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Short communication

Validation of a quantitative NMR method for suspected counterfeit products exemplified on determination of benzethonium chloride in grapefruit seed extracts

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ABSTRACT

A ^{1}H-nuclear magnetic resonance (NMR) spectroscopy method for quantitative determination of benzethonium chloride (BTC) as a constituent of grapefruit seed extract was developed. The method was validated, assessing its specificity, linearity, range, and precision, as well as accuracy, limit of quantification and robustness. The method includes quantification using an internal reference standard, 1,3,5-trimethoxybenzene, and regarded as simple, rapid, and easy to implement. A commercial grapefruit seed extract was studied and the experiments were performed on spectrometers operating at two different fields, 300 and 600 MHz for proton frequencies, the former with a broad band (BB) probe and the latter equipped with both a BB probe and a CryoProbe™. The concentration average for the product sample was 78.0, 77.8 and 78.4 mg/ml using the 300 BB probe, the 600 MHz BB probe and CryoProbe™, respectively. The standard deviation and relative standard deviation (R.S.D., in parenthesis) for the average concentrations was 0.2 (0.3%), 0.3 (0.4%) and 0.3 mg/ml (0.4%), respectively.

1. Introduction

Quantitative nuclear magnetic resonance spectroscopy (QNMR) is a technique which is increasingly used and has the advantages as being a rapid primary method of measurement with simple sample preparation and versatility as well as having a non-destructive nature [1–3]. Since NMR can be used both in the identification and quantification it is an ideal method in the determination of an unknown sample as in this case an adulterated natural product. Once an appropriate internal reference standard and solvent have been chosen the quantification including sample preparation takes only 3–4 h.

In a case reported to the Swedish Medical Products Agency (MPA) two patients prescribed to warfarin experienced adverse effects upon self-medication, using a commercially available GSE product [4]. The preservative *N*-benzyl-*N*,*N*-dimethyl-2-[2-[4-(1,1,3,3-tetramethylbutyl)-phenoxy]ethoxy]ethanammonium chloride or benzethonium chloride (BTC) was found to be the major constituents of the GSE product.

This is to our knowledge the first validation of a QNMR method for suspected counterfeit natural products and we have chosen to demonstrate this on GSE but in principle the same type of method could be used for any natural product or drug substance. The method is validated in aspects of selectivity, linearity, range, precision, accuracy, limit of quantification and robustness. Also a comparison was made a of the results obtained on two different spectrometers, operating at 300 and 600 MHz ^{1}H frequencies and using two different probes for the higher field instrument.

2. Experimental

2.1. Materials and chemicals

The product, GSE was bought from internet. The product was a solution with declared ingredients grapefruit seed extract (33%), glycerol and water. Benzethonium chloride 99.8% and 1,3,5-trimethoxybenzene 99.9% were purchased from Sigma–Aldrich, Stockholm, Sweden, and Merck, Darmstadt, Germany, respectively.

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Deuterated solvent, methanol-d_4 (99.8%), was obtained from Armar Chemicals, Döttingen, Switzerland. Disposable 5 mm NMR tubes, HIP-7, were from Wilmad-LabGlass, Buena, NJ, USA.

2.2. NMR spectroscopy and sample preparation

All the ^{1}H and ^{13}C NMR spectra have been acquired at 25 °C using Bruker Avance spectrometers operating at 300.13 and 600.13 MHz proton frequencies (Bruker Biospin AG, Faellanden, Switzerland). The 600 MHz spectrometer was equipped with either a 5 mm Z-gradient TCI (H/C/N) CryoProbe™ using a ^{1}H 90° pulse width of 7.9 μs or a 5 mm Z-gradient broad band (BB) probe using a ^{1}H 90° pulse width of 13.4 μs. The 300 MHz spectrometer was equipped with a 5 mm Z-gradient BB probe using a ^{1}H 90° pulse width of 7.2 μs. The excitation frequency was set to 6.17 ppm. All processing and spectra handling have been performed using Topspin 1.3, 2.0 or 2.1 program suite (Bruker Biospin GmbH, Rheinstetten, Germany). Calibration of the chemical shift scale was performed by adjusting the residual methanol ^{1}H signal to 3.31 ppm and ^{13}C signal to 49.15 ppm, respectively. The two-dimensional experiments ^{1}H, ^{13}C heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC), ^{1}H, ^{1}H correlation spectroscopy (COSY) and diffusion ordered spectroscopy (DOSY) [5,6] were performed on the 600 MHz spectrometer using the cryogenic probe. The DOSY was performed using the LED [7] technique with a bipolar gradient pulse pair, two 1.1 ms spoil gradients, and 5 ms spoil gradient delays using the following experimental settings: spectral width 10 ppm, 8 K complex data points, δ 2 ms, Δ 100 ms, 5 s repetition time, and 16 DOSY increments using 5–95% of the maximum gradient amplitude (5.35 G/cmA).

One-dimensional (1D) spectra recorded for the purpose of quantification had 64 scans of 64 K complex data points were recorded with a 30° pulse length and a 90 s repetition delay. The pre-acquisition delay was 5 or 6 μs. Each free induction decay (FID) was multiplied with a 0.3 Hz exponential line-broadening factor but not zero-filled prior to Fourier transformation. Phasing and integration were performed manually after baseline correction using first or second order polynomial functions. Wherever needed adjustment of the integral was done manually with the software functions BIAS and SLOPE. For the two 600 MHz probes integration regions around the signals were chosen to cover carbon-13 satellites but for the 300 MHz due to the lower resolution instead the integration region was chosen according to Rabenstein and Keire: 32× the signal full width at half height ($v_{1/2}$) on both sides of the signal in order to obtain 99% of the signal [8].

Each sample of the commercial GSE product was prepared by diluting 100 μl of the product to 1 ml with methanol-d_4. For determination of the performance, *i.e.* linearity and range 10 samples containing between 2 and 28 mg pure BTC was prepared in 1 ml methanol-d_4. For each sample, exact weights close to 2 mg of the internal standard, 1,3,5-trimethoxybenzene, was added. Calibrated Hamilton GasTight™ syringes of 100 μl volume were used for the volume measurements and weighing was performed on a microbalance Mettler-Toledo UMT2 (Mettler-Toledo GmbH, Greifensee, Switzerland) with a precision of 0.0001 mg using metal weighing pans. The solutions were agitated on a test tube whirly-mixer and ultrasonicated until the solutions were clear. Finally the ready solutions were transferred to NMR tubes.

3. Results and discussion

3.1. Identification of benzethonium chloride

The identification of BTC is described elsewhere [4]. It was observed that the spectra recorded for commercial samples and reference BTC were identical except for the signals originating from other ingredients, *i.e.* water and glycerol and propylene glycol. A major advantage for the identification of the major ingredients of the product was the DOSY experiment which displays the analytes with different diffusion coefficients on different rows the enabling separation of signals from different compounds (Fig. 1). The ^{1}H and ^{13}C chemical shifts in methanol-d_4 were assigned using COSY, HSQC and HMBC experiments (data not shown). The chemical shifts proved to be in fair agreement with previously reported chemical shifts for benzethonium chloride in chloroform-*d* [9].

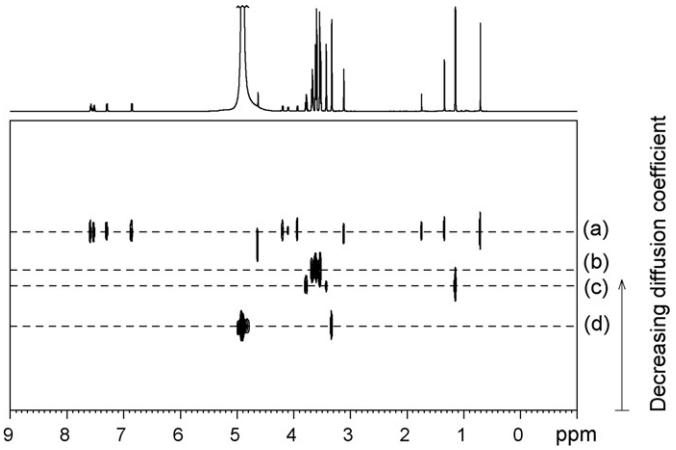

Fig. 1. The high resolution DOSY spectrum of the commercial GSE product. The dashed lines are placed in order to guide the eye for the observation of different components of the mixture with respect to their diffusion coefficients. From low to high diffusion coefficients, lines show the signals from benzethonium chloride (a), glycerol (b), propylene glycol (c), and water/NMR solvent (d), respectively.

3.2. Quantitative NMR method

The signal intensity of a known amount of an internal reference standard (IS) was compared to the area of the peaks originating from the analyte. In the current study, the IS chosen was 1,3,5-trimethoxybenzene, since it supplies a well-separated signal (6.07 ppm) without any interference from benzethonium chloride in the integration region. Among the other common internal standards frequently used in our lab this was the best candidate in aspects of both solubility and the chemical shifts of the different protons compared to BTC and other substances in the samples. A disadvantage was the proximity of this resonance to that of water and hydroxyl resonances since in cases of poor shimming (optimised automatically) the water peak slightly affected the measured value of the IS.

For BTC, the doublet at 6.85 ppm, originating from the two equivalent protons in the 2-position of the phenoxy-ring, was used, since this peak appears well-separated from other signals. The ^{1}H NMR spectrum of GSE in methanol-d_4 shows a well-separated doublet of BTC and the singlet of the internal standard 1,3,5-trimethoxybenzene (Fig. 2). In QNMR, often singlet signals are used; however the methyl singlets of BTC were obscured by some low concentration impurities which made this region inappropriate to integrate.

3.3. Validation

3.3.1. Selectivity

The spectrum of the blank solvent sample did not show any extra signal except for the two accounted signals of methanol-d_4 itself at 3.31 and 4.86 ppm, *i.e.* there is no signal present in the frequency region (6.0–7.7 ppm) where the integration of the peaks for quan-

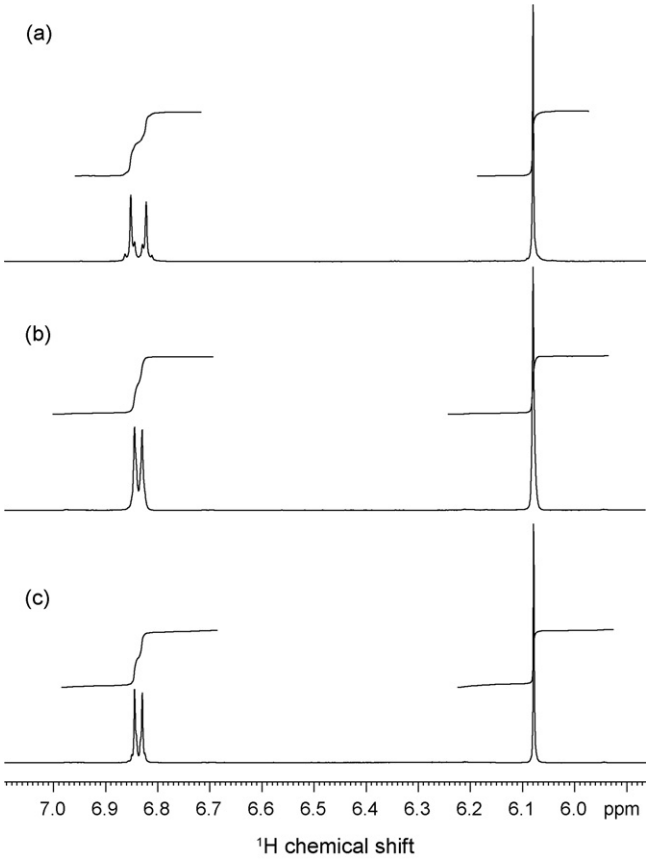

Fig. 2. Region of the ^{1}H NMR spectra of the GSE product containing the signals from 2-phenoxy proton of BTC (left) and the internal standard (right) recorded on (a) the 300 MHz BB probe, (b) the 600 MHz BB probe and (c) the 600 MHz cryogenic probe. The integration regions used for quantification are indicated for each peak.

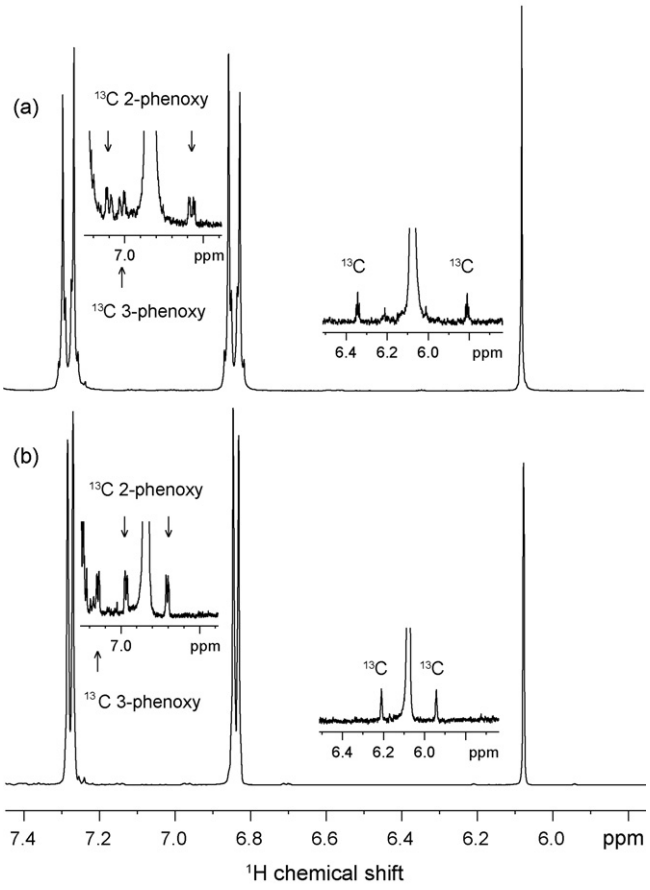

Fig. 3. Region of the ^{1}H NMR spectra recorded on a sample containing BTC (3-phenoxy and 2-phenoxy and the IS peaks (left to right)) on (a) the 300 MHz and (b) the 600 MHz BB probes. Expansions show the crossed over ^{13}C satellites of the 3- and 2-phenoxy signals as well as the greater dispersion of signals on the 600 MHz instrument.

titative determination were performed. For the sample with only internal standard, 1,3,5-trimethoxybenzene, a very small peak (*ca* 0.05% of the main peak at 6.08 ppm) has been observed at 6.17 ppm, within the integration region of the internal standard. No correction has been applied for this.

Since the spectral dispersion is directly proportional to the magnetic field strength of the instrument, *i.e.* the distance between two unique peaks at a 600 MHz instrument is twice that of a 300 MHz instrument. Due to this fact the carbon-13 satellites of the BTC phenoxy proton signals are crossed so that the satellite of the 2-phenoxy is inside the 3-phenoxy proton integration region and vice versa (Fig. 3). In order to obtain 99% of the peak area $32 \times \nu_{1/2}$ Hz on each side of the peak was integrated [8]. The concentrations calculated from the experiments on the 300 MHz instrument were consistent with the results from measurements on the 600 MHz spectrometer (see below).

All the peaks from the samples of benzethonium chloride as well as the commercial GSE product have been identified, with the exception for small peaks at 6.17–6.21 ppm within the integration region of the internal standard. The area of this disturbance was 0.8% of the main peak of the internal standard. No correction for this was applied in this work.

3.3.2. Linearity and range

To assess the linearity and the measuring range, 10 samples with different concentrations of benzethonium chloride (30–400% of the expected content in the commercial samples (*ca* 70 mg/ml)) and an exact weight of about 2 mg of IS was prepared. The experimental values were plotted against the gravimetric values give linear regression with coefficients of determination of 0.9999 for both broad band and cryogenic probes (Table 1). The maximum deviation (residual) for any experimental value is 1.7% with respect to the corresponding value on the fitted regression line of each probe.

An indication that the measuring range is exceeded would be a non-linear recovery compared to the gravimetric values. Another sign of this would be a broadening of the peaks which could result from a highly concentrated solution having a higher viscosity which may influence relaxation processes. Neither of these conditions was reached and the method is considered valid for the whole concentration range.

3.3.3. Precision and intermediate precision

The precision was assessed by sampling a stock solution of the GSE product and IS, diluted to 6 ml with methanol-d_4. The 6 identical samples was analyzed on each of the three probes and the concentration calculated was 78.0, 77.8 and 78.4 mg/ml on the BB 300, BB 600 and CryoProbe^{TM} 600 MHz, respectively. The standard deviation and relative standard deviation (R.S.D.) for the measurements were 0.2 (0.3%), 0.3 (0.4%) and 0.3 (0.4%), respectively.

The intermediate precision was determined in a similar matter but the measurements were performed on two different occasions. Three samples made from a stock solution of GSE with different amounts of IS were prepared and analyzed on 1 day and a new

Table 1
Gravimetric and experimental values for the 10 samples used in linearity testing

Sample	Gravimetric (mg)	300 MHz broad band probe (mg)	600 MHz broad band probe (mg)	600 MHz cryogenic probe (mg)
1	2.101	2.02	2.06	2.08
2	3.509	3.46	3.49	3.66
3	5.558	5.50	5.50	5.53
4	6.303	6.23	6.26	6.31
5	7.032	6.97	6.97	7.00
6	7.683	7.50	7.50	7.55
7	8.368	8.28	8.30	8.39
8	14.047	13.86	13.86	13.97
9	20.952	20.89	20.90	20.89
10	28.154	28.25	28.12	28.01
k		1.0045	1.0001	0.9936
m		−0.1227	−0.0765	0.0344
R^2		0.9999	0.9999	0.9999

The lower part of the table comprises the linear regression and coefficient of determination for each probe.

set of three new samples were prepared and analyzed the second day. Day 1, the average concentration of the three samples was 78.9 ± 1.1 (1.4%) and 78.8 ± 1.6 (2.0%) mg/ml on day 2 for the 300 MHz. The BB probe on the 600 MHz gave 75.6 ± 0.4 (R.S.D.: 0.5%) and 77.2 ± 0.6 mg/ml (R.S.D.: 0.8%) on day 1 and day 2, respectively.

3.3.4. Accuracy

The accuracy was assessed by preparing three samples of known amount of BTC (4.47, 6.88, 21.55 mg) and the IS and subsequently analyzing the samples (BB 600 MHz) and compare the gravimetric to experimental values. The recovery of BTC in each sample was 99.6%, 101.3% and 99.4% of the gravimetrical amounts, average 100.1% and a standard deviation of 1.0%.

3.3.5. Limit of quantification

Since the main purpose in this study was quantification of benzethonium chloride in commercial products, we decided to focus on limit of quantification rather than detection. Limit of quantification was assessed by studying the S/N and was investigated using the sample with the minimum amount (2.101 mg) of benzethonium chloride (corresponding to about 20 mg/ml (2%) in a commercial product). The lowest S/N observed was 336 for the 300 MHz spectrometer. However this was still above what is considered to be required; according to literature, for an uncertainty of 1% the S/N should be at least 150–250 [2,8].

3.3.6. Robustness

As it has been previously shown the biggest factor of influence on the quality of a QNMR analysis is the handling of the NMR data by different operators [2]. Integration of peaks as well as phase and baseline corrections are the most subjective parts of the method. This has been tested by letting three operators, A, B and C, run the analysis on a common data set, which was recorded using the BB probe at 600 MHz, and comparing the results. In order to ensure the identical concentrations, the six samples were made by using a stock solution of the commercial product. The average of six analyses, standard deviation and relative standard deviation values were for operator A 78.6, 0.9 mg/ml, 1.2%, for operator B 75.8, 1.3 mg/ml, and 1.7%, and for operator C 76.2, 0.8 mg/ml, and 1.0%, all respectively. This shows that integration done by different operators have a large influence on the results from a QNMR method and emphasises the importance of a precise and detailed protocol for NMR data processing.

4. Conclusion

The QNMR method employed herein proved to be rapid as well as easy to implement. The different aspects of performance of the method, such as linearity, precision and accuracy, satisfied our requirements well. Furthermore, any modern NMR equipment operating at a field of 300 MHz or more may be used, assuming that suitable processing of data is performed. Considering the simplicity, reliability, simultaneous identification and quantification, and the fact that no reference compound of the same kind as the unknown is needed, QNMR has a high potential in analysis of suspected counterfeit products.

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