry were used (Holzgrabe & Malet-Martino, 2011; Panusa et al., 2007). So, illegal addition and/or counterfeit in herbal medicines are still a worldwide problem especially in developing countries (Cockburn et al., 2005; Wiest et al., 2014).

In 2019, a literature of The Lancet revealed the tip of the iceberg of the counterfeit herbal medicines in Yongfeng county of Jiangxi province, China (Mose & Bygum, 2019). After 2 years later, some so-called herbal creams were still manufactured in some districts of Jiangxi province. Recently, we got some so-called herbal kids creams (Figure S1, Suplementary Material) manufactured in Zhangshu, another city of Jiangxi province from "Taobao.com". They were advertised as 100% natural and labelled herbal ingredients including Colla apis, Matricara recutita, Shea butter, Opuntia stricta, Radix sophorae flavescentis, Radix stemonae. To determine whether the illegal adulterant ingredients were still existed in the herbal kids creams, a high-throughput method (Jian et al., 2021) was firstly employed to detect 63 glucocorticoids, but none were detected. Owing to fast-acting, we have always suspected that these unscrupulous businessmen would really change their ways. But it is very difficult to know precisely what ingredients they are illegally adding without any clues. So, we next separated the main ingredients in the creams by column chromatography and elucidated their structure by NMR technique. The results showed that there were a large amount of unlabeled Terbinafine hydrochloride, Methylparaben and Propylparaben in the creams. In order to determine the content of these illegally added ingredients in the creams, it is essential to establish a simple, sensitive and accurate analytical method. A literature survey revealed that several analytical methods have been published for the assay of Terbinafine hydrochloride, Methylparaben and Propylparaben (Priyanka & Sk, 2014; Dewani et al., 2015; Bhosale, & Nikalje, 2017). These methods utilized buffer solutions to stabilize the analytes. Buffered mobile phases can provide stability to analytes,

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[1] *Guizhou Engineering Research Center of Industrial Key-technology for Dendrobium Nobile, School of Pharmacy, Zunyi Medical University, Zunyi, China*
[2] *Department of Pharmacy, Tianjin Baodi Hospital, Baodi Clinical College of Tianjin Medical University, Tianjin, China*
[3] *Guizhou Keytrum Bio-Tech Co. Ltd., Guiyang, China*
*Corresponding author: sxq722000@163.com
[#] *These authors contributed equally.*

but also significantly increases the rapid aging of stationary phases, especially when using direct dissolution methods to prepare analytical sample solutions, again bringing a large amount of ointment matrix into the stationary phase and requiring timely maintenance of the LC system. In the present, we thus developed the single HPLC-PDF method for the estimation of these illegal ingredients in the creams to reliably separate the three ingredients within a short analysis time and without buffer solutions. The established method was simple, sensitive and accurate, and would be potentially used to monitor the illegal addition of local cream formulations precisely.

2 Materials and methods

2.1 Materials and chemicals

Cream for baby (for kids, batch no. 20210406, 20210418, 20210513, 20210528, manufactured by Jiangxi Huifa industrial Co., Ltd, Zhangshu, China) were purchased from "Taobao.com". The Terbinafine hydrochloride, Methylparaben and Propylparaben reference standards were obtained from Chengdu Lemeitian pharmaceutical technology Co., Ltd (Chengdu, China). Their purities were higher than 98.0% detected by the HPLC method.

Acetonitrile (HPLC grade, Merck, Germany) was employed as the mobile phase. All other reagents (analytical grade) were purchased from commercially sources. Water was purified by a Milli-Q water purification system.

2.2 Instrument parameters

The illegal glucocorticoids were detected by an Agilent 1290 UPLC system coupled with a SCIEX Triple TOF 4600 mass spectrometer operated in an ESI mode. The chromatography and mass parameters were optimized according to the literature (Jian et al., 2021).

The illegal antifungals analysis was performed by a Waters HPLC system consisted with Waters 2995 controller and 2998 PDA detector. An Elite Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm) was employed for the separation. Acetonitrile and water were used as mobile phases A and B, respectively. The elution gradient was set as follows: 0-15 min, 35% of B; 15-20 min, 35 to 85% of B; 20-30 min, 85% of B; and equilibrated for 10 min before the next injection. The elution rate, column temperature, injection volume, and detective wavelength were set at 1.0 mL/min, 25 °C, 10 μL and 250 nm, respectively. Data were collected by Waters Empower Chemstation Software. NMR spectra were recorded on a Varian INOVA AS 400 instrument (Agilent, Santa Clara, USA) with TMS as internal standard.

2.3 Illegal ingredients isolation

The cream for baby (180 g) was added calcium carbonate powder (360 g) and uniform grinded, and then extracted by ultrasonication in methanol (1 L). The residue was obtained after reduced pressure evaporation, and then subjected to an AB-8 macroporous adsorption resin column and eluted with H_2O, 10% MeOH, 50% MeOH and 80% MeOH, successively to afford ten fractions (Fr. 1-10). Frs. 3, 5, and 8 were separated and purified by Sephadex LH-20 to yield compounds **1** (15 mg), **2** (18 mg), and **3** (13 mg), respectively (Figure 1).

2.4 Sample preparation

The cream for baby (2.0 g) was grinded with calcium carbonate powder (8.0 g) into a homogeneous powder mixture, The powder (2.5 g) was accurately weighed and extracted by ultrasonication in methanol (25 mL) for 30 min. The extracted sample solution was filtered with a 0.22 μm membrane for the HPLC analysis.

The reference standards of Methylparaben, Propylparaben and Terbinafine hydrochloride were dissolved in MeOH to yield a mixed standard stock solution with concentrations of 316.6 μg/mL, 378.0 μg/mL and 487.8 μg/mL, respectively. The working standard solutions for calibration curves and recovery were obtained by diluting the stock solutions and filtered with a 0.22 μm membrane before HPLC analysis.

2.5 Method validation

In The HPLC method validation, the limit of detection (LOD), limit of quantification (LOQ), linearity, precision, repeatability, recovery and stability were examined.

3 Results and discussions

3.1 Detection of illegal glucocorticoids

63 Glucocorticoids including Hydrocortisone, Betamethasone, Dexamethasone, Diethylstilbestrol, Estradiol, Megestrol acetate, Clomiphene dipropionate, Ansinede, Beclomethasone dipropionate, Beclomethasone, Beclomethasone acetate, Beclomethasone dipropionate, Budinet, Difcot, Dexamethasone acetate, Clometasone, Difluoro diacetate, Clomiolone, Clomiolone acetate, Fluorohydropine, Fluhydrocortisone acetate, Fluticasone propionate, Fluoroacetate, Methylprednisolone, Methylprednisolone acetate, Hasined, Cortisone, Mometasone Furoate, Cortisone acetate, Clobetazone butyrate, Clobetasol propionate, Prednisolone

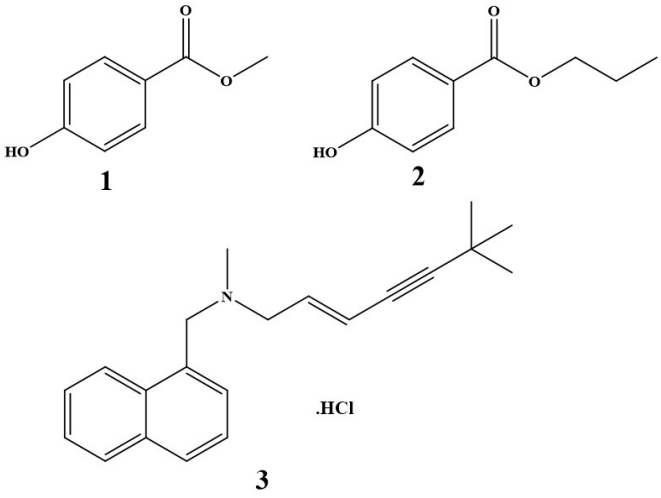

Figure 1. The structrues of three illegal ingredients. (**1**, Methylparaben; **2**, Propylparaben; **3**, Terbinafine hydrochloride)

acetate, Hydrocortisone butyrate, Prednisolone ester, Prednisone, Prednisone acetate, prednisolone, Hydrocortisone Acetate, Hydrocortisone valerate, Triamcinolone acetonide, Triamcinolone acetonide 21-acetate, Triamcinolonediacetate, Triamcinolone, Betamethasone 17-valerate, Progesterone Prog, Estriol, Estrone, Methyltestosterone, Fluocinolone acetonide, Desonide, Halometasone, Diflucortolone valerate, 9a-fluoroprednisolone, Halobetasol Propionate, Ciclesonide, Loteprednol, Flunisolide, Testosterone, Progesterone Acetate, Chlormadinone acetate, Hydroxyprogesterone caproate, Evonorgestrel, Gestrinone were tested by UPLC-MS method according to the literature (Jian et al., 2021) and Food and Drug Administration (2015a), but no glucocorticoids were detected in the so-called herbal cream (Table S1). Did the results indicate that those counterfeit herbal medicine manufacturer forsaked heresy and returned to the truth? After all, glucocorticoids were usually the main illegal addition ingredients in herbal products. So, we next separated and elucidated the main ingredients in the creams by column chromatography and NMR technique.

3.2 Characterization of compounds 1-3

Methylparaben (**1**) white needles, ESI-MS m/z: 151[M-H]$^-$. ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.78 (2H, d, *J* =8.0 Hz, H-3, 5), 6.71 (2H, d, *J* =8.0 Hz, H-2, 6), 3.79 (3H, s, H-8) (Figure S2, Suplementary Material); ^{13}C-NMR (100 MHz, CD_3OD) δ: 167.5 (C-7), 165.1 (C-1), 131.2 (C-3, 5), 118.6 (C-4), 115.6 (C-2, 6), 50.7 (C-8) (Figure S3, Suplementary Material) (Lin et al, 2020).

Propylparaben (**2**) white needles, ESI-MS m/z: 179[M-H]$^-$. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.94 (2H, d, *J* =8.0 Hz, H-3, 5), 6.86 (2H, d, *J* =8.0 Hz, H-2, 6), 4.25 (2H, t, *J*= 8.0 Hz, H-8), 1.78 (2H, m, H-9), 1.00 (3H, t, *J*= 8.0 Hz, H-10) (Figure S4, Suplementary Material); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 166.9 (C-7), 160.2 (C-1), 131.8 (C-3, 5), 122.5 (C-4), 115.1 (C-2, 6), 66.4 (C-8), 22.1 (C-9), 10.5 (C-10) (Figure S5, Suplementary Material) (Pouchert & Behnke, 1993).

Terbinafine hydrochloride (**3**) white needles, ESI-MS m/z: 292 [M-HCl+H]$^+$. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 12.65 (1H, br, NH^+), 8.04~7.57 (7H, m, Ar-H), 6.33 (1H, m, -CH=CH), 5.83 (1H, brd, *J*= 16.0 Hz, -CH=CH), 4.72 (2H, m, $ArCH_2N$), 3.83 (2H, m, NCH_2CH=), 2.59 (3H, d, *J*= 4.0 Hz, NCH_3), 1.21 (9H, s, t-Bu) (Figure S6, Suplementary Material); ^{13}C-NMR (100 MHz, $CDCl_3$); δ: 133.8, 131.9, 131.6, 131.0, 129.4, 128.1, 127.6, 126.4, 125.6, 124.3, 122.5, 121.1, 103.0, 75.6, 57.6, 54.2, 38.7, 30.6, 27.9 (Figure S7, Suplementary Material) (Han et al., 2001).

3.3 Quantitation of illegal ingredients

Optimization of the extraction method

The cream is very difficult to extract due to abundant ointment base. In additon, the cream is't suitable for heating extraction. So, a large amount of $CaCO_3$ powder was employed to scatter the cream and then extracted by ultrasonication. Three extraction factors including solvents, solvent volume and extraction time were evaluated to afford the most efficient extraction parameters. At last, the mixed samples extracted by ultrasonication in 25 mL of methanol for 30 min were selected for the extraction method.

Optimization of HPLC parameters

To get an effective resolution, HPLC parameters including chromatography column, temperature, mobile phases and elution gradient were examined. At last, the Hypersil ODS2 C_{18} column (4.6mm × 250 mm, 5 μm) was employed for the LC separation. According to UV absorption of three compounds **1**-**3**, the detective wavelength was set at 250 nm. The typical HPLC chromatograms of the mixed standards and samples were shown in Figure 2.

Validation of the developed method

The HPLC analytical method was validated in terms of precision, linearity, accuracy and stability. The linear equation (e.g., $y = ax + b$) was generated by plotting the peak area (y) versus the concentration of each analyte (x; μg/mL). The limit of detection (LOD) and the limit of quantification (LOQ) were determined based on a S/N ratio of 3 and 10, respectively (Table 1). All analytes exhibited linearity over the range tested, with R^2 ranging 0.9997 to 1.0000. The LODs and LOQs for the three ingredients were in the range of 0.10-0.28 μg/mL and 0.32-0.76 μg/mL, respectively.

Precision was assessed by intra-day and inter-day variability. The intra-day variability was examined by six consecutive replicates in one day. The inter-day variability was evaluated by three consecutive replicates over three days. The variations (RSD%) of intra-day and inter-day variability were shown in Table 2. The total intra-day and inter-day variations did not exceed 1.89%. The repeatability was investigated by six replicates, and the variation in repeatability did not exceed 1.21%. The stability of sample solution was assessed at 0, 2, 4, 8, 12, and 24 hr. The variations of peak areas of the three analyzed ingredients

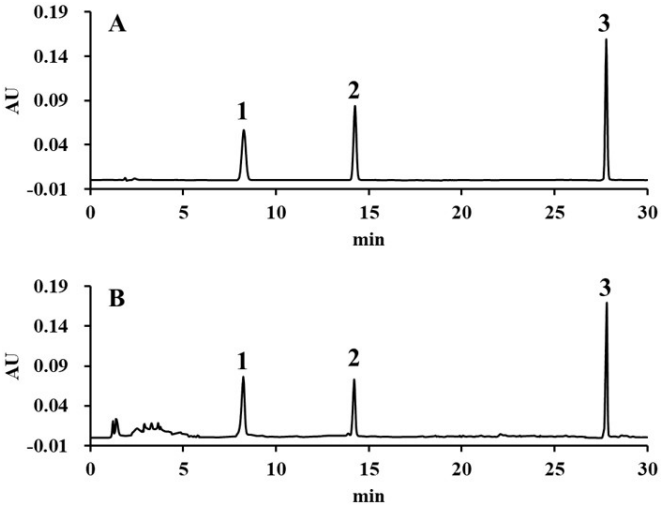

Figure 2. The typical HPLC chromatograms of the mixed standards and Legu® creams samples. (A the reference standards; B the sample of Legu® creams; **1**, Methylparaben; **2**, Propylparaben; **3**, Terbinafine hydrochloride).

Table 1. Linearity and sensitivity of the HPLC analysis

No.	Compound name	Calibration curve[a]	R^2	Linear range(μg/mL)	LOD[b](μg/mL)	LOQ[c](μg/mL)
1	Methylparaben	y = 37436x + 27325	0.9997	0.76~316.60	0.28	0.76
2	Propylparaben	y = 41868x - 15252	1.0000	0.36~378.00	0.10	0.36
3	Terbinafine hydrochloride	y = 35612x - 37316	0.9998	0.32~487.80	0.10	0.32

[a]y is the peak area and x is the concentration of compound (μg/mL); [b]LOD refers to the limit of detection, S/N = 3:1; [c]LOQ refers to the limit of quantification, S/N =10:1.

Table 2. The results of precision, repeatability, and stability (n=6).

No.	Intra-day (RSD, %, n=6)[a]			Inter-day (n=6)			Repeatability (n=6)	Stability (n=6, 18h)
	low	medium	high	low	medium	high	RSD (%)	RSD (%)
1	1.18	1.05	0.78	1.89	1.61	1.09	1.06	2.38
2	1.31	0.97	0.69	1.37	1.02	0.84	1.13	1.29
3	1.31	1.14	0.68	1.43	1.21	0.91	1.21	2.11

[a]RSD% = (SD/mean) × 100%. RSD: Relative Sandard Deviation.

in the samples did not exceed 2.38%. The results indicated that the three ingredients in the samples were stable for at least 24 hr.

The accuracy of the HPLC method was assessed by a recovery test. Three different concentrations of standard solutions (80%, 100%, and 120%) were added into the samples. The recovery of each spiked reference standard was calculated according to the formula recovery % = [(found amount-original amount)/spiked amount] × 100% (Tan et al., 2020). The recoveries of the three analyzed compounds ranged 98.52–100.76% (Table 3).

The validation results indicated that the established HPLC-PDA method was accurate and precise for the determination of the three illegal ingredients in the herbal cream.

Table 3. Recovery of the targets (n=3).

No.	Original (mg)	Spiked (mg)	Found (mg)	Recovery (%)[a]	RSD (%)[b]
1	1.30	1.04	2.33	99.04 ± 2.91	2.73
	1.32	1.32	2.65	100.76 ± 2.87	2.36
	1.30	1.56	2.84	98.72 ± 3.34	3.03
2	1.56	1.25	2.80	99.20 ± 3.18	2.97
	1.58	1.58	3.17	100.63 ± 2.97	2.21
	1.56	1.87	3.44	100.53 ± 3.45	2.14
3	2.00	1.60	3.58	98.75 ± 2.64	3.04
	2.03	2.03	4.03	98.52 ± 3.22	2.88
	2.00	2.40	4.38	99.17 ± 2.98	2.91

[a]Recovery% = [(found amount - original amount)/spiked amount] × 100%; [b]RSD% = (SD/mean) × 100%. RSD: Relative Sandard Deviation.

Sample assay

In the present study, three illegal addition constituents including Terbinafine hydrochloride, Methylparaben and Propylparaben were found in the Legu® cream, an adulterated Chinese herbal cream for kids and assayed by a validated HPLC-PDA method. The concentration of the three illegal constituents were shown in Table 4.

Table 4. The contents of three illegal addition constituents in the Legu® cream (mg/g, mean ± SD, n = 3).

No.	20210406	20210418	20210513	20210528
1	5.27 ± 0.03	6.38 ± 0.04	7.12 ± 0.02	5.33 ± 0.02
2	6.31 ± 0.05	8.23 ± 0.04	6.95 ± 0.02	8.39 ± 0.06
3	8.12 ± 0.03	25.10 ± 0.03	14.36 ± 0.06	21.66 ± 0.04

Terbinafine hydrochloride, an allylamine antifungal agent, has been available for the treatment of dermatophytic infections and onychomycosis more than twenty years. Terbinafine hydrochloride has serious side effects in clinic such as inducing subcutaneous lupus erythematosus (SCLE), Stevens-Johnson syndrome, toxic epidermal necrolysis, and psoriasis, etc. (Mayser, 2016). So, the illegal addition of terbinafine hydrochloride in herbal products provided a relatively high risk. In addition, the terbinafine hydrochloride content is only 1% in approved marketed creams, such as Dingke® terbinafine hydrochloride creams manufactured by Qilu Pharmaceutical Co. Whereas, the terbinafine hydrochloride content is up to 2.51% in so-called herbal cream for baby, and its content varies greatly from batch to batch.

Methylparaben and Propylparaben are two commonly nipagin ester preservatives with strong bacteriostatic effects and used as pharmaceutic aid (antifungal) in foods and cosmetics. According to the Food and Drug Administration (2015b), the upper limit of nipagin ester preservatives that can be used is single ester: 0.4%, mixed ester: 0.8%. The content of methylparaben and propylparaben in this so-called herbal creams also far exceeds the limits.

Since the frenzied illegal addition in Chinese herbal products came to light by a Lancet literature in 2019, the local government once adopted a strict management and control on the production of Chinese herbal products. In the past, glucocorticoids were

the major illegal added ingredients in topical formulations. Today, there are well-established high-throughput analytical methods for glucocorticoids. However, the road is high one feet evil spirit is high one a unit of length, we can never know in advance what kind of illegal ingredients will be added by unscrupulous traders. For example, in the present, we found that excess amount of unlabeled Terbinafine hydrochloride, Methylparaben and Propylparaben were illegally added in the baby creams. These illegal added ingredients were accurately identified by NMR. A method for simultaneous determination of the content of these three components was established, and the new analytical method does not require the use of buffers, and the sample preparation method identifies and removes as much of the formulation matrix as possible, reducing the effect on the stationary phase and extending the instrument life and maintenance intervals. In addition, the method is simple, reliable and accurate for the determination of Terbinafine hydrochloride, Methylparaben and Propylparaben.

4 Conclusion

Illegal addition and/or counterfeit in herbal medicines are still a seriously worldwide problems especially with the development of internet commerce in developing countries. The way of illegal addition is unceasing changing. There are now sophisticated high-throughput analytical methods for the simultaneous detection of dozens of glucocorticoids (Jian et al., 2021; Shi et al., 2020), leading to a gradual reduction in the glucocorticoids that were commonly illegal added in the past. However, non-hormonal drugs and even large doses of preservatives have been found to be used in these adulterated herbal products, especially in products for children, and may become a new way to illegally add counterfeit. Hence, there is an urgent need to establish a comprehensive high-throughput detection method for illegally added non-hormonal drugs in adulterated herbal products. In the present, a simple and accurate HPLC-PDA analytic method was developed to simultaneously quantify three illegally added ingredients, including Terbinafine hydrochloride, Methylparaben and Propylparaben, in an adulterated herbal product for kids. This established HPLC method is helpful to improve the regulation of counterfeit in herbal medicines on the market, to a certain extent.

Conflicts of interest

All the authors declare that there are no conflicts of interest.

Data Availability

The research data generated from this study is included within the article.

Acknowledgements

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. The results of detections of 63 Glucocorticoids in the Legu® cream (batch no. 20210406)
Figure S1. The packaging of Legu® creams.
Figure S2. The 1H-NMR mapping of Methylparaben.
Figure S3. The 13C-NMR mapping of Methylparaben.
Figure S4. The 1H-NMR mapping of Propylparaben.
Figure S5. The 13C-NMR mapping of Propylparaben.
Figure S6. The 1H-NMR mapping of Terbinafine hydrochloride.
Figure S7. The 13C-NMR mapping of Terbinafine hydrochloride.

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