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^{1}H-qNMR for direct quantification of stachydrine in *Leonurus japonicus* and *L. cardiaca*

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ABSTRACT

^{1}H-qNMR-spectroscopy was successfully applied to quantify the pharmacologically active alkaloid stachydrine ((2S)-1,1-dimethylpyrrolidinium-2-carboxylic-acid) in aerial parts of *Leonurus japonicus* (*Leonuri* herba, yimucao; Chin.Ph.2010, DAB2012) which are used in TCM and Kampo for the treatment of various gynaecological and cardiovascular disorders. Pharmacological publications on this betaine describe cardiovascular, hypotensive, and tissue-protective effects. However, its pharmacopeial analytics poses severe difficulties as it does not contain any chromophore suitable for HPLC–UV-detection. Nine samples from three countries were prepared as decoctions and freeze-dried. ^{1}H-NMR-spectra were recorded in D_2O. The direct-quantitative ^{1}H-qNMR-procedure was carried out using the N-CH_3-singlet at δ 3.03 ppm in comparison to the δ 6.18 ppm singlet of the two vinylic protons of maleic-acid, which was identified as a most favourable internal standard. The quantification limit of stachydrine was 0.44 mg/g drug material. Neither reference-compounds for calibration-curves nor sample-pre-purification was necessary. This protocol revealed stachydrine contents in the range from 0.09 up to 1.01% (w/w) for the tested yimucao samples. Furthermore, between 0.18 and 0.21% of stachydrine was found in the *L. japonicus* fruit-drug (*Leonuri* fructus, chongweizi; Chin.Ph.2010) which was examined for this constituent for the first time. In four co-investigated samples of the closely related and similarly used European herb *Leonurus cardiaca* Ph.Eur., even higher contents up to 1.55% were attested. The presented quantitative ^{1}H-qNMR-method was shown to be precise with respect to concentration, and yielded highly reproducible data in a series of inter-day repetitions. Methodically, ^{1}H-qNMR may be a powerful tool for quality assurance of 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 already identified as a dominant constituent of *Leonurus japonicus* Houtt. [1] about 60 years ago, and has thus been discussed as an important contributor to the overall pharmacological activity of this Traditional Chinese Medicine (TCM) and Kampo drug [2]. Its structure was elucidated as N-dimethyl-L-proline (Fig. 1) by classical chemical structure analysis when it was first discovered and was later confirmed by several NMR examinations [3–5]. Although molecular pharmacological data for the betaine itself are scarce, it was found in one of these few publications that intravenous injections of 0.01–0.1 g/kg bodyweight

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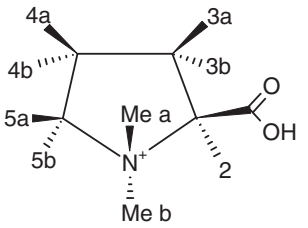

Position	Me a	Me b	2	3a	3b	4a	4b	5a	5b
δ [3]	3.03	3.25	4.10	2.40	2.40	2.40	2.40	3.60	3.60
δ [5]	3.02	3.22	4.29	2.52	2.15	2.35	2.15	3.67	3.53
δ (observed)	3.03	3.22	4.23	2.26	2.45	2.09	2.09	3.65	3.50
Type of peak (observed)	s	s	dd	m	m	m	m	m	m
Molecular group	CH_3	CH_3	H	H	H	H	H	H	H

s = singlet dd = double doublet m = multiplet

Fig. 1. Observed chemical shift (δ) values for each proton of the stachydrine molecule in comparison with existing literature. Grey underlined fields mark chemical shifts that were not correctly assigned in an earlier study [5]. This publication also reported the signals at δ 2.35 and δ 4.29 as triplets. However, higher resolution of state of the art NMR technology revealed these as a multiplet and a double doublet, respectively. For a detailed discussion of the measuring conditions see Fig. 5, Supplementary data, and text.

stachydrine in aqueous solution exerted profound positive chronotropic and negative inotropic effects in dogs [6]. 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 [7].

Analytically, the Chinese Pharmacopoeia (Chin.Ph.) [8] resorted in its 2000 edition for quality control of yimucao (*Leonuri* herba; Chin.Ph.; German Pharmacopoeia (DAB 2012)) 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. However, this protocol has been repeatedly described as irreproducible in experimental literature [9–11]. The Chin.Ph. 2010 edition has proceeded to an RP-HPLC method using a rare evaporative light scattering detector to circumvent the absence of a chromophore in the stachydrine molecule. No quantitative examinations are mentioned for the fruit drug chongweizi (Chin.Ph.). On the other hand, the DAB [12] 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 Ph.Eur. quality standards for *Leonurus cardiaca* L., a closely related plant that is used in Europe in a similar fashion for the treatment of heart conditions [13,14] and gynaecological disorders [15].

As the area of each signal is directly proportional to the number of contributing nuclei [16], ^{1}H-qNMR spectroscopy is ideal for the chemical characterisation of active constituents of medicinal plants, especially in cases where the lack of chromophoric or fluorophoric groups makes conventional UV detection impossible, such as in the case of artemisinin in *Artemisia annua* [17]. In the present paper we describe the application of ^{1}H-qNMR for quantitative determination of stachydrine in *L. japonicus* and *L. cardiaca* pharmaceutical crude drug samples.

2. Material and methods

2.1. Plant material

The officinal aerial parts (*Leonuri* herba, yimucao; Chin.Ph., DAB) and fruits (*Leonuri* fructus; chongweizi; Chin.Ph.) of *L. japonicus* as well as *Leonuri cardiacae* herba (Ph.Eur.) were purchased from the sources listed in Table 1 and controlled for their quality according to the requirements of the Chin.Ph. or Ph.Eur. respectively, the regulations of which were met by all analysed samples, which were all 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*.

2.2. 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.

2.3. Isolation and characterisation of the stachydrine reference standard

For purification of stachydrine, 1.5 g of aqueous extract from yimucao sample H, corresponding to 6 g of herbal drug material, 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