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Identification of adulterants in a Chinese herbal medicine by LC–HRMS and LC–MS–SPE/NMR and comparative *in vivo* study with standards in a hypertensive rat model

Julie Regitze Kesting[a], JingQi Huang[b], Dan Sørensen[b,*]

[a] *Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark*
[b] *Merck Frosst Centre for Therapeutic Research, Merck Frosst Canada Ltd., 16711 Trans Canada Highway, Kirkland, Québec H9H 3L1, Canada*

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ABSTRACT

Based on anecdotal evidence of anti-hypertensive effect of Gold Nine Soft Capsules, an *in vivo* study of this complex Chinese "herbal-based" medicine was initiated. Dosage of the content of Gold Nine capsules in spontaneous hypertensive rats showed a remarkably good effect. This led to further investigation of the components of the preparation and eventual identification of three known anti-hypertensive drugs; amlodipine, indapamide and valsartan, which were not declared on the label. Compounds were rapidly identified using LC–HRMS and LC–MS–SPE/NMR, quantified by HPLC, and the *in vivo* activity of a combination of commercially purchased standards was shown to be equivalent to that of the capsule content. Adulteration of herbal remedies and dietary supplements with synthetic drugs is an increasing problem that may lead to serious adverse effects. LC–MS–SPE/NMR as a method for the rapid identification of such adulterants is highlighted in this case study.

1. Introduction

Many people rely on herbal medicines for the treatment of various ailments and disease states and, often anecdotal, evidence of their efficacy warrants further scientific studies of the natural products and their mechanisms of action. The unfortunate fact, impeding proper scientific studies of the health benefit claims of products like Traditional Chinese Medicines (TCM), either in the form of raw natural materials or natural components mixed and formulated according to documented tradition as Chinese Patent Medicines (CPM), is that many of these herbal preparations may have been adulterated with synthetic drugs [1–3]. In the work described here, the measurable effect of Gold Nine Soft Capsules, a Chinese "herbal-based" medicine intended for treatment of hypertension, led to further investigations of this product. The investigation was initiated based on the assumption that one or more natural products were responsible for the effect. However, at a very early stage, it became clear that the Gold Nine capsules were adulterated with synthetic drugs. Hyphenation of liquid chromatography (LC) with mass spectrometry (MS), solid phase extraction (SPE), and nuclear magnetic resonance (NMR) has been widely accepted as a tool for dereplication and discovery of natural products from complex mixtures of plant and endophytic origin [4–10]. Application of these hyphenated methods in the present work also proved to be a rapid and efficient tool for the identification of adulterants in the Gold Nine capsules. In the Gold Nine capsules, three anti-hypertensive drugs, amlodipine (calcium channel blocking agent), indapamide (diuretic), and valsartan (Angiotensin II receptor antagonist) [11], were identified by LC–HRMS and NMR. Combined with *in vivo* studies, it was evident that the efficacy of the Gold Nine capsules could be attributed to the synthetic compounds alone.

2. Experimental

2.1. Gold Nine Soft Capsules

Gold Nine Soft Capsules were manufactured by Hong Kong Five-Circle Medicinal Industry Co. Ltd. and purchased from Ponglia Pharmacy, Shangdon province, China. The composition of the capsules was listed: Chinese Caterpillar Fungus, Garter Snake, Monascus anka, Earthworm, Barbary Wolfberry Fruit, Common

* Corresponding author. Tel.: +1 514 428 2797; fax: +1 514 428 4900.
E-mail address: dan_sorensen@merck.com (D. Sørensen).

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Yam Rhizome, Spina Date Seed, Dahurian Angelica Root, Nutmeg, Tangerine Peel, Salad Oil, etc., as a total of 500 mg per capsule. Dosage recommended by the manufacturer is 1–2 capsules per day.

2.2. Chemicals

Authentic standards were purchased from commercial sources: indapamide from LKT Laboratories Inc. (St. Paul, MO, USA), amlodipine from Toronto Research Chemicals (North York, ON, Canada), and valsartan from Altan Corporation (Orange, CT, USA). HPLC-grade "Optima" acetonitrile from Fischer Scientific Canada (Ottawa, ON, Canada) and milli-Q filtered water were used for chromatography and as general solvents. Deuterated solvents CD_3CN and DMSO-d_6 (both 99.9 atom% d) were purchased from C/D/N Isotopes Inc. (Pointe-Claire, QC, Canada). 0.5% methyl cellulose (aq) was prepared from methyl cellulose purchased from Sigma–Aldrich Canada Ltd. (Oakville, ON, Canada).

2.3. Identification of adulterants

For LC–HRMS analysis, one capsule was cut open and the content was dissolved in 3 mL DMSO-d_6 (deuterated DMSO was used to enable additional analysis of the crude content by NMR). The solution was filtered and 1 μL was injected into the chromatographic system. For LC–MS–SPE/NMR analysis, the same solution was used for trapping and identification of valsartan and indapamide (one injection of 10 μL and three injections of 15 μL, respectively) while a more concentrated solution of one capsule in 1 mL DMSO-d_6 was used for trapping and identification of amlodipine (one 20 μL injection). All samples were filtered through 0.45 μm Waters GHP (13 mm) syringe filter prior to injections.

2.3.1. LC–HRMS

The liquid chromatography–high resolution mass spectrometry (LC–HRMS) system consisted of an Agilent 1200 series HPLC, equipped with a G1312A binary pump and a G1315C Diode Array Detector (DAD SL), connected to an Agilent G1969A LC/MSD Time-of-flight mass spectrometer (MS). Separation was achieved on a ZORBAX Eclipse Plus (C-18; 1.8 μm) 3.0 mm × 50 mm HPLC column with a 1 mL/min gradient of acetonitrile/water + 0.1% formic acid (10:90–95:5 over 5 min, held at 95:5 for 1 min). The MS instrument was operated with electrospray ionization (ESI) in both positive and negative mode for initial screening, and just positive mode for further investigation, with the following settings: drying gas temperature, 350 °C, nebulizer pressure, 60 psi, drying gas, 13 L/min, and spray voltage, 4000 V.

2.3.2. LC–MS–SPE/NMR

The hyphenated liquid chromatography–mass spectrometry–solid phase extraction (LC–MS–SPE) system consisted of an Agilent 1100 series HPLC with a G1315B Diode Array Detector (DAD), Knauer WellChrom K-120 solvent pump (post-column dilution), Accurate ICP-04-20 flow-splitter by LC Packings, Bruker Esquire 4000 ion trap mass spectrometer (MS), Bruker/Spark Prospekt II LC–SPE–NMR interface module with N_2-blanketing of the SPE compartment and an additional GP resin cartridge placed in a clamp holder in the waste outlet leading to an Agilent G1315A Variable Wavelength Detector (VWD) and was managed by Bruker HyStar/EsquireControl software v.3.0. Separation was achieved on a Phenomenex Max-RP (C-12; 4 μm) 4.6 mm × 250 mm HPLC column, using a 0.75 mL/min gradient of acetonitrile/water + 0.1% formic acid (20:80–95:5 over 20 min, held at 95:5 for 5 min). A constant post-column makeup flow of water (1.25 mL/min) was added to reduce the solvent strength for trapping on Spark Hysphere Resin GP 2 mm × 10 mm SPE cartridges. The flow splitter directed 5% into the MS and 95% into the DAD and the SPE module. A preliminary non-trapping "scouting" analysis was performed to determine potential retention on SPE cartridges and the absorbance threshold levels at 300 nm for automated trapping of peaks. To facilitate the SPE/NMR transfer, cartridges were dried with pressurized N_2 for 45 min prior to elution. Trapped peaks were eluted into 3 mm (335-PP) NMR tubes from Wilmad, using 165 μL of CD_3CN. NMR spectra were acquired on a Varian Inova 600 spectrometer equipped with 5 mm HCN PFG Cold Probe operating at 599.92 MHz for ^{1}H NMR. Chemical shifts were referenced to the residual solvent peak (1.94 ppm for CD_3CN). For indapamide and valsartan the ^{1}H NMR spectra were acquired using 512 scans, whereas 1K scans were acquired to obtain ^{1}H NMR data for amlodipine. For valsartan, additional gradient selected COSY (nt = 8, ni = 128), HSQC (nt = 16, ni = 128) and HMBC (nt = 48, ni = 200) spectra were acquired. All NMR experiments were performed at 25 °C.

2.4. Quantification of adulterants

2 mg of commercial reference compounds, valsartan, indapamide, and amlodipine, were accurately weighed and dissolved in acetonitrile/water 1:1, then subsequently diluted in series to obtain five different solutions containing in concentrations ranging from 0.02–0.2 mg/mL for valsartan, and 0.02–0.2 mg/mL for amlodipine and indapamide. The following procedure for dissolution and analysis was used for both intact capsules and content alone, but to obtain and measure the content, the capsules were cut open and emptied and the oily content was weighed: to either two intact capsules or the weighed content of two capsules in 100 mL volumetric flasks, 30 mL of water was added and the mixtures were stirred for 30 min. 30 mL of acetonitrile was then added to each flask and the mixtures were stirred for 30 min. The flasks were topped up with acetonitrile/water 1:1 to a total volume of 100 mL.

2.4.1. HPLC

Diode array UV chromatograms (190–400 nm) from injections of 2 μL of each sample of the standard solution series were acquired on the LC–HRMS system using the conditions described in Section 2.3.1. Each sample was injected in duplicate. For evaluating reproducibility of the injections, two concentrations of each compound were injected in triplicate. Accuracy of the method was determined by analyzing four samples with known concentrations for each compound in triplicate. Samples for evaluating precision and accuracy were prepared in concentrations within the range used in construction of the standard curves. Each dissolution sample of capsules or capsule content was centrifuged to remove dust particles and fiber residues, and duplicates of 2 μL were injected into the LC–HRMS to acquire diode array UV chromatograms (190–400 nm). All samples were filtered through 0.45 μm GHP (13 mm) syringe filters prior to injection.

2.5. In vivo studies

All experimental procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care and were also approved by an internal Animal Care Committee. Dosing solutions were prepared in 0.5% methyl cellulose (aq). For the preparation of solutions, 600 mg capsule content was weighed and diluted with 10 mL 0.5% methyl cellulose (aq) to prepare an arbitrary 60 mg/mL dosing solution and pure reference standards were used in concentrations matching the actual drug levels as determined experimentally.

2.5.1. Probe implantation and dosing experiments

Male Spontaneous Hypertensive Rats (SHR), 4–6-month-old, were obtained from Charles River Laboratories and were kept in an environment with temperature and humidity control and a 12 h

light–dark cycle. Standard rat chow and water was freely accessible to the animals. Under general anesthesia using 3% isoflurane, SHRs were implanted with telemetry probes (TA11-PAC40, Data Sciences Inc.). Following surgery, the animals were single-housed and left to recover for at least 1 week before initiating studies. During the post-recovery period, the animals underwent acclimation to the dosing procedures for 1 week to prevent handling-induced stress during experimental studies. The arterial blood pressure and the heart rate were monitored continuously by a computerized data acquisition system (Data Sciences Inc.). Telemetrized animals were grouped randomly with emphasis on balancing their MAP (mean arterial pressure) to match the baseline level between groups. Three animals per treatment group were used in a total of six groups, including the vehicle control. All animals (non-fasting) received treatment or vehicle, as a single oral dose administered by gavage, in a volume of 5 mL/kg bodyweight. Animals received either vehicle (0.5% methyl cellulose) or Gold Nine Soft Capsule content (300 mg/kg) or synthetic compounds as a single or combination treatment in a dose corresponding to 300 mg/kg of the capsules (amlodipine 0.81 mg/kg, indapamide 0.84 mg/kg and valsartan 19.30 mg/kg). Physical parameters including blood pressure (systolic as well as diastolic), heart rate (HR), animal activity and pulse pressure were recorded continuously for 24 h prior to and 7 days following administration. These experiments were carried out in a double-blinded fashion with each step, from preparation of samples to dosing of animals as well as data analysis, carried out by different individuals.

2.5.2. Data acquisition and analysis

Data were acquired, displayed, and analyzed using a computer based data acquisition system from Data Sciences Inc. (DSI) [12]. Data were recorded for 10 s in 5-min intervals and were averaged into 1 h measurements using Dataquest A.R.T. Gold Analysis v.4.0 software. Baseline levels were defined as the average MAP measured for 24 h prior to dosing as well as the mean of the vehicle control group. The maximal changes in MAP (ΔMAP) and HR, and duration of the effect, were determined by comparison with control values. At the starting point, all animals had similar values for all measured physiological parameters. The data were collected up to 168 h after administration, but given that, for some of the compounds, the duration of the effect was not extended beyond 96 h, data are only presented up to that time point. To obtain valid results for the duration and intensity of the effect of capsules and synthetics, ABC (Area Between Curves) for treated and control groups were also calculated for the consecutive effective days after dosing. Cardiovascular variables were analyzed using a one-way ANOVA analysis following Bonferroni's multiple comparison.

3. Results and discussion

3.1. Identification of adulterants

A two-tier approach was used for the identification of the adulterants. An initial screening by LC–HRMS was performed to identify compounds of interest and, in order to rule out that they were not isobaric analogues and to unambiguously confirm the identity of the compounds, they were subsequently isolated and analyzed by LC–MS–SPE/NMR. As a method of isolation, LC–MS–SPE provides rapid access to pure material for further analytical studies. One advantage of using LC–MS–SPE/NMR, as compared to LC–MS or GC–MS, which has often been the method of choice for routine analysis, is that full structural information is available. For herbal remedies adulterated with known synthetic drugs, LC–MS and GC–MS, as well as a range of other analytical methods, are highly valuable methods that have been used extensively in the analysis of adulterants in herbal medicines. Often detection is based on comparison with reference standards or extensive databases that allow for rapid detection of adulterants [13–15]. The disadvantage of these methods is not only that they require a prior idea of which synthetic drug to look for, as well as acquisition of a vast amount of data for reference, but also that structural analogues of known compounds have been used for adulteration to evade detection by LC–MS [3,16–18]. As the databases may not include these analogues, acquisition of MS data or comparison of retention times may not be sufficient. NMR is the only analytical technique which provides full structural information from novel compounds. In this case, LC–MS–SPE/NMR allowed us to rapidly identify the adulterants without any need for reference standards.

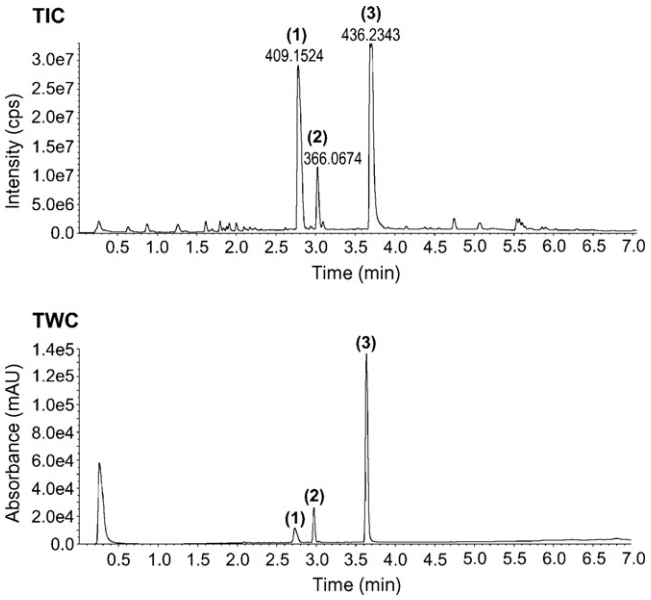

Fig. 1. LC–HRMS of Gold Nine Soft Capsules. Top: total ion chromatogram, positive mode, with high-resolution mass indicated for each peak. Lower: total wavelength chromatogram (UV). (1) Amlodipine, (2) indapamide, and (3) valsartan.

3.1.1. LC–HRMS

Analysis of sample solutions by LC–HRMS, using the conditions described in Section 2.3.1, revealed primarily three peaks in both the UV and MS chromatograms and resulted in immediate suspicion of adulteration of the alleged herbal preparation (Fig. 1).

Likely molecular formulas were calculated to fit within a 1 ppm error margin of the m/z of the $[M+H]^+$ ions and their isotopic pattern distributions were used to further validate selections. A combined literature search for known anti-hypertensive drugs and molecular formulas (peak 1 w., R_t = 2.72 min: $C_{20}H_{25}ClN_2O_5$, peak 2 w., R_t = 2.97 min: $C_{16}H_{16}ClN_3O_3S$, and peak 3 w., R_t = 3.63 min: $C_{24}H_{29}N_5O_3$) suggested that the constituents were indeed synthetic adulterants as the molecular formulas were identical to those of: (1) amlodipine (HRESIMS m/z 409.1524 $[M+H]^+$, calcd for $[C_{20}H_{26}ClN_2O_5]^+$, 409.15248), (2) indapamide (HRESIMS m/z 366.0674 $[M+H]^+$, calcd for $[C_{16}H_{17}ClN_3O_3S]^+$, 366.06737), and (3) valsartan (HRESIMS m/z 436.2343 $[M+H]^+$, calcd for $[C_{24}H_{30}N_5O_3]^+$ 436.23432) [11].

3.1.2. LC–MS–SPE/NMR

To assess retention of compounds during the preliminary "scouting" analysis, a custom modification to instrumentation was made: an additional GP SPE cartridge was placed in a clamp holder between the waste outlet of the Prospekt device and the variable wavelength detector. This allowed for the evaluation of the trapping potential of peaks during the scouting analysis. After the

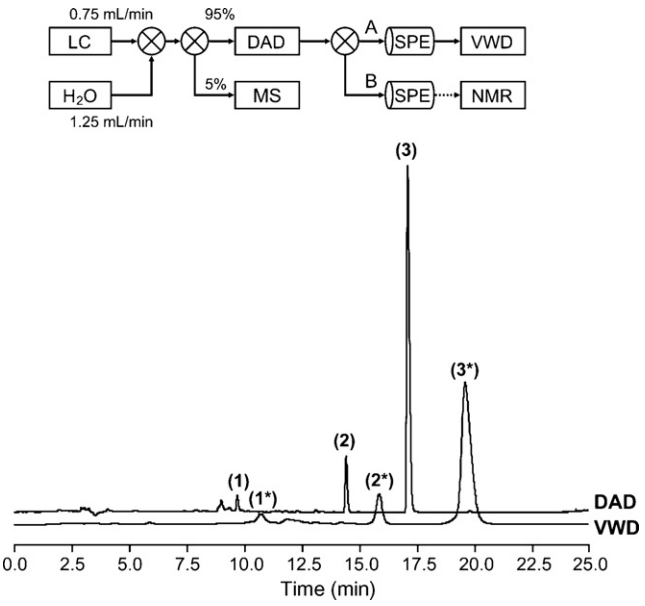

Fig. 2. Schematic of the LC–MS–SPE/NMR system and UV chromatogram (300 nm) showing the retention delay (*) caused by an SPE-cartridge as detected by the variable wavelength detector used in scouting mode (A). The actual trapping mode on SPE for NMR is (B). (1) Amlodipine, (2) indapamide, and (3) valsartan.

eluents were diluted by post-column water addition and detected once by UV and MS, they were directed through the cartridge and detected by the second UV detector, which provided information about the delay and "trapping" potential for all peaks in the chromatogram. The ratio of post-column water addition can thus be adequately adjusted and the maximum number of multiple trapping on single SPE cartridges can be estimated by comparison of the two UV chromatograms (Fig. 2).

For example, it would be expected that one or possibly two repeated trappings of (1) could be done on one SPE cartridge before break-through, while it may be possible to perform three to four repeated trappings of (3) under these chromatographic conditions. In this particular case, a single trapping afforded (3) and a triple trapping yielded (2). As many repeated trappings of (1) was not deemed feasible, a more concentrated sample and single trapping was required. Another advantage of having an additional cartridge is that the fluctuations in back-pressure that are often observed when changing valve positions for trapping of peaks are avoided. Regardless of whether or not peaks are being trapped, the back-pressure remains the same. Trappings of peaks on GP SPE cartridges were triggered by absorbance threshold levels at 300 nm. For isolated compounds, ^{1}H NMR spectra were acquired for (1) and (2) and ^{1}H as well as 2D NMR spectra were acquired for (3), which by comparison to reference data [19–21] led to rapid and confident identification of structures of amlodipine, indapamide, and valsartan (Fig. 3). ^{1}H NMR data for these compounds in CD_3CN solution are reported here. As described in Ref. [19] two rotamers of valsartan were observed in the ^{1}H NMR spectrum, but only chemical shift values for the major rotamer are given in this report.

Amlodipine (1): ^{1}H NMR (CD_3CN, 599.92 MHz) δ 8.02 (1H, s, NH), 7.43 (1H, dd, $J_{H\text{-}6',H\text{-}5'}$ = 7.6 Hz, $J_{H\text{-}6',H\text{-}4'}$ = 1.6 Hz, H-6′), 7.27 (1H, dd, $J_{H\text{-}3',H\text{-}4'}$ = 8.0 Hz, $J_{H\text{-}3',H\text{-}5'}$ = 1.2 Hz, H-3′), 7.22 (1H, td, $J_{H\text{-}5',H\text{-}6'}$ = $J_{H\text{-}5',H\text{-}4'}$ = 7.6 Hz, $J_{H\text{-}5',H\text{-}3'}$ = 1.2 Hz, H-5′), 7.12 (1H, td, $J_{H\text{-}4',H\text{-}3'}$ = $J_{H\text{-}4',H\text{-}5'}$ = 7.6 Hz, $J_{H\text{-}4',H\text{-}6'}$ = 1.6 Hz, H-4′), 5.37 (1H, s, H-4), 4.72 (1H, d, $J_{H\text{-}7a,H\text{-}7b}$ = 14.75 Hz, H-7a), 4.63 (1H, d, $J_{H\text{-}7b,H\text{-}7a}$ = 14.75 Hz, H-7b), 4.01 (2H, m, H-11), 3.75 (2H, m, H-8), 3.55 (3H, s, H-14), 3.21 (2H, t, $J_{H\text{-}9,H\text{-}8}$ = 4.6 Hz, H-9), 2.33 (3H, s, H-15), 1.14 (3H, t, $J_{H\text{-}12,H\text{-}11}$ = 7.1 Hz, H-12).

Indapamide (2): ^{1}H NMR (CD_3CN, 599.92 MHz) δ 8.86 (1H, s, NH), 8.49 (1H, d, $J_{H\text{-}11,H\text{-}9}$ = 2.2 Hz, H-11), 8.02 (1H, dd, $J_{H\text{-}9,H\text{-}10}$ = 8.2 Hz, $J_{H\text{-}9,H\text{-}11}$ = 2.2 Hz, H-9), 7.72 (1H, d, $J_{H\text{-}10,H\text{-}9}$ = 8.3 Hz, H-10), 7.12 (1H, d, $J_{H\text{-}4,H\text{-}5}$ = 7.5 Hz, H-4), 7.06 (1H, dd, $J_{H\text{-}6,H\text{-}7}$ = 8.0 Hz, $J_{H\text{-}6,H\text{-}5}$ = 7.0 Hz, H-6), 6.79 (1H, dd, $J_{H\text{-}5,H\text{-}4}$ = 7.5 Hz, $J_{H\text{-}5,H\text{-}6}$ = 7.0 Hz, H-5), 6.57 (1H, d, $J_{H\text{-}7,H\text{-}6}$ = 8.0 Hz, H-7), 5.99 (2H, s, NH_2), 4.02 (1H, m, H-1), 3.19 (1H, dd, $J_{H\text{-}3a,H\text{-}3b}$ = 15.4 Hz, $J_{H\text{-}3a,H\text{-}1}$ = 8.1 Hz, H-3a), 2.63 (1H, dd, $J_{H\text{-}3b,H\text{-}3a}$ = 15.4 Hz, $J_{H\text{-}3b,H\text{-}1}$ = 11.0 Hz, H-3b), 1.36 (3H, d, $J_{H\text{-}2,H\text{-}1}$ = 6.2 Hz, H-2).

Valsartan (3): ^{1}H NMR major conformer (CD_3CN, 599.92 MHz) δ 7.75 (1H, m, H-20), 7.67 (1H, m, H-22), 7.56 (2H, m, H-19/H-21), 7.22 (2H, d, $J_{H\text{-}13/H\text{-}17,H\text{-}14/H\text{-}16}$ = 8.06 Hz, H-13/H-17), 7.14 (2H, d, $J_{H\text{-}14/H\text{-}16,H\text{-}13/H\text{-}17}$ = 8.06 Hz, H-14/H-16), 4.80 (1H, d, $J_{H\text{-}11a,H\text{-}11b}$ = 16.98 Hz, H-11a), 4.49 (1H, d, $J_{H\text{-}11b,H11a}$ = 16.98 Hz, H-11b), 3.98 (1H, d, $J_{H\text{-}6,H\text{-}7}$ = 10.4 Hz, H-6), 2.46 (1H, m, H-7), 2.38 (2H, m, H-4), 1.57 (1H, m, H-3a), 1.38–1.26 (3H, m, H-3b, H-2), 0.95 (3H, d, $J_{H\text{-}9,H\text{-}7}$ = 6.6 Hz, H-9), 0.86 (3H, m, H-1, H-8).

3.2. Quantification

For each adulterant, a local absorbance maximum in the UV spectrum was chosen as the optimum wavelength for quantification: 360 nm for amlodipine, 240 nm for indapamide, and 250 nm for valsartan. Linearity was observed in the analyzed range of concentrations for all three compounds. Equations for standard curves were $y = 1113.4x + 0.7944$ for amlodipine ($r^2 = 0.9998$), $y = 3860.8x + 14.043$ ($r^2 = 0.9986$) for indapamide, and $y = 1856.8x + 41.802$ ($r^2 = 0.9972$) for valsartan, where y represents the peak area, expressed in mAu × min and x represents the concentration expressed in mg/mL. Reproducibility of peak areas was excellent with R.S.D. values for repeated injections determined as 0.14–3.36%, 0.53–0.79%, and 0.19–0.28% for amlodipine, indapamide, and valsartan, respectively. The accuracy of the analysis was analyzed as described in Section 2.5.2 and calculated concentrations of amlodipine were 97.79 ± 3.40% of the actual concentration, for indapamide they were 100.08 ± 5.47%, and

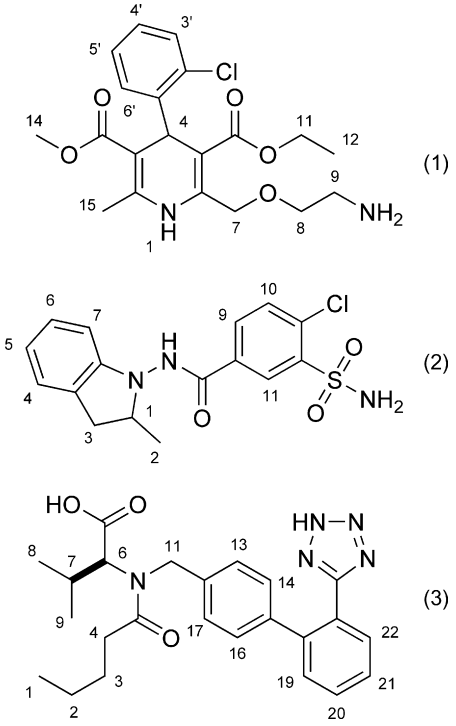

Fig. 3. Chemical structures with atom numbering used for NMR data assignment. (1) Amlodipine, (2) indapamide, and (3) valsartan.

Table 1
Mean arterial blood pressure and heart rate for all treatment groups.

Baseline (−24 h)				Post dosing (+96 h)			
Treatment	Mean	Std. dev.	B.M.C. test	Treatment	Mean	Std. dev.	B.M.C. test
Mean arterial blood pressure							
Vehicle	166.7	5.40	–	Vehicle	165	5.78	–
Capsule	163.3	7.04	$P > 0.05$	Capsule	147.3	9.08	$P < 0.001$[a]
Combination	166.5	6.04	$P > 0.05$	Combination	145.7	11.21	$P < 0.001$[a]
Amlodipine	165.5	5.73	$P > 0.05$	Amlodipine	163.4	6.83	$P > 0.05$
Indapamide	164.7	4.71	$P > 0.05$	Indapamide	159.9	7.06	$P < 0.001$[a]
Valsartan	165.2	4.26	$P > 0.05$	Valsartan	151.7	9.08	$P < 0.001$[a]
One-way ANOVA, $P = 0.3232$				One-way ANOVA, $P < 0.0001$			
Heart rate							
Vehicle	325.1	32.44	–	Vehicle	326.6	35.1	–
Capsule	321	34.28	$P > 0.05$	Capsule	327.8	38.56	$P > 0.05$
Combination	328.3	38.19	$P > 0.05$	Combination	335.9	38.43	$P > 0.05$
Amlodipine	324.8	39.19	$P > 0.05$	Amlodipine	326.1	36.02	$P > 0.05$
Indapamide	318.8	33.94	$P > 0.05$	Indapamide	321.8	34.83	$P > 0.05$
Valsartan	320.8	32.73	$P > 0.05$	Valsartan	329.5	34.38	$P > 0.05$
One-way ANOVA, $P = 0.9423$				One-way ANOVA, $P = 0.153$			

Bonferroni's multiple comparison vs. vehicle.
[a] Significant difference.

Table 2
Max. change in mean arterial blood pressure (ΔMAP) in comparison with baseline control.

Treatment (5 mL/kg)	Dose (mg/kg)	Max. ΔMAP	ABC, 72 h	ABC, 96 h
0.5% methyl cellulose (aq)	Vehicle	N/A	N/A	N/A
Gold Nine Soft Capsules	300 mpk	27.5 ± 2.7	1169 ± 149.6	1314.3 ± 216.9
Combination of synthetics	See doses below	40.6 ± 8.5	1784 ± 350.9	1538.9 ± 302.2
Amlodipine	0.81 mpk	16.5 ± 5.1	103.2 ± 83.0	N/A
Indapamide	0.84 mpk	15.6 ± 5.4	339 ± 72.3	N/A
Valsartan	19.30 mpk	30.4 ± 2.8	1064.5 ± 141.5	1137.4 ± 145.7

for valsartan 105.81 ± 3.40%. Actual concentration measurements were within the ranges used for standard curves. Each 300 mg of the extracted capsule contents, which was equivalent to the dose used in the *in vivo* studies, contained 0.78 ± 0.06 mg amlodipine, 0.78 ± 0.02 mg indapamide, and 18.47 ± 1.03 mg valsartan (R.S.D. 7.12%, 2.57% and 5.60%, respectively). Assuming that each capsule was consistently formulated with 500 mg of content, this would correspond to approximately 1.3 mg amlodipine, 1.3 mg indapamide, and 30.8 mg valsartan per capsule. Actual measurements showed that each intact capsule contained 1.52 ± 0.10 mg amlodipine, 1.52 ± 0.07 mg indapamide, and 40.46 ± 2.44 mg valsartan (R.S.D. 6.48%, 4.58%, and 6.03%, respectively). It may be speculated that the high levels of valsartan are not completely soluble in the oily formulation and have been partially absorbed by the gelatin shell of the intact capsule.

3.3. *In vivo studies*

Blood pressure levels were collected from conscious and unrestrained SHRs using the radio-telemetry probes. At baseline level (untreated rats and vehicle control group), rats were clearly hypertensive and the data collected for 24 h prior to dosing showed no significant difference in mean values of blood pressure or heart rate between groups.

Following a single oral dose of 300 mg Gold Nine Soft Capsules per kg, the maximal ΔMAP and ABC values were comparable to values from groups receiving a combination treatment of amlodipine, indapamide, and valsartan (see Tables 1 and 2 as well as Fig. 4 for detailed data). Also, the duration of the effect was similar for these two groups with a one-way ANOVA analysis showing no significant difference between treatment with either capsules or combination of the three synthetic standards ($P > 0.05$). A trend in increase of the heart rate immediately after dosing was observed for a short period in animals treated with capsules or the combination, but it is not statistically significant (Fig. 4).

The three synthetic drugs found to be the major components of the capsules were also tested individually in SHRs and results showed that a single oral dose of either indapamide or valsartan gave a significant reduction in BP, whereas administration of amlodipine did not give a statistically significant change compared to vehicle treatment. Especially in the animals receiving a single dose of valsartan, the blood pressure lowering effect, as well as the duration of this effect, was remarkably higher than for single doses of amlodipine and indapamide. The ΔMAP for the group treated with valsartan was similar to ΔMAP in the group receiving capsules, but the duration of the effect was significantly longer in the capsule-treated group.

These results suggest that combination therapy involving more than one anti-hypertensive agent might improve the duration of the effect more than it improves the actual maximal effect. As there is no significant difference in the effect observed in animals treated with either capsules or the combination of pure drugs, the results from the *in vivo* experiments show that the anti-hypertensive effect of Gold Nine Soft Capsules can be attributed solely to the presence of amlodipine, indapamide and valsartan in the capsules. In this study valsartan was dosed to the animals in a much higher concentration than in previous reports [22] and doses were generally determined by a preliminary analytical study of capsule content. In-house pharmacokinetic studies (data not shown) confirmed that slight variations in administered doses of valsartan (19.30 mg/kg or 18.65 mg/kg) did not lead to significant differences in actual drug exposures. It is obvious that valsartan is responsible for a major part of the anti-hypertensive effect observed in Gold Nine Soft Capsules, but amlodipine and indapamide might also play a role in either an additive or synergistic way. In order to determine these mechanisms, rigorous preclinical and clinical studies would need to be established, which is not within the scope of this investigation.

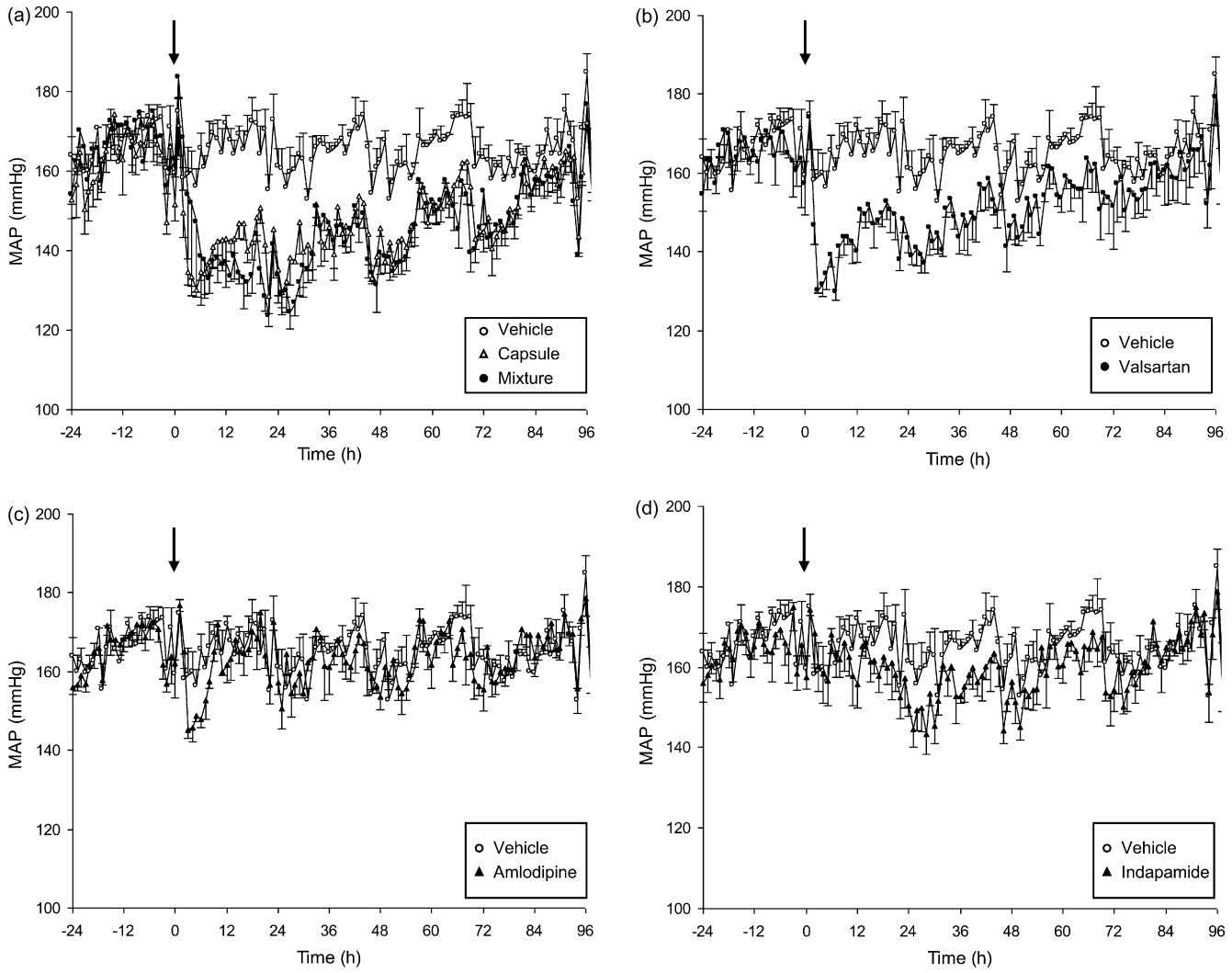

Fig. 4. Changes in mean arterial blood pressure following administration of a single dose of vehicle, Gold Nine capsules or synthetic standards to telemetrized spontaneously hypertensive rats (SHR), $n = 3$ in each group. (a) Vehicle (0.5% methyl cellulose) vs. capsules (300 mg/kg) vs. combination treatment of synthetic standards (corresponding to 300 mg/kg capsule), (b) vehicle vs. valsartan (19 mg/kg), (c) vehicle vs. amlodipine (0.81 mg/kg), and (d) vehicle vs. indapamide (0.84 mg/kg). Data shown are the means of groups per time-point for up to 96 h after administration and are expressed as mean ± S.E.M. For amlodipine, there is no significant effect, whereas a significant effect is observed for indapamide as well as for valsartan. It is also shown that there is no significant difference between Gold Nine Soft Capsules and the combination treatment using the synthetic compounds.

4. Conclusion

LC–MS–SPE/NMR is a rapid and reliable method for identification and structure verification and/or structure elucidation of compounds from mixtures like plant or microbial extracts or herbal remedies, in this case, adulterated with synthetic drugs. The possibility of rapidly obtaining high-quality NMR data from small amounts of compounds is an advantage that makes LC–MS–SPE/NMR a powerful analytical tool – not just in natural product research, but potentially also in various other fields of analytical chemistry. With an increased number of cases of adulteration with not only known synthetic drugs, but novel analogues hereof, we anticipate that NMR will be required more often to reveal such fraudulence. None of the active drugs found in the Gold Nine Soft Capsules were mentioned on the label and consumers had no knowledge of the drugs that they were actually exposed to. They could be subject to the risks of drug–drug interactions with other medications, potential overdosage from simultaneously using similar prescribed anti-hypertensive drugs, or experiencing un-attributable side-effects that pose a threat to their health. If one or more of the drugs found as adulterants in this study were contra-indicated for individual patients, consumption of Gold Nine Soft Capsules could have led to serious adverse effects of unknown origins, as has been reported for other adulterated herbal preparations [23,24].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2009.09.043.

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