Department of Pharmaceutical Biology[1], Institute of Pharmacy, University of Leipzig; Schaper & Brümmer GmbH[2], Salzgitter-Ringelheim, Germany

Stachydrine in *Leonurus cardiaca*, *Leonurus japonicus*, *Leonotis leonurus*: detection and quantification by instrumental HPTLC and ^{1}H-qNMR analyses

K. Kuchta[1], R. B. Volk[2], H. W. Rauwald[1]

Received December 6, 2012, accepted February 22, 2013

Prof. Dr. Hans Wilhelm Rauwald, Department of Pharmaceutical Biology, University of Leipzig, Johannisallee 21-23, 04103 Leipzig, Germany
rauwald@rz.uni-leipzig.de

Dedicated to Prof. Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.

Pharmazie 68: 534–540 (2013) *doi: 10.1691/ph.2013.6527*

Stachydrine ((2*S*)-1,1-dimethylpyrrolidinium-2-carboxylic acid) may be regarded as an essential active principle of the aerial parts of *Leonurus japonicus* Houtt. (*Leonuri* herba, yimucao; Chin.Ph., DAB) which are used in TCM and Kampo for the treatment of various gynaecological and cardiovascular disorders. Medically and botanically closely related *Lamioideae* drugs are the fruits of *L. japonicus* (*Leonuri* fructus, chongweizi; Chin.Ph.), the aerial parts of European *Leonurus cardiaca* L. (*Leonuri cardiacae* herba; Ph.Eur.) as well as the aerial parts of their South African relative *Leonotis leonurus* (L.) R.Br. (*Leonotis leonuri* herba). Regarding *L. cardiaca*, stachydrine might be an exceptionally interesting constituent as Dragendorff-positive substances like stachydrine were found to be enriched in an antiarrhythmic *L. cardiaca* refined extract, which was most recently developed via bioassay guided fractionation. The few pharmacological publications on this betaine do indeed describe cardiovascular, hypotensive, and tissue protective effects. However, its pharmacopeial analytics poses a severe difficulty, as it does not contain any chromophoric group suitable for customary HPLC-UV detection. For quality control of yimucao according to Chin.Ph. the entirety of its N-containing compounds is photometrically quantified after Reinecke's complexation. Unfortunately, this method suffers from a relatively low reproducibility. Since no reliable quantification method for stachydrine is available up to now, a highly reproducible instrumental HPTLC method was newly developed, using postchromatographic derivatization by Vágújfalvi reagent, thus changing non absorbing stachydrine into a detectable derivative at 517 nm, and an automatic HPTLC system with scanner and analysis software (winCATS). This method was shown to be precise with respect to concentration and yielded highly reproducible data over numerous inter-day repetitions. Not only did the independent evaluation of the scanned HPTLC sheets for stachydrine peak area and height result in almost identical values for all samples, but also the results of a parallel-developed direct quantitative ^{1}H-NMR procedure using its N-CH_3 singlet δ 3.03 ppm in comparison with the singlet of the two vinylic protons of the internal standard maleic acid at δ 6.18 ppm were always within the standard deviation of the HPTLC data. These measurements of 12 drug samples revealed stachydrine contents (w/w) of 0.2 to 1.0 % for the *L. japonicus* aerial parts, 0.6 to 1.5 % for the *L. cardiaca* aerial parts, 6.7 % for the antiarrhythmic refined extract of *L. cardiaca*, and 0.3 % for the aerial parts of *Leonotis leonurus*, while both *L. japonicus* and *L. cardiaca* fruits contained, on average, 0.2 %. Furthermore, stachydrine was found for the first time as a constituent of *L. japonicus* and *L. cardiaca* fruits as well as *Leonotis leonurus*. Methodically, instrumental HPTLC may be a powerful tool for quality assurance for stachydrine containing plants and herbal drugs, especially for industrial routine protocols.

1. Introduction

Stachydrine ((2S) - 1, 1 - dimethylpyrrolidinium - 2 - carboxylic acid resp. N-dimethyl-L-proline), a chemotaxonomic marker of the *Lamioideae* subfamily, was firstly described as a constituent of both European *Leonurus cardiaca* (van Eijk 1952) and East Asian *Leonurus japonicus* (Hung 1959) about 60 years ago. As the alkaloid fraction of *L. japonicus* extracts, which appears to be dominated by stachydrine, has been reported as the most active (Villamar et al. 1994), this betaine has been discussed as a possibly important contributor to the overall pharmacological activity of not only the TCM/Kampo drug (Luo 2003; Villamar et al. 1994) but also for *L. cardiaca* (Nahrstedt 1985). Although molecular pharmacological data for the betaine itself are scarce,

Abbreviations: HPTLC, High Performance Thin Layer Chromatography; LCH, *Leonuri cardiacae* herba; LCS, *Leonuri cardiacae* fructus; LCRE, *Leonurus cardiaca* refined extract from aerial parts; LJH, *Leonuri japonici* herba; LJS, *Leonuri japonici* fructus; LLH, *Leonotidis leonuri* herba; PRC, China; Jap, Japan; Ger, Germany; Chin.Ph., Chinese Pharmacopoeia; DAB, German Pharmacopoeia; Ph.Eur, European Pharmacopoeia.

it was found in one of these few publications that intravenous injections of 0.01 - 0.1 g/kg bodyweight stachydrine in aqueous solution exerted profound positive chronotropic and negative inotropic effects in dogs (Akopov et al. 1958). Most recently, it was described to effectively ameliorate the decline in cell viability of human umbilical vein endothelial cells after injuries induced by anoxia-reoxygenation (Yin et al. 2010). These findings are especially interesting, as we have only just proven that a newly developed refined extract of *L. cardiaca* exhibits profound hypotensive and tissue protective effects on isolated rabbit hearts in Langendorff setup and that Dragendorff-positive substances such as stachydrine were enriched in this preparation (Ritter et al. 2010). Analytically, the Chin.Ph. (Anonymous 2000) resorts for quality control of yimucao (*Leonuri herba*; Chin.Ph., DAB) to the photometrical measurement of its overall content in N-containing compounds, complexated with Reinecke salt ($NH_4[Cr(SCN)_4(NH_3)_2]$) and calculated as stachydrine. On the other hand, the DAB (Anonymous 2010) does not mention stachydrine and prescribes a total flavonoid content of at least 0.3 % of the dry weight, calculated as hyperoside, analogues to the quality standards for *L. cardiaca* according to Ph.Eur. Since the photometric Chin.Ph. method appears relatively unspecific for stachydrine, an HPLC approach has been proposed (Chao et al. 2004) which did however display conceptual deficits and proved not reproducible as discussed later.

The paper at hand presents alternative methods of reproducible stachydrine quantification in plant drug material using instrumental HPTLC technology – especially designed for analytes not containing a chromophore – in comparison with ^{1}H-qNMR spectroscopy which is by its very nature a quantitative means of chemical analysis as the area of each signal is directly proportional to the number of contributing nuclei (Berger et al. 2004). These methods were applied for measuring the stachydrine content in twelve aerial part and fruit samples of *L. cardiaca* and *L. japonicus* as well as in a sample of the closely related *Leonotis leonurus* (L.) R.Br. which is used in Muthi (Traditional Southern African Medicine) with very similar indications (Watt et al. 1962; Hutchings et al. 1996). Its cardioactivity has recently been demonstrated using a Langendorff isolated rat heart model (Burger et al. 2008). Up to now, no research into the presence or content of stachydrine in plants of the genus *Leonotis* or in fruits of *Leonurus* spec. has ever been reported.

2. Investigations, results and discussions

As there is currently no reproducible method for routine quantification of stachydrine available, the development of such a procedure was defined as the aim of the present research project, applied to the five Lamioideae drugs *Leonuri cardiacae herba* and *fructus*, *L. japonici herba* and *fructus*, *Leonotidis leonuri herba*, as well as to the special preparation *L. cardiaca* refined extract from aerial parts (Ritter et al. 2010). As for the detection and quantification methods published so far in Chin.Ph., both turned out to be unworkable in the current course of experiments. The same is true for the HPLC method by Chao et al. (2004) using a specialised YMC-Park cyano phase column with a complicated aqueous mobile phase containing 10 % MeOH, 0.00125 mol/l of the ion-pair forming agent SDS, and 0.05 % of reactive perchloric acid. In the paper at hand, the use of a CN stationary phase – both with and without ion-pair forming agents – could not achieve a baseline separation of stachydrine in the extracts as described below. This may be correlated with the fact that only short exerts of HPL-chromatograms are depicted (Chao et al. 2004). However, the most significant problem with this HPLC method is the inability to inject raw extracts directly. Instead, a special measurement solution analogous to the photometric Chin.Ph. method for measuring the total alkaloid content of yimucao had to be laboriously prepared with each step resulting in losses and thus additional systematic error.

According to Chin.Ph. stachydrine can be identified in *Leonuri herba* (*L. japonicus*) via TLC using a stationary phase of silica gel G and a mobile phase of *n*-butanol:concentrated HCl_{aq}: H_2O (8:2:1) with Dragendorff spray as after development derivatisation (Anonymous 2000). It has to be mentioned that for identity control of *Leonuri cardiacae herba* (Ph.Eur.) a similar TLC examination on stachydrine was also described in Wichtl et al. (2002). However, it likewise proved to be inappropriate (e.g. charcoal; aluminiumoxide: basicity not declared). In preliminary experiments to the presented project, these approaches proved unworkable with customary aluminium TLC sheets that dissolve in this mobile phase, thus making expensive glass plates mandatory. Even on these, stachydrine zones after detection were much broader and much less clearly defined as with the newly developed mobile phase MeOH:CH_2Cl_2:NH_3 25 % (8:2:3), which could also be applied to much cheaper aluminium TLC sheets without any problems. Besides the aforementioned TLC system, Chin.Ph. prescribes the photometric determination of the alkaloid content of the yimucao drug. Therefore, the total alkaloids are complexated with Reinecke salt $NH_4[Cr(SCN)_4(NH_3)_2]$ in hydrochloric acid solution and the total absorbance measured at 525 nm against 0.1 M hydrochloric acid. The total alkaloid content is calculated as stachydrine and must not be lower than 0.40 % in officinal material. This way of measurement is however not specific for the betaine but reacts to all N-containing compounds. In contrast to the LJH drug, no qualitative or quantitative examinations are prescribed for its fruit drug chongweizi (LJS) (Anonymous 2000). The detection and quantification methods for stachydrine in the Chin.Ph. monograph for *Leonuri* herba have not only been shown to be highly impractical in our own laboratory. The problem was also addressed in a recent publication stating that *".. . with as well the TLC detection as the quantitative determination of stachydrine according to the Chin.Ph. both impractical and questionable in their methodological setup, this constituent could not be examined further (Bomme et al. 2006)"*, or simply *".. . the assay for stachydrine as described in the monograph Herba Leonuri japonici was not reproducible (Heuberger et al. 2010)"*.

Consequently, in order to facilitate routine examination of this prominent constituent of both the TCM drug and European LCH, a state of the art HPTLC protocol has been developed. Originally the term "high performance thin-layer chromatography" (HPTLC) was used to describe every thin-layer chromatography performed on plates with small particles having an average size of 5 μm and narrow size distribution as opposed to TLC material of 15 μm average particle size and broader size distribution. These "high performance TLC sheets" thus offer a significantly higher peak resolution and shorter developing times. Furthermore, standard TLC is used for mostly manually performed analyses whereas HPTLC also includes the use of instruments for all steps of the chromatographic process. These have dramatically advanced over the recent decade leading to a strong improvement of performance and reliability of planar chromatography to the level of any other relevant analytical method (Reich et al. 2009; see also *3.5.1*). Only the use of these specially developed HPTLC plates in combination with automatic sample application, scanning, and data analysis as described above makes the presented novel procedure for stachydrine detection and quantification workable. Early in the course of our preliminary studies, the customary Dragendorff reagent of Chin.Ph. proved insufficient for detecting the proline derivative stachydrine with reproducible colour intensity, an effect that has also

Table 1: Occurrence and content of stachydrine in all examined drug samples of *Leonurus cardiaca*, *Leonurus japonicus*, *Leonotis leonurus*

	Company	Batch number	Origin	Extract % (w/w) in the drug	Stachydrine % (w/w) in the drug			
					HPTLC peak area	HPTLC peak height	qNMR	Ø
LCH A	Galke	10477	Ger	24.17	1.48	1.55	1.55	1.53
LCH B	Caesar & Loretz	32054354	Ger	21.96	1.13	1.17	1.13	1.14
LCH C	Galke Organic Farming	0000010957	Ger	26.86	0.54	0.50	0.63	0.56
LCH D	Botanical Garden	-----	Leipzig	9.53	0.67	0.67	0.65	0.66
LCRE	Pharmaceutical Biology, Leipzig University (Ritter et al. 2010)			-----	6.58	6.93	6.69	6.73
LCS	Botanical Garden	-----	Leipzig	7.53	0.22	0.22	0.24	0.23
LJH A	Galke	0000011580	Ger	23.16	1.05	1.07	1.01	1.04
LJH B	Kräuterhof Frieß	E7558A	State of Bavaria (Ger)	24.66	1.04	1.04	0.96	1.01
LJH C	Nakajima Shouyaku	11112A075	Jap	13.59	0.39	0.37	0.43	0.40
LJH D	SinoPhytoMed	13964K262-4-100	Province of Jiangsu (PRC)	11.95	0.16	0.15	0.18	0.16
LJS A	SinoPhytoMed	13963K263-4-100	Province of Anhui (PRC)	9.28	0.15	0.13	0.18	0.15
LJS B	Uchida Wakanyaku	7B33213	Jap	10.48	0.19	0.17	0.21	0.19
LLH	Botanical Garden	-----	Leipzig	13.45	0.32	0.30	0.28	0.30

Samples were obtained from China (PRC), Japan (Jap), and Germany (Ger). In the case of the Chinese and German samples the name of the exact region is given in all cases where this information could be obtained.

been observed by Bomme et al. (2006). After further unsuccessful trials with Pt-reagent (for composition see Wagner et al. 1983) and elemental iodine, Vágújfalvi reagent was shown to detect stachydrine on TLC and HPTLC sheets with high reproducibility. Thus, applying the above-mentioned techniques, the predominant *Leonurus* constituent stachydrine has been reproducibly quantified for the first time (Table 1, Fig. 1).

In order to provide a backup method that can be used as an external prove of the correctness of the HPTLC data, ^{1}H-qNMR was comparatively used, as the area of each signal is directly proportional to the number of contributing nuclei (Berger et al. 2004; Malz 2008). However, in order to relate this area to the concentration of an individual compound, an internal standard of known concentration has to be added to the solution for comparison of its NMR peak with the one of the analyte. In the paper at hand, maleic acid proved most appropriate for this purpose. Stachydrine ^{1}H-qNMR spectra were recorded upon dissolution of the freeze-dried samples in D_2O. The analysis was carried out by comparing the integral of the $-CH_3$ singlet signal of stachydrine at δ 3.03 ppm with the integral of the singlet signal of the two vinylic protons of the internal maleic acid standard, at δ 6.18 ppm. The contents of stachydrine in the twelve examined drug specimen and the *L. cardiaca* refined extract calculated from as well qNMR as both HPTLC heights and area values were all in good accordance between the three different measurement approaches as shown in the Table. Usual trading samples of *L. cardiaca* (Ph.Eur.) contain significantly more stachydrine than all national and international trading samples of *L. japonicus* (Chin.Ph., DAB). Only the fresh plant and the organically cultivated drug did yield lower stachydrine contents than the *L. japonicus* samples from unspecified German or Bavarian (German) cultivation but their contents were still above those of the drug harvested in Japan. In contrast to the aerial parts, no significant differences in stachydrine content could be observed for the fruits of the two species. As far as *Leonotis leonurus* is concerned, the sample did contain only half as much stachydrine as the *L. cardiaca* plant grown on the same area. Nevertheless, it still had a higher content of stachydrine than all fruit samples. This finding is especially interesting, as plants of the genus *Leonotis* have not been examined for the presence of stachydrine previously. Concerning the minimal stachydrine content of 0.40 % according to Chin.Ph. it can be stated that with the exception of the Chinese herb from Jiangsu (Table) all aerial part samples of both species were in accordance with the requirements, with the Japanese sample fulfilling the requirements to a point. The examined *Leonotis leonurus* plant ranked clearly below this minimal content. None of the examined fruits did contain more than 0.40 % of stachydrine but, as stated above, Chin.Ph. does not require any test on this compound for the fruit drug.

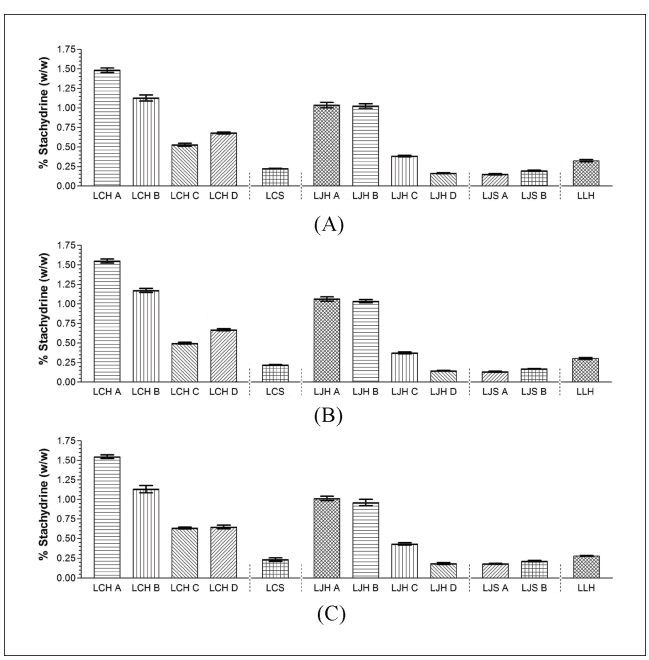


Fig. 1: Stachydrine content (w/w) in all examined drug samples, determined by HPTLC peak area (A), HPTLC peak heights (B), and ^{1}H-qNMR (C). For identification of the individual samples see Table. The standard deviation of the raw data is given in the form of error bars

Among the *L. japonicus* samples, the highest and very similar contents were found in the drug from not further specified German cultivation obtained from Galke (LJH A) and the material from Bavarian cultivation obtained from "Kräuterhof Gerhard Frieß" in the town of Abenberg (LJH B) which contained 1.04 and 1.01 % of stachydrine respectively. The high stachydrine contents in the two drug samples from German cultivation are especially interesting as it was observed in patients that the effect of dynamisation of the 'xue' according to Chin.Ph. appears clearly more intensive than the effects of Chinese reference samples (Bomme 2008). A possible explanation for this observation may be that, as the study at hand demonstrates, the stachydrine content in the Bavarian drug (LJH B) is almost five times as high as in the sample that was purchased from China by the professional TCM drug import company SinoPhytoMed (LJH D). A further observation leading to the question of a potential contribution of stachydrine to the pharmacological effect of the examined drugs is that among all investigated samples the *L. cardiaca* refined extract (LCRE) that was developed by bio-guided fractionation (Ritter et al. 2010) had the highest content of stachydrine with 6.73 % in the average of all measuring systems. The herb sample LCH B from which LCRE was prepared did contain 1.14 %. The enrichment was achieved to a large degree by the first aqueous extraction step, as the aqueous extract of LCH B did already contain 5.16 % of stachydrine also, as demonstrated above, a significant further enrichment did occur during the preparation of the refined extract. The highest stachydrine content in all primary raw drug samples was found in LCH A with 1.53 %. These observations are especially interesting as they may, together with its strong accumulation in the before mentioned bioassay (Ritter et al. 2010), hint to the potential role of this betaine contributing to the antianginal, hypotensive, and central nervous activities of *L. cardiaca* herb and its antiarrhythmic refined extract LCRE. For example, stachydrine has already been discussed as a component of *L. cardiaca* with potentially sedative activity (Nahrstedt 1985). This statement was however not grounded on experimental data, but on the observation that stachydrine is found in several medicinal plants, regarded as sedative in folk medicine, with the members of the genus *Stachys* – like all test samples belonging to the Lamioideae – being the most prominent ones.

As mentioned above, the experimental procedures given in the Chin.Ph. for *Leonuri herba*, both the TLC identification of stachydrine and the photometric determination of the total alkaloid content of the drug, have been shown to be hardly reproducible (Bomme et al. 2006) and the development of more precise methods is necessary. However, the lack of a chromophore in stachydrine hampers its determination via standard methods such as UV detection. In such a case, either a qNMR or a quantitative HPTLC, combined with a derivatisation of the analyte, could be an alternative tool for quantification as demonstrated previously for HPTLC analysis of artemisinin (Widmer et al. 2007). Both alternatives were realized in this study, and both proved to be suitable for the quantitative detection of the analyte stachydrine. In the paper at hand new qualitative and quantitative HPTLC and ^{1}H-qNMR analyses for the determination of stachydrine are described for the first time applied to different plant drug materials of *Leonurus cardiaca*, *Leonurus japonicus*, and *Leonotis leonurus*, also for the first time. Both methods yielded constantly reproducible data in accordance with those of the parallel measurement on a totally different physical basis. However, of these two methods HPTLC is certainly more qualified for routine quantitative analyses on the industrial scale, not least because of a lower instrumental complexity. Finally, the occurrence of stachydrine is detected for the first time in *Leonuri japonici fructus* (chongweizi), *L. cardiacae* fructus, and in *Leonotis* spec. (*Leonotis leonurus*).

3. Experimental

3.1. Plant material

The officinal aerial parts (*Leonuri* herba, yimucao; Chin.Ph., DAB) and fruits (*Leonuri* fructus; chongweizi; Chin.Ph.) of *Leonurus japonicus* as well as *Leonuri cardiacae* herba (Ph.Eur.) were purchased from the sources listed in the Table and controlled for their quality according to the requirements of the Chin.Ph. or Ph.Eur. respectively, regulations which were met by all analysed samples, which were in complete accordance to co-investigated authentic voucher specimen deposited in the herbarium of the Institute of Special Botany, Leipzig University under the registration number LZ 203412 for *L. japonicus* and under the EDV registration number 167244 for *L. cardiaca*. *Leonotis leonurus* was cultivated at the gardens of botany of Leipzig University. Fresh flowering aerial parts were collected for examination. A voucher specimen of these was deposited in the above-mentioned herbarium under the registration number LZ 203520.

3.2. Extract preparation

Pulverised drug material (6.00 g) was extracted with boiling water (120 ml) for 60 min under reflux. The resulting infusion was filtered under vacuum until the residue was dry and the liquid was clear. It was subsequently evaporated to dryness and lyophilised. The resulting dry extract was weighted in order to calculate the drug extract ratio. Finally, all extracts were powdered and stored in sealed glass flasks at −20 °C. For details concerning the preparation of the *L. cardiaca* refined extract (LCRE) see Ritter et al. (2010).

3.3. Isolation of stachydrine

For purification of stachydrine, LCRE (0.5 g) was fractioned by preparative RP18 MPLC (Büchi, Flawil, CH; C-615 gradient pumping station; two C-605 pumps; Sepacore C-690 glass column No. 19675; RP-C18 packing by Merck, Darmstadt) with water-acetonitrile gradients as mobile phases at 4 ml/min. An aliquot of the lyophilised enriched stachydrine fractions, obtained from the first MPLC eluates, was dissolved in water in a concentration of 50 mg/ml for further purification by preparative silica TLC. Bands with a length of 1 cm, each containing 1.5 mg of the purified extract, divided by 0.5 cm empty space were applied to 20 x 20 cm TLC silica gel 60 F_{254} sheets (Merck, Darmstadt) with 1 cm distance to the rim. These TLC sheets were placed in a development chamber saturated with the mobile phase MeOH: CH_2Cl_2: NH_3 25 % (8: 2: 3). The TLC run was allowed to proceed to a total propagation of 18.0 cm. Following careful evaporation of the mobile phase, one sample band was cut out of each TLC sheet with a carpet knife and sprayed with Vágújfalvi solution, revealing stachydrine as a red zone at $R_f = 0.53$ (Fig. 1). The areas around this position on the rest of the TLC sheets were cut out, the silica gel scratched of and extracted with a solution of MeOH: CH_2Cl_2 (1: 1) for three hours under constant shaking. The resulting solution was filtered and subsequently evaporated to dryness to afford about 5 mg of stachydrine.

3.4. Stachydrine reference substance

In addition to *3.3*., commercial stachydrine as a reference compound was purchased from Phytolab, Vestenbergsgreuth (100 % purity) and proved to be identical to the sample isolated from the drug as described above. Identity, purity, and content were determined by positive ESI-MS ($[M]^+ = m/z$ 144) as well as by ^{1}H- and ^{13}C-NMR experiments as described above and were found to be in accordance with the declared purity.

3.5. HPTLC

3.5.1. General and specific chromatographic conditions

Although planar chromatography is primarily regarded as a qualitative technique, it can also offer precise and accurate quantitative data (Reich et al. 2009), as evaluation of planar chromatograms by high end scanning densitometry can today be regarded as a standard technique, fully equivalent to modern photo diode array detectors in HPLC (Dammertz et al. 2001; Apers et al. 2006; Skarkova et al. 2008). As a quantitative tool, HPTLC in combination with classical TLC detection solutions is of particular advantage if the analyte does not contain a chromophore, which is the case for stachydrine.

In the HPTLC procedure developed in the presented project, all samples were applied to 10 x 20 cm HPTLC silica gel 60 F_{254} plates (Merck) by an Automatic TLC Sampler III (CAMAG, Muttenz, Switzerland). Developed and derivatised plates were scanned by a TLC Scanner 3 (CAMAG) and analysed with the winCATS Planar Chromatography Manager software (CAMAG), version 1.4.4, which was also used for controlling the entire automated HPTLC procedure including its calibration and validation.

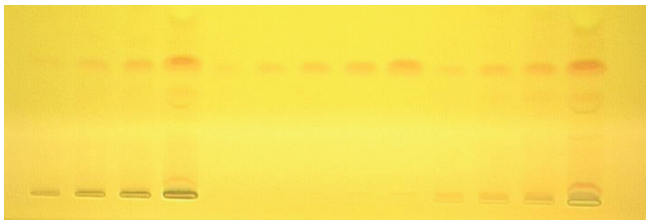


Fig. 2: HPTLC of *L. cardiaca* raw extract (sample LCH B, four rows on the left), stachydrine reference substance (five rows in the centre), and the antiarrhythmic *L. cardiaca* refined extract (sample LCRE, four rows on the right). For details concerning measurement conditions see text

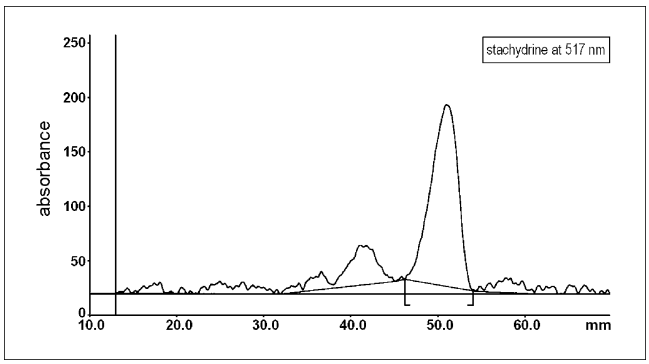


Fig. 3: HPTLC chromatogram of a representative run of 80 µg of sample LCH B (Table 1) in the described HPTLC system, scanned at 517 nm after derivatisation with Vágújfalvi reagent. Baseline correction is marked with a straight line

3.5.2. *Sample preparation*

An amount of approximately 22.00 mg of dried extract was exactly weighted and dissolved in a respective amount of water, resulting in a concentration of exactly 20.00 mg/ml. All samples were sonicated for 5 min in order to facilitate easier dissolution and centrifuged for 15 min with 145 rotations/s for removing insoluble compounds from the extract. Finally, the solution was filtered through a Chromafil PET-20/15 MS filter, thus preventing plugging of the sample application syringe of the automatic TLC sampler. The stachydrine standard was dissolved in water with a concentration of 2.00 mg/ml. Three bands with a length of 8.0 mm containing 60, 80, and 100 µg of the extract sample were applied by a CAMAG Automatic TLC Sampler III to 10 x 20 cm HPTLC silica gel 60 F_{254} plates. On the same plate, four identical standard lines were applied containing amounts of 1.00, 3.00, 5.00, and 7.00 µg stachydrine. With a distance of 15 mm to the left and 10 mm to the lower edge of the plate as well as 5 mm of free space between the lines, there was enough space on each HPTLC plate for 13 lines. Thus, three different extract samples with three lines each could be measured on every single plate using an identical set of four lines of stachydrine standard. This procedure was repeated on n = 3 independent plates for each sample resulting in a total amount of 9 measurements per extract. After evaporation of the solvent, the HPTLC plate was placed in a Horizontal-Entwicklungskammer 2 (20 × 10 cm) TLC chamber (CAMAG), saturated with the development reagent MeOH: CH_2Cl_2: NH_3 25 % (8: 2: 3). After 10 min of fumigation the HPTLC run was started and allowed to proceed to a total propagation of 8.5 cm. Following the complete evaporation of the development solution, the TLC plate was dipped for three seconds into Vágújfalvi solution (for composition see Wagner et al. 1983) for after development derivatisation, using a DC-Tauch-fix II dipper (Lothar Baron Laborgeräte, Insel Reichenau). Subsequent to the evaporation of the Vágújfalvi reagent and the development of red spots at the position of stachydrine (Fig. 2), the HPTLC glass plate was scanned in a CAMAG TLC Scanner 3 at 517 nm, a wavelength identified in preliminary experiments (data not shown) as the absorption maximum of the reddish stachydrine derivate after treatment with Vágújfalvi reagent. All data were processed with the CAMAG winCATS Planar Chromatography Manager automatic analysis software, version 1.4.4, using an automatically calculated polynomial formula for the separate computation of stachydrine content from both peak heights and areas.

3.5.3. *Method calibration and validation*

In order to prove the suitability of the method for its intended use, different characteristics can be investigated during validation. In this work, purity of the stachydrine reference compound was proven as described above. The developed HPTLC method was highly specific with no other Dragendorff-positive constituents detected in the zone between R_f 0.45 and 0.60 (Fig. 3). In a dilution series, the detection limit was determined as well below 0.25 µg. After the completely developed plates were scanned with the TLC Scanner 3, stachydrine contents on the lanes of extract samples were calculated using a polynomial formula developed from the absorption values measured for the four standard concentrations. This mathematic approach was chosen over a linear correlation taking into account the amount of the alkaloid that is hidden in the depth of the HPLC layer and can thus not be measured by the scanning detector. All these calculations as well as the statistical analyses of their results were automatically performed by the winCATS Planar Chromatography Manager software according to the instructions of the manufacturer. In every case, the formula developed by the program displayed a correlation coefficient of at least 0.999, clearly sufficient for the approval of the method. Accuracy and precision of the newly developed HPTLC protocol was not only manifested by the low standard deviations derived form nine runs per sample (three plates with three concentrations each), but also by the fact that the evaluation of the scanned plates both for height and area of the stachydrine peaks yielded results in high accordance with the independently performed ^{1}H-qNMR measurements that are based on a completely different physical mode of operation (Table, Fig. 1). The fact that after all calculations the final results concerning the stachydrine contents in the drug material were in such high accordance although highly divergent amounts of extract were applied to the individuals HPLC lanes, nicely demonstrates the robustness of the presented approach. Intermediate precision was examined by repeating the analyses after several weeks without any noticeable changes of the outcome. After investigating all these characteristics, it could be concluded that the developed analytical method is suitable, i.e. precise, accurate, and specific, for its intended use. A control experiment using a serial dilution of pure stachydrine yielded a limit of detection of 0.1 % (w/w) stachydrine in extract samples.

3.6. *^{1}H-qNMR*

3.6.1. *General and specific NMR conditions*

The presented ^{1}H-qNMR measurements were carried out using a Mercury Plus 300 MHz spectrometer (Varian, Palo Alto). D_2O solvent and NMR glass tubes were obtained from Chemotrade, Leipzig. Maleic acid as an internal standard was purchased from Sigma, Taufkirchen (100 % purity, verified by ^{1}H-NMR as described below); concerning pure stachydrine for preliminary ^{1}H-qNMR experiments and calibration of the developed method see above.

3.6.2. *Determination of stachydrine by ^{1}H-qNMR*

For every measurement, approximately 22.0 mg of thoroughly dried extract were exactly weighed into a reaction tube and dissolved in 80 µl of a 10 mg/ml solution of maleic acid in D_2O and diluted with 620 µl of D_2O under intensive shaking. This measurement solution was filled into NMR glass tubes and the ^{1}H-spectrum of the entire solution was recorded with 128 repetitions at 300 MHz, following the standard protocol for ^{1}H-measurements according to the manufacturer. NMR processing for all samples included manual phase correction for each replicate and manual baseline correction over the entire spectral range. Peak integration was performed automatically using the NMR spectrometer software. For each sample, three replications were analysed. In every single spectrum, the peak of the three protons of an N-methyl group of the stachydrine molecule was clearly separated from all other peaks at about 3.0 ppm (Fig. 4), whereas the peak of the two vinylic protons of the maleic acid molecule was positioned outside of the extract resonances at 6.18 ppm (Fig. 5). The abovementioned concentrations guarantied that both peak areas ranked in the same order of magnitude.

The concentration of stachydrine was calculated from the following equation:

$$C_S = C_M \cdot \frac{I_S}{I_M} \cdot \frac{2}{3}$$

C_S is the molar concentration of stachydrine in the measurement solution, C_M is the molar concentration of maleic acid in the measurement solution, I_S signal intensity at 3.0 ppm (N-CH_3, stachydrine), and I_M is signal intensity at 6.18 ppm (HC = CH, maleic acid). Stachydrine contents in the drug samples as percent of dry weight were consequently calculated based on the above-calculated molar concentration, the molecular weight, and the respective drug extract ratios (Table). The quantification limit of stachydrine was 0.104 mg/ml. All measurements were performed in three repetitions with intervals of several days between the individual runs and new aliquots of the freeze-dried extracts dissolved for each individual measurement. Nevertheless, the presented quantitative ^{1}H-NMR method yielded highly reproducible data in a series of inter-day-repetitions in high accordance with the above-described HPTLC protocol. A control experiment

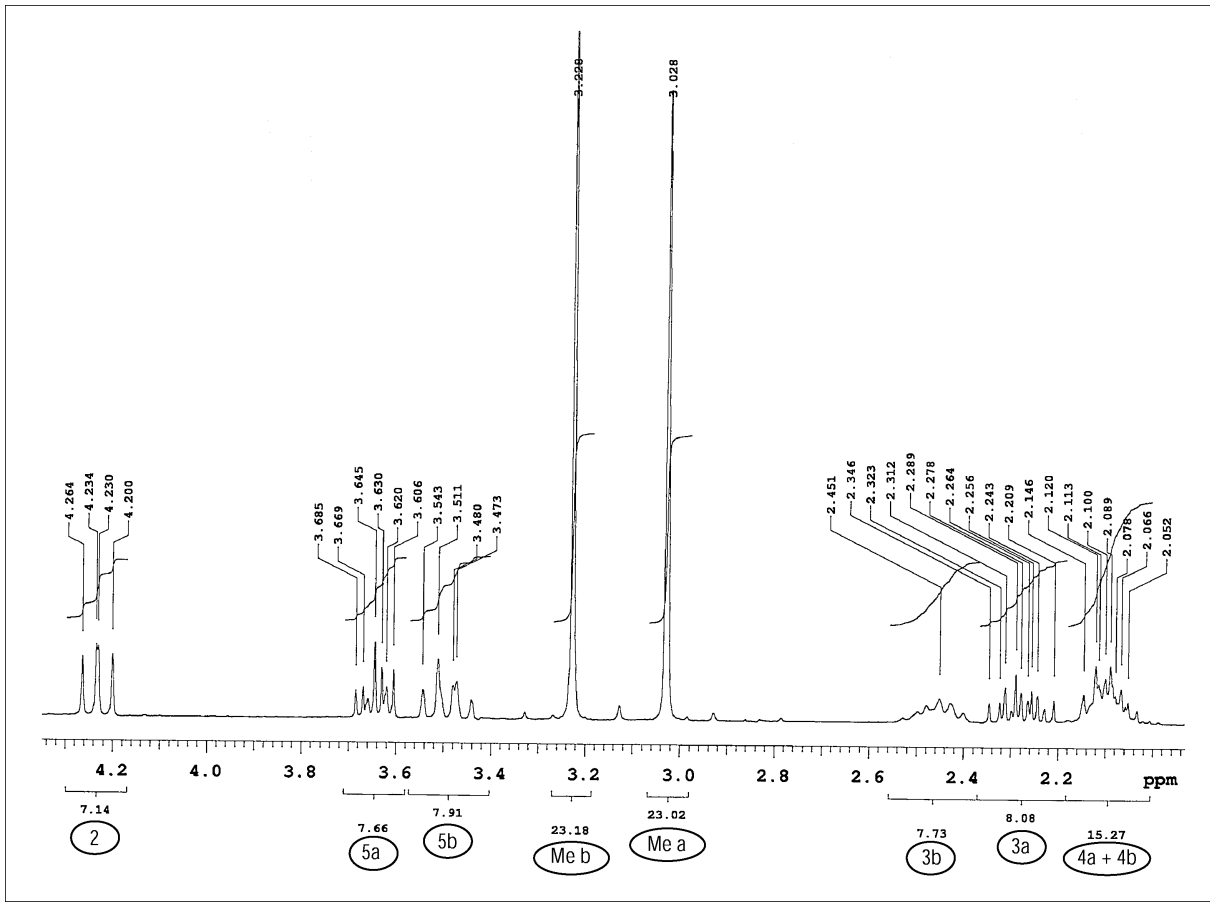


Fig. 4: 300 MHz ^{1}H NMR spectrum of stachydrine in D_2O; further measurement conditions see text. Assignment of signals is indicated by encircled numbers in accordance to the individual protons listed in Fig. 6

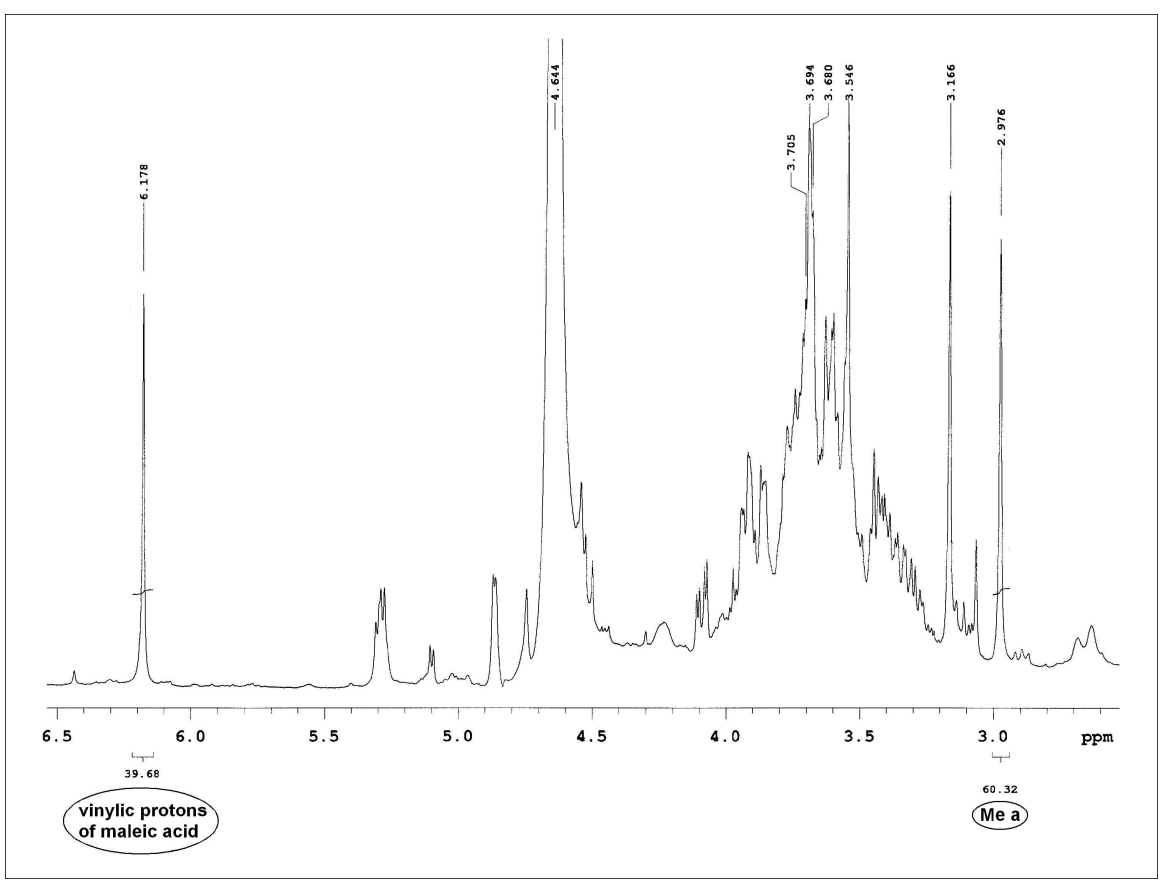
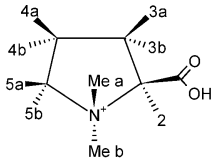


Fig. 5: Positions of the stachydrine N-CH_3 singlet at δ2.98 ppm and of the singlet of the two vinylic protons of maleic acid at δ 6.18 ppm, which was used as internal standard, in a representative ^{1}H-qNMR measurement of sample LJH B



Position	Me a	Me b	2	3a	3b	4a	4b	5a	5b
δ	3.03	3.22	4.23	2.26	2.45	2.09	2.09	3.65	3.50
Molecular group	CH_3	CH_3	H	H	H	H	H	H	H

Fig. 6: Observed chemical shifts (δ) for each proton of stachydrine. For a detailed discussion of the measuring conditions see text and Figs. 4/5

using a serial dilution of pure stachydrine yielded a limit of detection of 0.1 % (w/w) stachydrine in extract samples.

3.7. HPLC

In preliminary experiments, attempts to preproduce the published (Chao et al. 2004) HPLC method for stachydrine quantification were undertaken. All solvents used for HPLC were of gradient grade quality. A first attempt to reproduce the stachydrine HPLC-method described by (Chao et al. 2004) using a EC 250/4,6 Nucleodur 100-5 CN RP column, revealed it to be not reproducible, with unstable retention time and insufficient peak separation. In further preliminary experiments, several other HPLC columns such as a Shiseido ODS C18 column, a 250/4,6 Nucleodur 100-5 C18 ec column, or a 250/4 Nucleodur C18 Pyramid column (Macherey-Nagel, Düren) with the respective precolumns were tested, but none of these did yield any reproducible results.

Acknowledgements: Special thanks are due to the general management of Schaper & Brümmer for providing all materials for the HPTLC measurements as well as for free lodging (K.K.) in Salzgitter-Ringelheim. Ms Bogusława Busch and Ms Katrin Gehrs from Schaper & Brümmer are acknowledged for their excellent technical introduction into the HPTLC method. We also thank Prof. Dr. Kanji Hosono, CEO of the private Seikoen Hosono Clinic Kyoto, for sending the Japanese *L. japonicus* drug samples to our laboratory in Germany, as well as SinoPhytoMed for providing us with two samples of their *L. japonicus* drugs for free. Last but not least, Kenny Kuchta (Present address: Sanyo Gakuen University, Okayama, Japan) wants to express his deepest gratitude towards the "Studienstiftung des Deutschen Volkes" for providing a doctoral scholarship.

References

Akopov IE, Konovalova VA, Mansurov MM (1958) Pharmacology of stachydrine hydrochloride. Farmakol Toksikol 21: 44–46.

Anonymous (2000) Pharmacopoeia of the People's Republic of China. English Edition Vol 1. Monographs for the drugs Yimucao and Chongweizi. Chemical industry press, Beijing.

Anonymous (2010) DAB Monograph "*Leonuri herba* – Yimucao". In Dt Apoth Ztg 150: 1155–1156. /BAnz 34; 03.03.2010.

Apers S, Naessens T, Pieters L, Vlietinck A (2006) Densitometric thin-layer chromatographic determination of aescin in a herbal medicinal product containing *Aesculus* and *Vitis* dry extracts. J Chromatogr A 1112: 165–170.

Berger S, Braun S (2004) 200 and More NMR Experiments. Wiley-VCH, Weinheim, p. 315–317.

Bomme U (2008) A chance for better drug quality in CHM - documented and controlled cultivation of selected species for CHM. Zschr Phytother 29: 194–196.

Bomme U, Heubl G, Bauer R (2006) Erste Ergebnisse der Untersuchungen zur botanischen Charakterisierung sowie zum Ertragsverhalten und Inhaltsstoffspektrum verschiedener Herkünfte von *Prunella vulgaris* L., *Leonurus japonicus* Houtt. und *Sigesbeckia pubescens* Makino. Z Arzn Gew Pfl 11: 81–91.

Burger A, Kabatembe J (2008) Cardioactivity of 2 mg/ml *Leonotis leonurus* in the Langendorff rat heart not due to [K^+] and [Mg^{2+}]. Planta Med 74: 991.

Chao Z, Ma LL, Zhou XJ (2004) Determination of stachydrine and leonurine in Herba Leonuri by ion-pair reversed-phase high-performance liquid chromatography. Di Yi Jun Yi Da Xue Xue Bao 24: 1223–1226.

Dammertz W, Reich E (2001) Planar chromatography and densitometry. In Nyiredy S (Ed.) Planar Chromatography - A Retrospective View for the Third Millennium. Springer, Budapest, p. 234–246.

Heuberger H, Bauer R, Friedl F, Heubl G, Hummelsberger J, Nögel R, Seidenberger R, Torres-Londoño P (2010) Cultivation and breeding of Chinese medicinal plants in Germany. Planta Med 76: 1956–1962.

Hung SH (1959) Studies on the alkaloids of the Chinese drug I-Mu-Tsao, *Leonurus sibiricus* L. I. Stachydrine. Acta Pharm Sinica 7: 59–64.

Hutchings A, Scott AH, Lewis G, Cunningham A (1996) Zulu Medicinal Plants: An Inventory. University of KwaZulu-Natal Press, Pietermaritzburg, p. 266–267.

Luo X (2003) Compendium of Materia Medica, Book III /Vol 15, category of herbs (IV). Foreign Languages Press, Beijing, p. 1637–1644. A translation of the "Bencao Gangmu", first published by Li Shizhen in Beijing, 1593.

Malz F (2008) Quantitative NMR in the solution state NMR. In Holzgrabe U, Wawer I, Diehl B (Editors) NMR Spectroscopy in Pharmaceutical Analysis. Elsevier, Oxford, p. 43–62.

Nahrstedt A (1985) Drogen und Phytopharmaka mit sedierender Wirkung. Zschr Phytother 6: 101–109.

Reich E, Widmer V (2009) Plant analysis 2008 – planar chromatography. Planta Med 75: 711–718.

Ritter M, Melichar K, Strahler S, Kuchta K, Schulte J, Sartiani L, Cerbai E, Mugelli A, Mohr FW, Rauwald HW, Dhein S (2010) Cardiac and electrophysiological effects of primary and refined extracts from *Leonurus cardiaca* L. (Ph.Eur.). Planta Med 76: 572–582.

Skarkova J, Ostry V, Ruprich J (2008) Instrumental HPTLC determination of α-solanine and α-chaconine in peeled potato tubers. J Planar Chromatogr 21: 113–117.

van Eijk JL (1952) Phytochemical Investigations of *Leonurus cardiaca* and *Senecio vulgaris*. Pharm Weekblad 87: 38–41.

Villamar AA, Asseleih LMC, Rodarte ME (1994) Atlas de las Plantas de la Medicina Tradicional Mexicana Vol 1. Instituto Nacional Indigenista, México DF, p. 114–115.

Wagner H, Bladt S, Zgainski EM (1983) Drogenanalyse: Dünnschichtchromatographische Analyse von Arzneidrogen. Springer, Berlin, p. 301.

Watt JM, Breyer-Brandwijk MG (1962) The medicinal and poisonous plants of Southern and Eastern Africa. E & S Livingstone, Edinburgh.

Wichtl M (2002). Teedrogen und Phytopharmaka, ein Handbuch für die Praxis auf wissenschaftlicher Grundlage. Wissenschaftliche Verlagsgesellschaft, Stuttgart, p. 333–334.

Widmer V, Handloser D, Reich E (2007) Quantitative HPTLC analysis of artemisinin in dried *Artemisia annua* L.: A practical approach. J Liq Chromatogr Related Technol 30: 2209–2219.

Yin J, Zhang ZW, Yu WJ, Liao JY, Luo XG, Shen YJ (2010) Stachydrine, a major constituent of the Chinese herb *Leonurus heterophyllus* Sweet, ameliorates human umbilical vein endothelial cells injury induced by anoxia-reoxygenation. Am J Chin Med 38: 157–171.