JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2007; **42**: 929–939
Published online 7 June 2007 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/jms.1230

JMS

Synthesis and characterization of succinylcholine-d_{18} and succinylmonocholine-d_3 designed for simultaneous use as internal standards in mass spectrometric analyses

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Received 16 January 2007; Accepted 12 April 2007

Succinylcholine (SUX) is a routinely used yet potentially lethal depolarizing muscle relaxant, the detection of which poses severe problems to the clinical or forensic analyst: within a few minutes after its *in vivo* administration, SUX is broken down via succinylmonocholine (SMC) to yield the endogenous substances succinic acid and choline. For quantification of SUX and SMC in biological matrices using mass spectrometric detection, appropriate internal standards, i.e. deuterated analogs of the above substances, are indispensable but not commercially available. Internal standards for both substances were hence tailored to fit the analytical needs. The two-step synthesis and subsequent characterization of SUX-d_{18} and SMC-d_3 using a combination of nuclear magnetic resonance (NMR) spectroscopy, fast atom bombardment mass spectroscopy (FAB-MS) and high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) are described. SUX-d_{18} was synthesized by reacting ethanolamine and iodomethane-d_3 in a first quaternization step to choline-d_9, which in turn was esterified with succinyldichloride to yield the final product. SMC-d_3 was produced by esterification of succinic acid anhydride with dimethylaminoethanol, yielding desmethyl-SMC as intermediate product. The latter was then reacted with iodomethane-d_3 to obtain SMC-d_3. ^{1}H- and ^{13}C-NMR data support the identity and purity as well as the designated deuteration of both preparations, findings which were further confirmed by FAB-MS as well as HPLC-MS/MS. Owing to a thoughtful design, the obtained substances SUX-d_{18} and SMC-d_3 feature different deuteration patterns at their trimethylamine moieties, and thus finally offer the possibility to simultaneously quantify SUX and SMC in clinical as well as forensic samples using isotope dilution mass spectrometry. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: succinylcholine; succinylmonocholine; synthesis; internal standard; isotope dilution; mass spectrometry

INTRODUCTION

The bis-quaternary amine succinylcholine (SUX) structurally resembles the endogenous neurotransmitter acetylcholine (ACh) and, as the latter, leads to an activation of post-synaptic ACh-receptors. SUX is rapidly released by these receptors, but compared to the native substrate ACh only slowly degraded by the specific acetylcholinesterase (EC 3.1.1.7), thus resulting in a prolonged depolarization and desensitization of the postsynaptic membrane. In the clinical setting in which SUX is routinely employed as a muscle relaxant during anesthetics, the concomitant respiratory depression can be compensated by artificial respiration. If respiratory assistance is not provided, a clinically effective dose will cause hypoxic brain damage and finally lead to death.

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SUX is degraded by the unspecific plasma cholinesterase (butyrylesterase; EC 3.1.1.8) to yield succinylmonocholine (SMC), and finally, after complete hydrolyzation of ester bonds, one molecule of succinic acid and two molecules of choline.

The monitoring of SUX levels in a clinical setting and especially the use of SUX as an asphyxiating agent in (attempted) homicides or suicides confront the analyst with several problems: firstly SUX is broken down within a few minutes after its *in vivo* administration,[1–7] and secondly the postmortem detection of the more stable metabolite SMC can no longer be regarded as a proof of SUX administration, as it was recently found in SUX-negative control tissues.[8,9] The analyte's sensitivity to heat and alkaline conditions as well as its pronounced hydrophilicity due to its precharged character pose further problems, as these features complicate SUX extraction.[10,11]

Various techniques have been employed for the detection of SUX ranging from thin-layer chromatography[12,13]

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and gas chromatography of succinate esters[12] to HPLC with electrochemical[1,3] or fluorescence detection,[5] as well as to several approaches using mass spectrometric detection such as, for example, GC-MS of derivatized extracts,[14,15] DI/EI-MS[16] and HPLC-ESI-MS.[11] Lately, also tandem-MS techniques like DI/ESI-MS/MS[6,7,10] and HPLC-MS/MS(MS)[9,17] have been reported. Only a minority of the latter authors employing a mass spectrometric detection method did use deuterated internal standards for quantification of SUX, i.e. SUX-d_6,[6,7] SUX-d_8[9] and SUX-d_{12}.[14,15]

SMC has less often been the subject of research interest; thus only a few authors have reported detection methods for this metabolite, ranging from thin-layer chromatography[13] to modern mass spectrometric methods like HPLC-ESI-MS[11] and HPLC-MS/MS(MS).[8,9] Only in two studies a deuterated internal standard, i.e. SMC-d_4, was used for the detection of SMC,[8,9] but in both cases SMC-d_4 was obtained through hydrolysis of SUX-d_8, thereby ruling out a parallel quantification of both analytes. To the knowledge of the authors, no simultaneous detection of SUX and SMC using isotope dilution mass spectrometry has been achieved yet.

As suitable internal standards are not commercially available, it was our immediate goal to synthesize deuterated analogs of SUX and SMC and ascertain their identity as well as their purity. Synthesis was designed in a way to ensure different deuteration patterns of the trimethylamine groups of both internal standards. Otherwise, owing to the above-mentioned degradation of SUX to SMC, the parallel use of both internal standards in a single sample must lead to erroneous data: if the degradation product of the deuterated SUX-analog is identical to the deuterated internal standard for SMC, it will drastically interfere with the quantification of the metabolite by causing an underestimation of SMC concentration. The chosen derivatives SUX-d_{18} and SMC-d_3 ensure a correct determination of concentrations of the metabolite as SMC-d_9, which is the expected product of SUX-d_{18} degradation, can easily be distinguished from the internal standard SMC-d_3 by mass spectrometric detection (Fig. 1).

Some authors have reported methods to synthesize SUX,[18–20] SUX-d_{12} (via choline-d_6),[14] and possible (deuterated) SUX precursors, as for example, choline-d_9[21] or desmethyl-SUX,[15], as well as SMC[22] and SMC-d_4 via hydrolysis of SUX-d_8.[8,9]

The present paper describes the successful synthesis of SUX-d_{18} and SMC-d_3 following modified methods of Forney *et al*.[14] and Phillips,[22] respectively. For the first time, deuterated internal standards for SUX and SMC were designed for simultaneous use, and assessment of the synthesis products was performed using elaborate analytical techniques such as NMR, FAB-MS and HPLC-MS/MS.

EXPERIMENTAL

Chemicals

All chemicals used for synthesis were of analytical grade and purchased from Sigma Aldrich, the only exception being iodomethane-d_3 which was obtained from Acros Organics.

Synthesis

SUX-d_{18} and SMC-d_3 were prepared via 2-step syntheses as detailed in the following sections.

SMC-d_3

Step 1 – Preparation of desmethyl-SMC. One equivalent (100 mmol, 10 g) of succinic acid anhydride and 1.12 equiv. (112.2 mmol, 10 g, 11.29 ml) of dimethylaminoethanol in 100 ml of dry acetone were refluxed for 3 h in an oil bath (75 °C). After the reaction, a small amount of white precipitate was filtered off and discarded. The remaining acetone filtrate was chilled on ice to crystallize the reaction product, which was then filtered off and vacuum-dried. The dry product was recrystallized from 5 ml of acetone. Before employing the intermediate desmethyl-SMC in the following reaction, its identity and purity were ascertained using NMR spectroscopy and FAB-MS (data not shown).

Step 2 – Alkylation of desmethyl-SMC yielding SMC-d_3. For the subsequent reaction, 1 equiv. (10 mmol, 1.9 g) of desmethyl-SMC and an excess of 3.2 equiv. (32 mmol, 4.65 g, 2 ml) of iodomethane-d_3 in 50 ml of dry acetone were refluxed for 24 h in an oil bath (75 °C). After completion of the reaction the solvent was distilled off, and the remaining residue was dried under vacuum. The product was then recrystallized from 10 ml of a 6 : 4 methanol : acetone (v/v) mixture and collected by filtration. To the filtrate 10 ml of diethyl ether were added to precipitate any solute product, which again was collected by filtration.

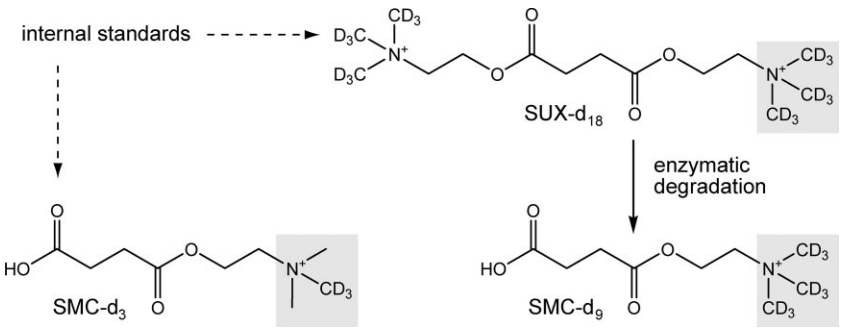

Figure 1. Synthesis design featuring the differently deuterated trimethylamine groups of both internal standard substances. Possible degradation of the internal standard SUX-d_{18} leads to SMC-d_9, a metabolite that is clearly distinguishable from the deuterated standard SMC-d_3 using mass spectrometric detection.

DOI: 10.1002/jms

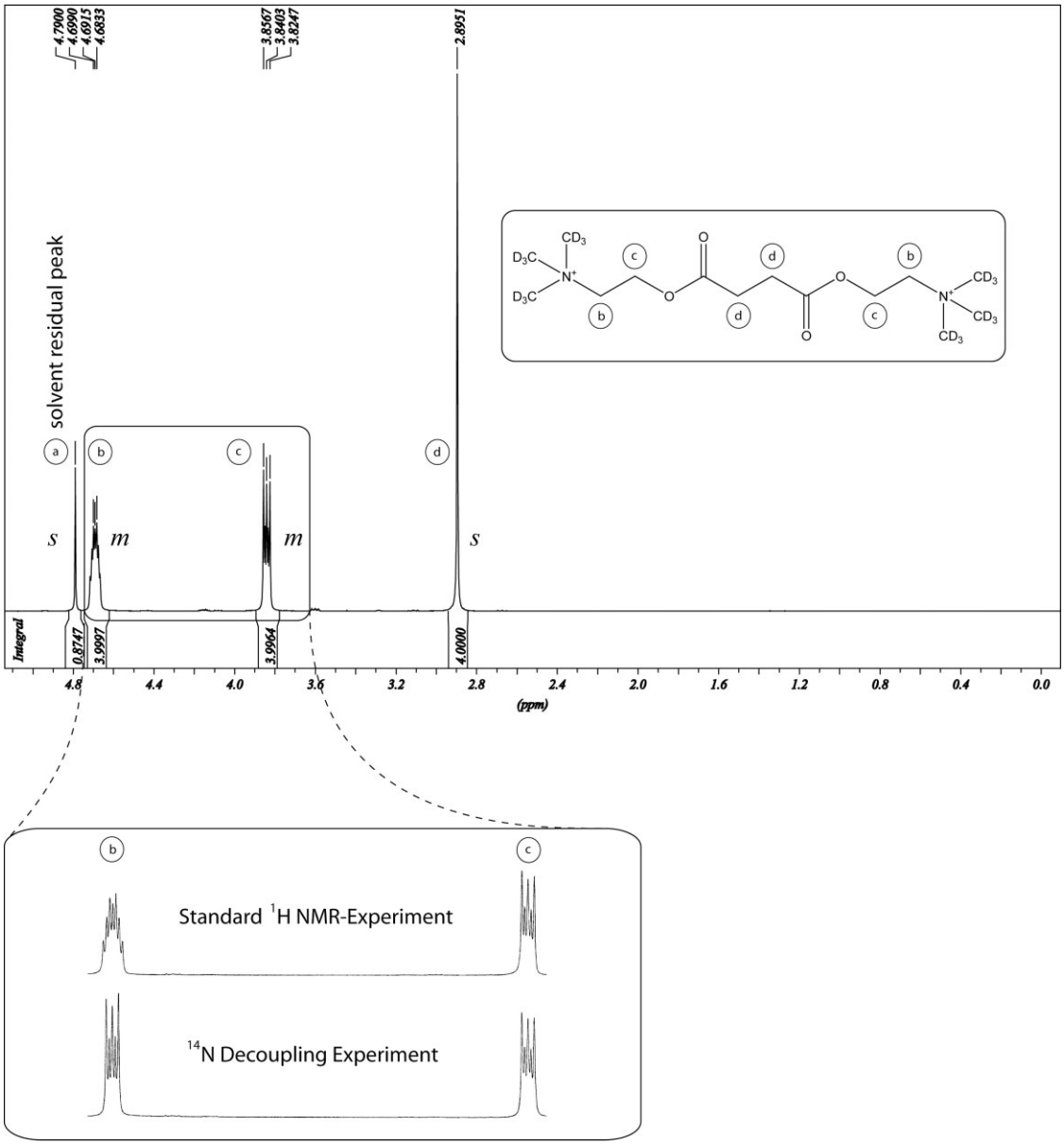

Figure 2. Top: ^{1}H-NMR spectrum of SUX-d_{18}. The structural formula of the target compound is shown in the box. NMR signals are assigned to distinct proton nuclei using matching letters. Signal multiplicity is described by s = singlet and m = multiplet. Bottom: Extraction of the indicated region from the above standard ^{1}H-NMR spectrum in superposition with the respective spectral region from a ^{14}N-decoupling experiment.

SUX-d_{18}

Step 1 – Preparation of choline-d_9. Choline-d_9 is commercially available from various sources; for budget reasons, however, we chose to synthesize it ourselves. One equiv. of ethanolamine (25 mmol, 1.53 g, 1.51 ml) and an excess of 3.2 equiv. (80 mmol, 11.6 g, 4.98 ml) of iodomethane-d_3 in 10 ml of methanol were stirred for 24 h at room temperature. Sodium methoxide (2.1 equiv., 52.5 mmol, 2.84 g) was dissolved in 25 ml of methanol and added in 7.5, 8.75 and 8.75 ml aliquots 1, 3.25 and 6.25 h after initiation of the reaction, respectively, i.e. when the pH of the reaction mixture had returned to neutral. Upon completion of the reaction, the solvent was distilled off and the remaining product was dried under vacuum. The residue was recrystallized twice from 15 and 12 ml of ethanol, respectively, and purified choline-d_9 iodide was collected by filtration. Before employing choline-d_9 in the following reaction, its identity and purity were ascertained using NMR spectroscopy and FAB-MS (data not shown).

Step 2 – Reaction of succinyldichloride with choline-d_9 yielding SUX-d_{18}. In a chilled reaction flask, 0.4 equiv. (1.68 mmol, 0.26 g, 185 µl) of succinyldichloride was added dropwise to 1 equiv. (4.2 mmol, 1 g) of choline-d_9 before addition of 10 ml of dry chloroform. The reaction mixture was slowly heated in an oil bath and then refluxed for 12 h at 60 °C. For product cleanup, the solvent was removed using a rotary evaporator and finally high vacuum. Together with 1 equiv. (4.2 mmol, 0.63 g) of NaI the dried product was dissolved in 450 ml of 1 : 2 methanol : ethanol (v/v). SUX-d_{18} iodide crystallized upon cooling of the reaction mixture,

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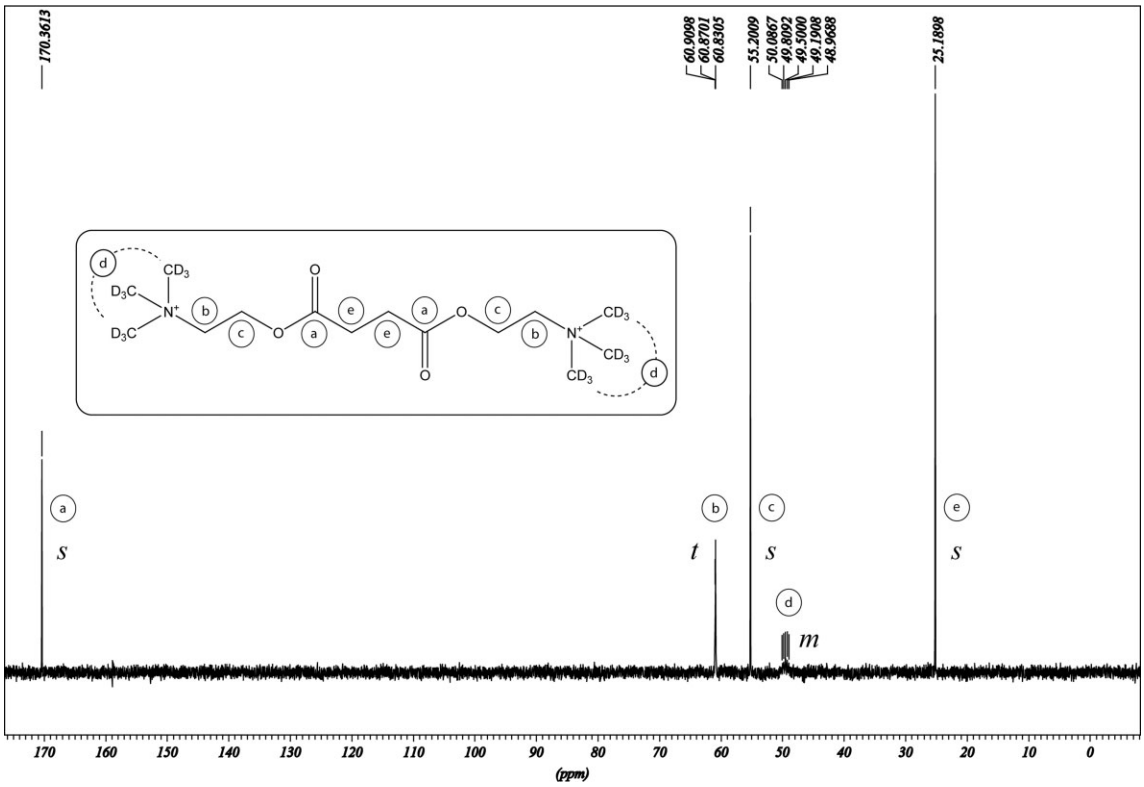

Figure 3. ^{13}C-NMR spectrum of SUX-d_{18}. The structural formula of the target compound is shown in the box. NMR signals are assigned to distinct carbon nuclei using matching letters. Signal multiplicity is described using s = singlet, t = triplet and m = multiplet.

and was collected by filtration. A subsequent cleanup step involved recrystallization of the product from 450 ml of a 1 : 2 methanol : ethanol (v/v) mixture and filtration of product crystals.

NMR

Spectra were recorded at room temperature using Bruker DPX 300 and DPX 400 instruments (Bruker, Rheinstetten, Germany). Chemical shifts are expressed in ppm relative to the standard tetramethylsilane and were obtained through referencing of solvent residual signals to the respective literature values.[23] Data were analyzed using 1D-WinNMR (Bruker Daltronic, Bremen, Germany). For NMR spectroscopy, SUX-d_{18} and SMC-d_3 were dissolved in D_2O and MeOD to yield concentrations of approx. 35 and 65 mg/ml, respectively. Both were measured on the DPX 300 instrument.

NMR analysis on the intermediates was performed using 75 and 95 mg/ml samples in MeOD for desmethyl-SMC and choline-d_9, respectively. Choline-d_9 and desmethyl-SMC were measured on the DPX 300 and DPX 400, respectively.

FAB-MS

The spectra were acquired on a Concept 1H (Kratos, Hofheim, Germany) instrument, using meta-nitrobenzyl alcohol (mNBA) as matrix. The amount of product employed for FAB-MS analysis was not exactly determined.

(HPLC-)ESI-MS/MS

The data were acquired using a PE Sciex API 2000 mass spectrometer in combination with an Agilent 1100 Series HPLC. For ESI-MS/MS, a turbo ion spray source was used in positive ion mode at a temperature of 350 °C and an ion spray voltage of 5000 V. Nitrogen was employed as the collision gas.

Product ion spectra were recorded in the multiple channel acquisition (MCA mode, 10 scans) using a 10 μg/ml methanolic stock solution of each compound at collision energies of 25 eV for both SMC and SMC-d_3, as well as 18 and 16 eV for SUX and SUX-d_{18}, respectively.

Subsequent mass spectrometric data were acquired in the multiple reaction monitoring (MRM) mode with the following transitions (quantifier in bold print, respective collision energies in parentheses):

SUX : **145.1** → **115.1** (15 eV); 145.2 → 79.2 (21 eV)

SUX-d_{18} : **154.0** → **120.0** (16 eV); 154.0 → 98.0 (21 eV)

SMC : **204.0** → **144.7** (20 eV); 204.0 → 98.6 (32 eV)

SMC-d_3 : **207.1** → **144.8** (22 eV); 207.1 → 98.8 (34 eV)

For separation, a Phenomenex Synergi Hydro RP (150 × 2 mm, 4 μm) column was used. Gradient elution using 5 mM ammonium formate in H_2O : acetonitrile 90 : 10 (v/v, solvent A) and 5 mM ammonium formate in H_2O : acetonitrile 10 : 90 (v/v, solvent B) was performed (0–3 min: 95% A/5% B; 3–5 min: 95% A → 5% A/5% B → 95% B; 5–8 min: 5% A/95%

DOI: 10.1002/jms

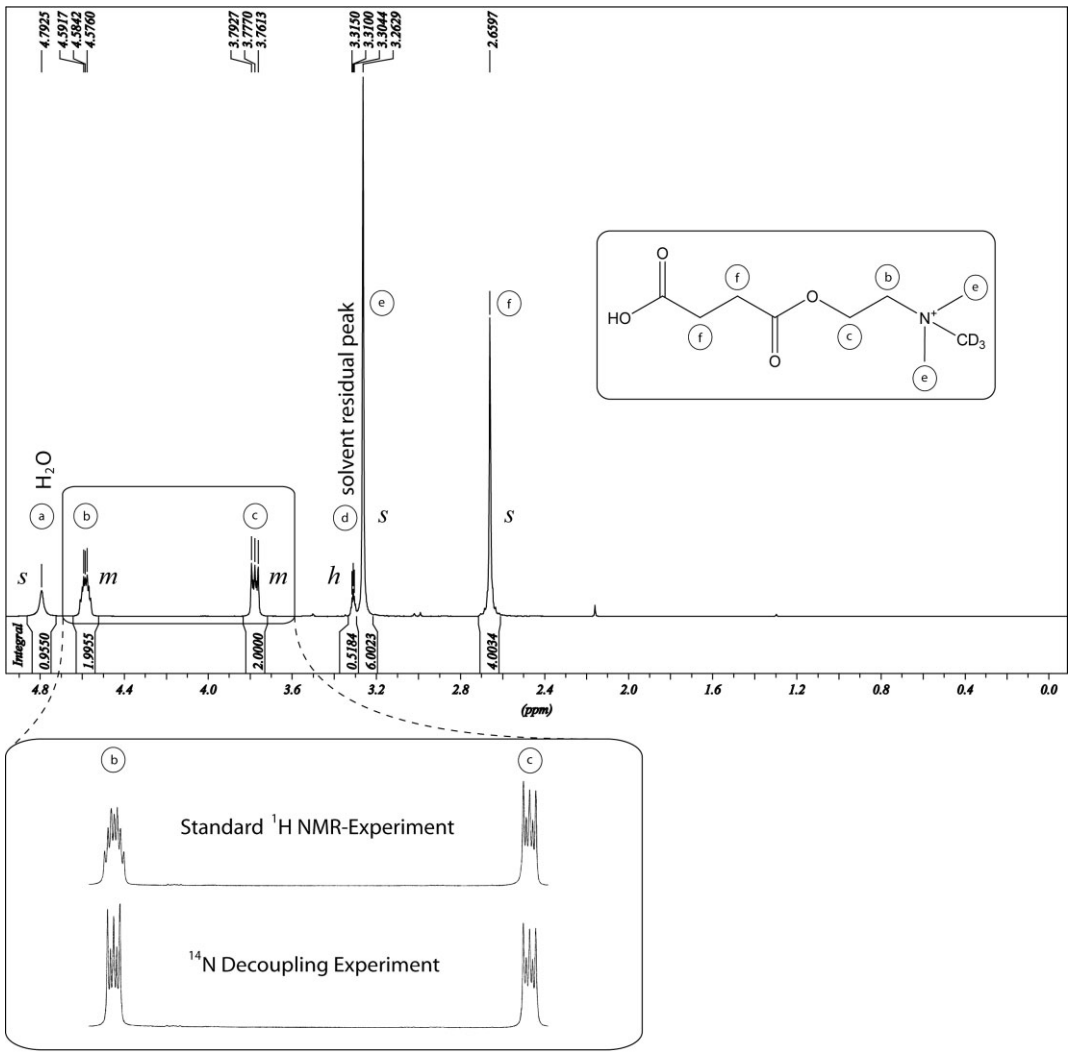

Figure 4. Top: ^{1}H-NMR spectrum of SMC-d_3. The structural formula of the target compound is shown in the box. NMR signals are assigned to distinct proton nuclei using matching letters. Signal multiplicity is described using s = singlet, h = heptet, and m = multiplet. Bottom: Extraction of the indicated region from above standard ^{1}H-NMR spectrum in superposition with the respective spectral region from a ^{14}N-decoupling experiment.

B; 8–10 min: 5% A → 95%A/95%B → 5% B; 10–13 min: 95% A/5% B) at a flow rate of 200 µl/min.

For the characterization of the synthesized products a 5 µl aliquot of a 1 µg/ml solution each of SUX-d_{18} and SMC-d_3 was injected for analysis.

Exemplary chromatograms were recorded by analyzing spiked urine and serum sample extracts. Serum was esterase-inactivated by addition of paraoxon. The aqueous sample matrix was spiked with 1 µg/ml each of SUX and SMC as well as the internal standard mix, yielding a final concentration of 1 µg/ml of both SUX-d_{18} and SMC-d_3. The extraction procedure is based on ion pairing reactions and employs a polymeric reversed phase solid-phase extraction (SPE) cartridge. Quantitation was performed by calculating the peak area (area under the curve, AUC) ratio of the analyte's to the respective internal standard's signal, and linear calibration curves were obtained for both biological matrices. Method development, full validation and testing of its applicability using biological samples are currently in progress and will be described elsewhere.

RESULTS

Recoveries

Final recovery for the intermediate desmethyl-SMC was determined to be 36.4%. Combined SMC-d_3 corresponded to a recovery of 73.0%. For the intermediate choline-d_9, a recovery of 62.5% was determined. At 22.2%, the recovery was lowest for synthesis of SUX-d_{18}; however, it has to be kept in mind that the methods were not optimized for high recoveries but, on the contrary, care was taken to obtain the cleanest possible material in each step.

NMR

SUX-d_{18} ^{1}H spectrum

For SUX-d_{18}, the ^{1}H spectrum comprises four distinct signals (Fig. 2). Integration of these signals proves that signal (a) with its odd integral value of 0.87 represents the solvent residual signal, and the remaining three signals, each with an even integral value of 4.00, can thus be attributed to the analyte. As already indicated by the above-mentioned integral values, four protons contribute to each of the latter signals. To

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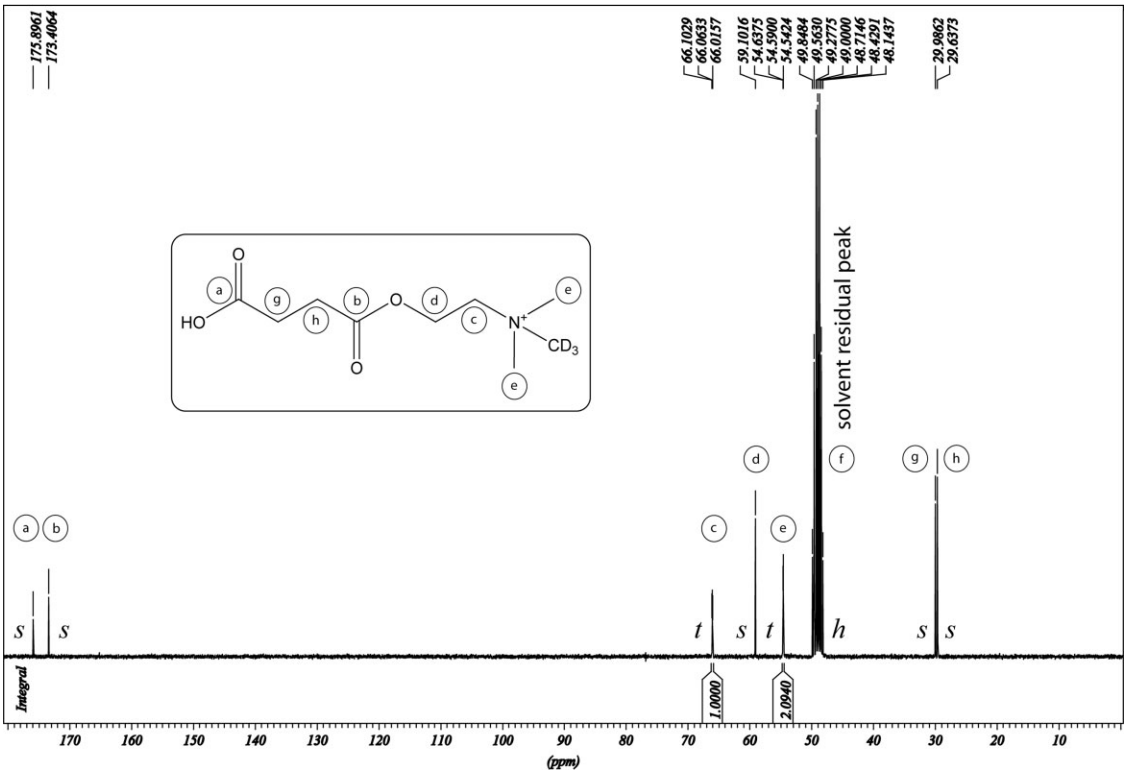

Figure 5. ^{13}C-NMR spectrum of SMC-d_3. The structural formula of the target compound is shown in the box. NMR signals are assigned to distinct carbon nuclei using matching letters. Signal multiplicity is described using s = singlet, t = triplet and h = heptet.

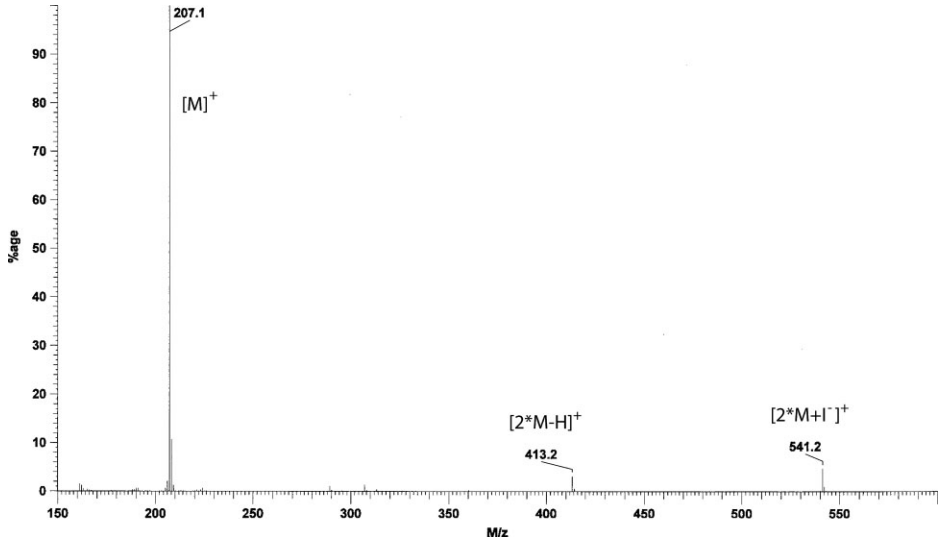

Figure 6. FAB-MS spectrum of SMC-d_3. The spectrum contains a main peak at *m/z* 207.1, corresponding to the isotopic molecular mass of the $[M]^+$ ion. The minor peaks in this spectrum can be attributed to the complexes $[2 \times M - H]^+$ and $[2 \times M + I]^+$. There is no evidence of incomplete deuteration or contamination of the product.

further assign the 12 protons in question, the chemical shift as well as the multiplicity data were used. The assignment of the four protons contributing to the singlet in (d) is unambiguous: only shielded protons, i.e. protons in a carbon-rich environment, can give a comparable upfield signal.

All other protons are bound to carbons, which in turn are bound to more electronegative heteroatoms, thus causing a deshielding effect that leads to higher values for δ, and the respective NMR signals can therefore be expected to be more downfield shifted. The final assignment of the (b) and (c) signals might seem surprising at first: the (c)-protons, in the vicinity of an oxygen atom, are usually predicted to result in more downfield signals as compared to the (b)-protons, which are closest to a (less electronegative) nitrogen. The presented assignment could however be confirmed using a ^{14}N-decoupling experiment, showing that upon suppression of ^{14}N-coupling effects the multiplicity of the signal in (b) resembles that in (c). It was thus proved that the protons

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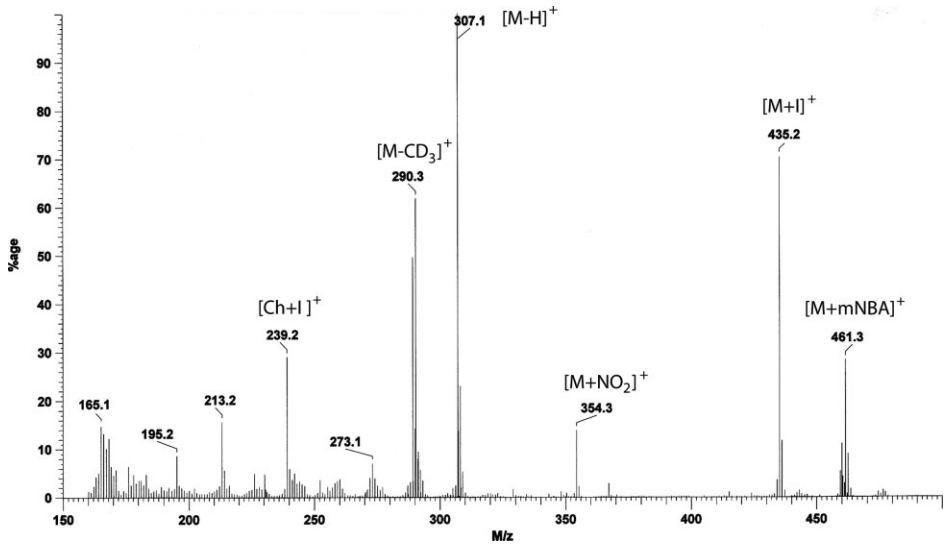

Figure 7. FAB-MS spectrum of SUX-d_{18}. The major mass peak at *m/z* 307.1 corresponds to $[M - H]^+$, and the other two major peaks can be attributed to $[M + I]^+$ at *m/z* 435.2 and $[M\text{-}CD_3]^+$ at *m/z* 290.3. The minor peaks correspond to complexes between the analyte and the matrix (*m/z* 461.3) or fragments of the matrix (*m/z* 354.3), as well as different fragments of the analyte or complexes thereof (e.g. iodo-adduct to choline at *m/z* 239.2). There is no sign of incomplete deuteration or contamination of the substance.

(b) are the ones in the closest vicinity to the nitrogen atom. The final assignment is summarized in Fig. 2, and it can be stated that this assignment is consistent with the chemical shift, integral as well as multiplicity data derived from the acquired spectrum. The same figure emphasizes the fact that no excess signals that would be indicative of organic impurities or incomplete deuteration are visible in the ^{1}H-NMR spectrum of SUX-d_{18}, and it can therefore be assumed that the product is of high purity.

SUX-d_{18} ^{13}C spectrum

The ^{13}C spectrum of SUX-d_{18} contains five discernible signals, all of which can be assigned to the analyte (Fig. 3). The signals in (a) and (e) can clearly be attributed to the carbons in the carboxyl and succinic acid moieties of the molecule, respectively: owing to the immediate vicinity of two oxygens, the chemical shifts for the nuclei in (a) are at the downfield end of the spectrum. In contrast, the carbon signals in (e), not being bound to more electronegative heteroatoms, can be found towards lower values for δ.

In ^{13}C-NMR the signal intensity, usually small due to low abundance and further signal splitting, is enhanced using proton decoupling. With this in mind, the multiplet signal in (d) can undoubtedly be assigned to the methyl carbons of the analyte; the six respective carbons are each bound to three deuterium atoms (nondecoupled) and one nitrogen atom (nondecoupled), and the ^{13}C signal is thus split multiple times and consequently barely rises above the baseline. The signals in (b) and (c) can be assigned using their respective multiplicity: the fact that the carbon signal in (b) is a triplet strongly suggests that the corresponding carbon nucleus, in contrast to the one in (c) yielding a singlet signal, is the one subjected to (^{13}C, ^{14}N) dipolar coupling, and therefore the one in the closest vicinity to the nitrogen atom. The complete assignment is depicted in Fig. 3. Also in the ^{13}C spectrum of SUX-d_{18} no excess signals indicative of organic impurities or incomplete deuteration are visible.

SMC-d_3 ^{1}H spectrum

The ^{1}H spectrum of SMC-d_3 contains six discernible signals (Fig. 4). Integration data established that signals (a) and (d) with their odd integral values of 0.96 and 0.52 are due to an H_2O trace impurity and solvent residual signal, respectively. Comparison of the chemical shifts with reference data[23] confirms this assignment. The remaining four signals can all be attributed to the analyte. Integral values were determined to be 2.0, 2.0, 6.0 and 4.0 for the signals in (b), (c), (e) and (f), respectively. Together with the chemical shift data, this leads to the conclusion that the signal in (f) is derived from the most shielded protons in the target compound, i.e. the protons of the succinic acid moiety. The six methyl protons give rise to the singlet in (e), this assignment being consistent with their expectedly lower shielding, i.e. the higher chemical shift of the resulting signal, due to the vicinal nitrogen. Two protons each contribute to the signals in (b) and (c), respectively, and in this case the same is true as for the ^{1}H spectrum of SUX-d_{18}: again it could be proven via a ^{14}N-decoupling experiment that the protons denoted by (b) are the ones in the closest vicinity to the nitrogen atom. The final assignment is summarized in Fig. 4, which further shows that no excess resonances indicative of organic impurities or incomplete deuteration were detectable in the ^{1}H-NMR spectrum.

SMC-d_3 ^{13}C spectrum

In the ^{13}C spectrum of SMC-d_3, eight well-resolved resonances were detected (Fig. 5), one of which, the heptet, represents the solvent residual signal. The remaining seven signals were assigned as follows: resonances (a) and (b) are derived from strongly deshielded carbon nuclei, i.e. the carboxy- and ester-group carbons, respectively. In contrast, the (g) and (h) signals can be attributed to the highly shielded carbons in the backbone of the succinic acid moiety. The remaining three signals can be assigned using a combination

DOI: 10.1002/jms

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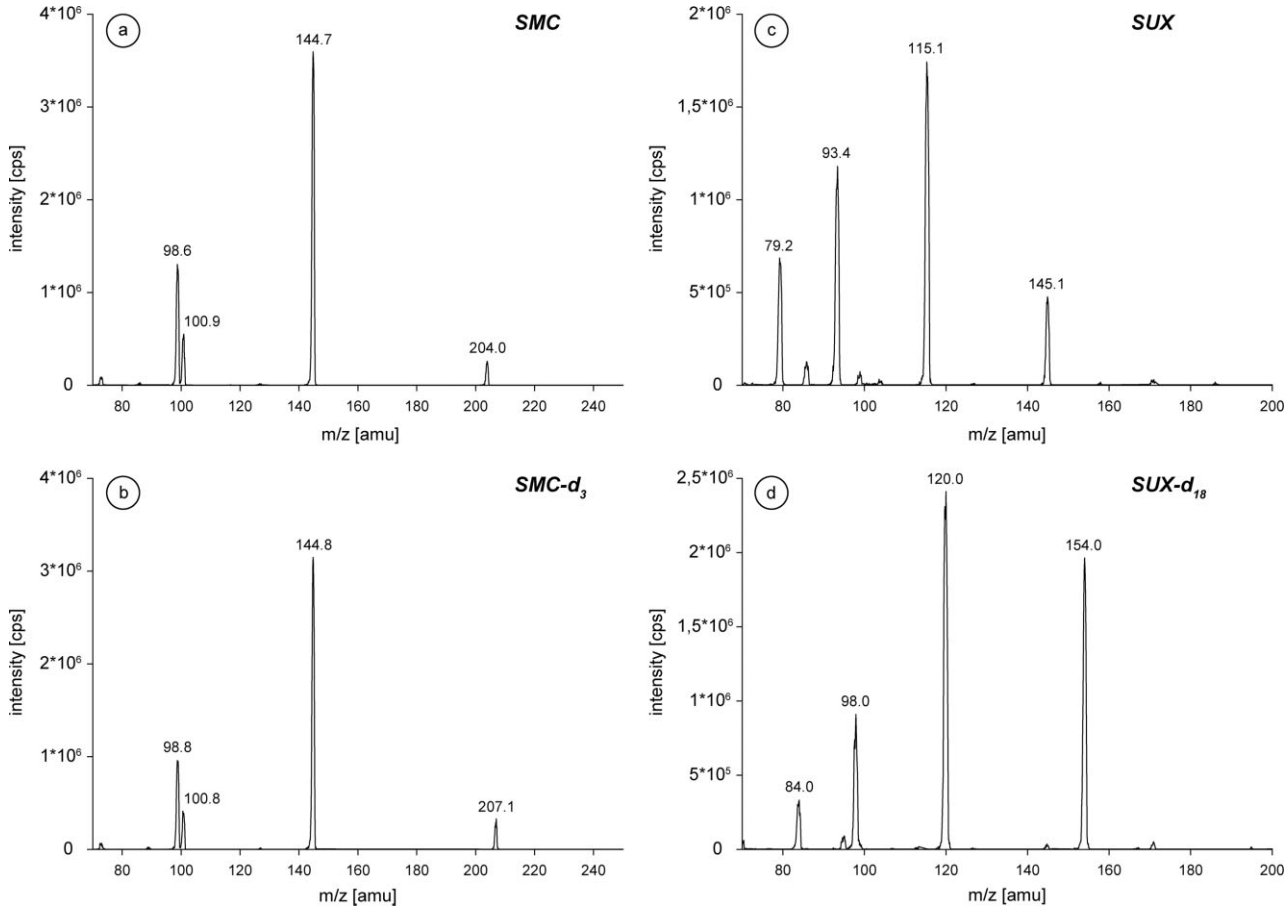

Figure 8. Product ion spectra of SMC (a), SMC-d_3 (b), SUX (c) and SUX-d_{18} (d). The figure emphasizes the differences in product ion patterns between the deuterated and the respective nondeuterated compound in mass spectrometric analysis, and indicates their applicability as internal standards in HPLC-MS/MS method development.

of multiplicity and integral data: The resonance in (d) is a singlet and can therefore be attributed to the carbon next to the ester bond. In contrast, the signals in (e) and (c) are split into triplets, signifying close proximity to the nitrogen atom. To further assign these resonances, the integral data was taken into account: integration of ^{13}C-NMR signals is not as meaningful as the integral data in ^{1}H spectra; it can, however, be used as a clue for assignment. In this case, the integration of the NMR signal in (e) yields a value that is about twice that of signal (c), and it can therefore be assumed that the two nondeuterated methyl carbons give rise to the former resonance (the deuterated methyl carbon would be nondecoupled and therefore barely contribute to the signal), while the latter is derived from the backbone carbon in the choline moiety next to the quaternary amine group. The final assignment is depicted in Fig. 5. The absence of excess signals in the presented spectrum confirms that the preparation is correctly deuterated and not contaminated with organic impurities.

FAB-MS

SMC-d_3

FAB-MS analysis of SMC-d_3 yields a spectrum with one main peak at m/z 207.1, which corresponds to the expected mass of the $[M]^+$ ion. The minor peaks in this spectrum can be attributed to the complexes $[2 \times M - H]^+$ and $[2 \times M + I]^+$. The interpretation of the SMC-d_3 FAB-MS spectrum is summarized in Fig. 6.

SUX-d_{18}

The FAB-MS spectrum of SUX-d_{18} is more complex: the major mass peak in this spectrum is visible at an m/z of 307.1 which corresponds to $[M - H]^+$, and the other two major peaks can be attributed to $[M + I]^+$ at m/z 435.2 and $[M - CD_3]^+$ at m/z 290.3. All other peaks are below 30% intensity: the ones featuring a higher m/z correspond to complexes between the target analyte and the matrix (m/z 461.3) or fragments of the matrix (m/z 354.3), while peaks in the low-mass region of the spectrum can be attributed to different fragments of the analyte or complexes thereof, e.g. the choline moiety of the product coupled to an iodine (m/z 239.2). The SUX-d_{18} FAB-MS spectrum as well as its interpretation is depicted in Fig. 7.

No signal indicative of incomplete deuteration or significant amounts of impurities was found in any of the two preparations.

(HPLC-)ESI-MS/MS

The product ion spectra of deuterated as well as nondeuterated SUX and SMC are depicted in Fig. 8(a–d). Comparing the respective signals at the approximate mass-to-charge ratios of m/z 145, 99 and 101, it becomes visible that SMC (Fig. 8(a)) and SMC-d_3 (Fig. 8(b)) break down into identical

DOI: 10.1002/jms

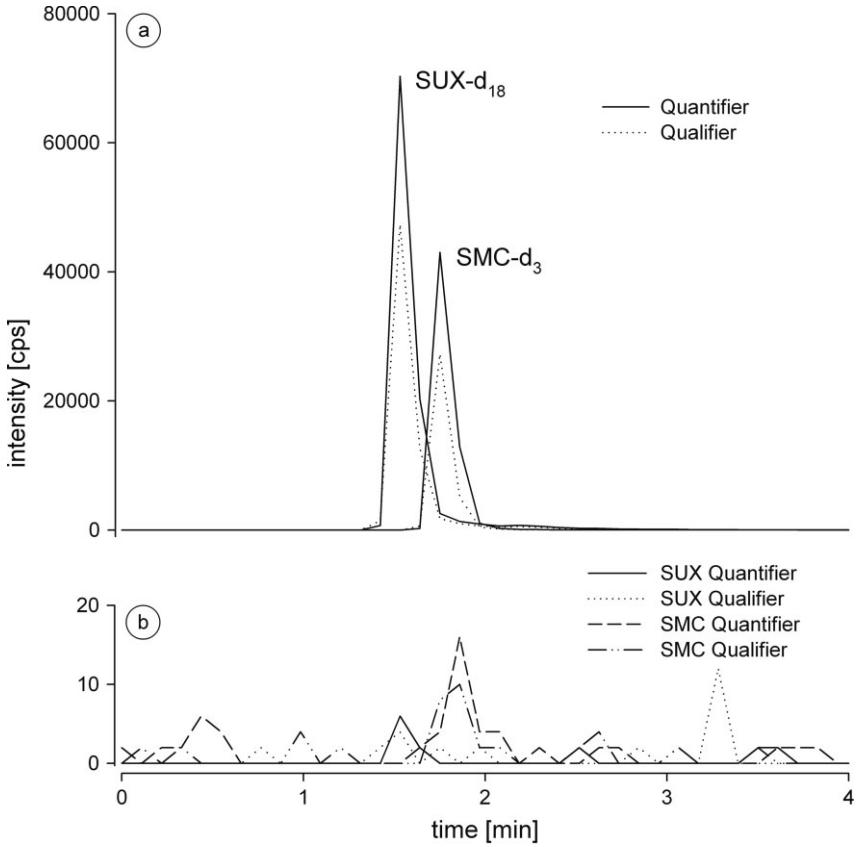

Figure 9. HPLC-MS/MS of an internal standard solution with 1 μg/ml of both SUX-d_{18} and SMC-d_3. Eight transitions (1 quantifier and 1 qualifier for each analyte) were monitored as detailed in 'Experimental'. (a) Both deuterated analogs yield good signal intensities for their respective quantifier as well as qualifier transitions. (b) In the same sample no nondeuterated SUX or SMC is detectable above the background noise.

fragments. These fragments are, however, derived from different precursor ions, i.e. m/z 204.0 for SMC *versus* m/z 207.1 for SMC-d_3, and distinguishable MRM transitions can therefore be defined. In contrast to the similar product ion spectra of SMC and SMC-d_3, the respective spectra of SUX (Fig. 8(c)) and SUX-d_{18} (Fig. 8(d)) are easily differentiated: the former, with a precursor ion of m/z 145.1, gives rise to product ions at m/z 115.1, 93.4 and 79.2, but the latter (m/z 154.0), in contrast, produces fragments of m/z 120.0, 98.0 and 84.0. The product ion spectra of the nondeuterated compounds and their deuterated analogs were subsequently used to define a set of unique MRM transitions for all substances.

As is visible from the diagram in Fig. 9(a), the synthesized compounds yield good signal intensities for their respective quantifier as well as the qualifier transition in HPLC-MS/MS. No nondeuterated SUX or SMC was detectable above the level of background noise (Fig. 9(b)).

Exemplary analyses of spiked serum (top) and urine (bottom) sample extracts are depicted in Fig. 10. It is shown that both nondeuterated analytes as well as their respective deuterated analogs yield well-defined signals in HPLC-MS/MS following sample extraction. The test samples were correctly quantified upon consulting with each applicable calibration curve (data not shown). Altogether, the presented HPLC-MS/MS results demonstrate the applicability of both SUX-d_{18} and SMC-d_3 as reliable internal standards in mass spectrometric analyses.

DISCUSSION

The synthesis and complete characterization of SMC-d_3 and SUX-d_{18} is reported. Synthesis was performed using two-step protocols and optimized for high purity of the resulting preparations. The products were confirmed to be chemically pure SMC-d_3 and SUX-d_{18} using ^{1}H- and ^{13}C-NMR spectroscopy as well as FAB-MS and HPLC-MS/MS: In NMR spectroscopy, all resonances could undoubtedly be assigned to defined nuclei or groups of nuclei. The integration data, i.e. the number of protons or, in one case, carbons giving rise to a certain signal, was always consistent with the respective target structure. Multiplicity of signals as well as ^{14}N-decoupling experiments were also used for assignment, and the results corresponded in all cases to the respective structural formula. It was shown that in none of the acquired spectra excess signals, i.e. evidence for organic impurities or incomplete deuteration, could be detected, findings that were confirmed by both mass spectrometric methods.

Product ion scans of deuterated as compared to nondeuterated compounds indicated the applicability of SUX-d_{18} and SMC-d_3 as internal standards for mass spectrometric analyses, and were used for the definition of unique MRM transitions. The simultaneous detection of SUX and SMC in biological media finally demonstrated the suitability of both SUX-d_{18} and SMC-d_3 as internal standards in the further development of an HPLC-MS/MS method.

DOI: 10.1002/jms

JMS

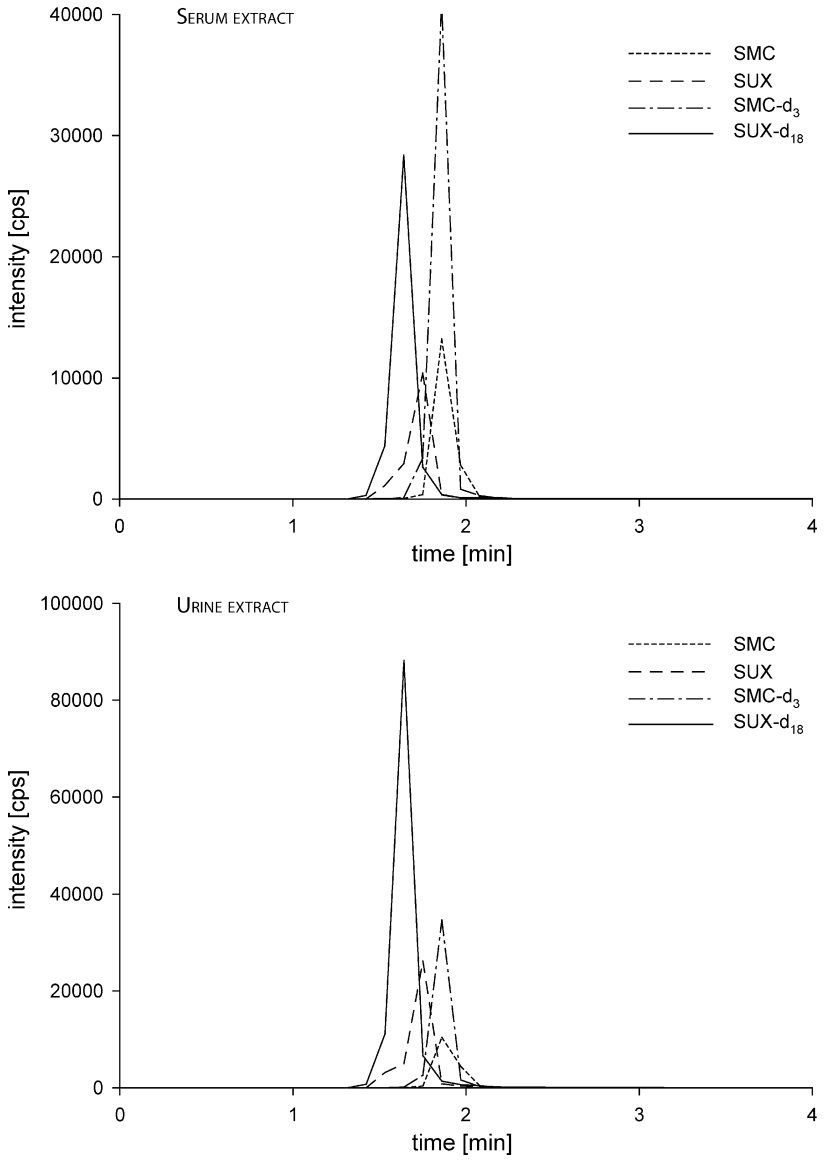

Figure 10. Exemplary HPLC-MS/MS chromatograms of spiked serum (top) and urine (bottom) sample extracts proving the applicability of the synthesized compounds as internal standards enabling the simultaneous detection of SUX and SMC. Each sample contained 1 μg/ml of both SUX and SMC. Internal standards were employed in concentrations of 1 μg/ml of each SUX-d_{18} as well as SMC-d_3. For purposes of clarity only the quantifier transition is shown for each of the four compounds.

For quantification of SUX and SMC in biological matrices using isotope dilution mass spectrometry, deuterated internal standards are indispensable but commercially unavailable. Owing to thoughtful design, SUX-d_{18} and SMC-d_3 feature different deuteration patterns at their trimethylamine moieties and therefore offer the unique possibility to simultaneously quantify SUX and its metabolite SMC for clinical and forensic purposes.

Acknowledgements

The authors would like to thank Prof. Dr M. Famulok and Dr S. Fusz from the Institute of Organic Chemistry of the University of Bonn for providing technical resources as well as expert guidance throughout this project.

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DOI: 10.1002/jms