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ORIGINAL ARTICLE

LC–MS assay for quantitative determination of cardio glycoside in human blood samples

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Abstract A method is described for liquid chromatography–mass spectrometry analysis of the cardio glycosides digoxin and digitoxin in biological samples. The method was optimized for use in the forensic field and, therefore, comprises the determination from whole blood and tissue samples. Sample cleanup by solid phase extraction (SPE) on a functionalized polymeric phase was sufficient to limit matrix suppression to <10% for all analytes. Chromatographic separation was achieved using an RP-8 column. Detection of the cardio glycosides was performed with electrospray ionization in the positive mode. The system was run in single ion monitoring mode, measuring the sodium adducts $(M+Na)^+$ of the analyte and of the internal standard, respectively. The method was fully validated for the analysis of blood samples and was also successfully applied in forensic cases. The method was accurate and precise over a linear concentration range up to 50 ng/g blood. Lower limit of quantitation was 0.2 ng/g for digoxin and 2 ng/g for digitoxin, respectively. As deuterated analyte was used as internal standard, we also present a new microwave-enhanced method for the fast preparation of the labelled analyte within 20 min.

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Introduction

Digitalis glycosides are used for the treatment of congestive heart failure. Clinical digoxin therapy requires strict drug monitoring due to its narrow therapeutic range of 0.5–2 ng/ml (digitoxin 10–35 ng/g). Because of this toxicity, it is also of forensic importance to know whether sample concentrations are within the therapeutic or toxic blood level [1, 2].

Several immunoassays are established methods in the clinical field, although the problem of cross-reactivity is well known [3, 4]. High-performance liquid chromatography (HPLC) coupled with mass spectrometry has been proven to be a specific and sensitive instrument for the determination of large molecules such as terpenoids [5] and cardenolides [6]. Several liquid chromatography–mass spectrometry (LC–MS) and LC–MS/MS methods are reported [7–9], but generally, they deal with the determination from serum or plasma samples. This matrix is often used in clinical screenings; in forensic cases, however, this material is normally not available. Determination has to be made out of blood or tissue samples. Only few publications concerning the determination of cardio glycosides deal with these complex matrices and their problems of sample cleanup [10, 11].

An easy method for the determination of cardio glycosides from blood samples was developed and is presented in this work. A simple cleanup procedure with the use of solid phase extraction (SPE) provides clean extracts. We also present a microwave-enhanced method for the preparation of labelled cardio glycosides, which were used as

internal standard (IS). This method proved its applicability in forensic cases.

Experimental

Chemicals and material

Chemicals used were digoxin, European Pharmacopoeia Chemical Reference Substance (EP CRS, purity 99.8%), and digitoxin, EP CRS (purity 99%) from Promochem (Wesel, Germany). A stock solution of 1 mg/ml was prepared in methanol and diluted for further use. For the preparation of the labelled analyte, digoxin (95%) from Sigma (Taufkirchen, Germany) and digitoxin (99%) from Fluka (Buchs, Switzerland) were used.

The labelled analytes were stored as solid substances in HPLC vials (100 μg each) and were diluted in methanol before use. The IS working solution contained labelled digoxin (50 ng/ml) and labelled digitoxin (500 ng/ml). All stock and working solutions as well as the IS substances were stored at −18°C.

Sodium acetate, acetic acid, and formic acid were obtained from Merck (Darmstadt, Germany), aqua bidest. from Waldeck (Münster, Germany). Isopropyl alcohol, acetone, and methanol, all of analytical grade, were from Lab Scan (Dublin, Ireland). SPE was performed on a functionalized polymeric phase (Bond Elut PPL, 100 mg) from Varian (Darmstadt, Germany). Acetate buffer for sample preparation consisted of 0.1 M sodium acetate, adjusted to pH=7.0 with acetic acid.

Chemicals used for the deuterations: 1,4-dioxane (99.5%), deuterium oxide (Uvasolv) from Merck, tetrahydrofuran (p.a.), and triethylamine (p.a.) from Fluka.

Blood samples/authentic samples

To assess validation data, blank blood samples were collected from seven healthy volunteers and spiked with the analytes, respectively. Blood, serum, urine, and gastric content from forensic cases with assumed cardio glycoside intoxications were determined with this method.

Apparatus

Microwave settings

A CEM Discover microwave (CEM, Matthews, NC) was used for the deuteration of the cardio glycosides. The closed vessel system allowed a maximum pressure up to 20 bars. The amount of energy (maximum 220 W) was regulated by a standard program (temperature set at 70°C). The run time was 20 min.

The successful hydrogen isotope exchange was verified by ^{1}H nuclear magnetic resonance (^{1}H-NMR) data (dimethyl sulfoxide-d6 [DMSO-d6]), which was attained using a Varian Mercury 400 plus spectrometer (400 MHz).

HPLC conditions and detector settings

A Waters Alliance 2695 separations module coupled to a Micromass Quattro micro with a Z-spray ion interface was used for all analyses (Table 1).

Chromatographic separation was achieved with a Waters X-Terra MS-C_8 analytical column (150×2.1 mm, 3.5 μm) and a corresponding pre-column. The chromatography was performed isocratically at a flow rate of 0.2 ml/min at 40°C column temperature. Mobile phase consisted of 0.1% formic acid in a mixture of 55% methanol and 45% water. Total run time was 30 min for each injection.

Materials and methods

Preparation of labelled glycosides

Substances and reagents were used as published by Kaiser et al. [12]: 1 mg glycoside was dissolved in a mixture of 1 ml 1,4-dioxane, 1 ml tetrahydrofuran, 1 ml deuterium oxide, and 0.5 ml triethylamine and stirred in a 10 ml pressurized microwave vial. During 20-min run time, the temperature of the vial did not exceed 70°C. After this procedure, the solution was cooled and portioned into HPLC vials (each 100 μg) and completely evaporated under a stream of nitrogen for storage. Before use, the deuterated substances were reconstituted in methanol.

Sample preparation

Blood, plasma, urine, and gastric content (each 0.5 g) were mixed with 2 ml 0.1 M sodium acetate buffer pH=7 and homogenized. After the addition of 50 μl IS working

Table 1 Method and ionization parameters using electrospray in the positive ionization mode

Compound	$(M+Na)^+$ m/z[a] Compound	$(M+Na)^+$ m/z[b] Internal Standard
Digoxin	803.4	807.4
Digitoxin	787.3	791.3
Capillary	3.3 kV	
Cone	60 V	
Source temperature	120°C	
Desolvation gas (N_2)	350°C, 300 l/h	

[a] Adduct of the C12 isotope
[b] Adduct of the C13 isotope

solution (d3-digoxin 50 ng/ml, d3-digitoxin 500 ng/ml), the mixture was homogenized and centrifuged at 4,000×*g* for 10 min.

Before the extraction, the SPE columns were conditioned with 3 ml methanol, 2 ml water, and 1 ml sodium acetate buffer. The sample supernatants were loaded on the activated columns and passed through under atmospheric pressure. The sample was washed with 3 ml sodium acetate buffer and dried under vacuum for 3 min. A second wash step with 2 ml of 20% isopropyl alcohol and 3 min drying followed. After adding 50 µl acetone, the columns were allowed to dry under vacuum for 10 min. The analyte was eluted with two aliquots of 1 ml acetone. Eluents were pooled and evaporated to dryness under a stream of nitrogen at 40°C. The dried residue was reconstituted with 100 µl of the mobile phase. A 10 µl aliquot of the sample was injected onto the LC–MS.

Validation procedure

Before the analysis of the forensic samples, the assay was validated with whole blood samples. Blood samples (0.5 g) were spiked with the required amount of analyte and prepared as described above. The data obtained were used to assess the calibration curve, accuracy and precision, limit of detection (LOD) and quantitation. Specificity was determined by testing six blank blood samples from different individuals without IS and two blank blood samples with IS according to forensic guidelines [13].

Ion suppression test and recovery

For the determination of matrix effects (ME), a systematic procedure according to Matuszewski et al. [14] was used. ME was evaluated by comparing analyte peak areas of neat standards prepared in the mobile phase (sample set A) with analyte peak areas of extracted blood samples spiked with the analyte after SPE (sample set B) at an equivalent concentration. The ME was expressed as a percentage (ME %) of the mean area of the neat samples (n=5). This method was also used to determine the recovery. For that purpose, analyte peak areas of extracted spiked samples (sample set C) were compared to the areas of sample set B. The recovery was expressed as a percentage of the mean area of sample set B.

Results

Synthesis and characterization of labelled glycosides

Recent advances in microwave-assisted synthesis revealed the possibility for quick and simple hydrogen isotope exchanges [15, 16]. Successful methods for the synthesis of deuterated digoxin and digitoxin have been described before [12, 17]. These former methods needed a reaction time of at least 24 h. We adopted the basic procedure and combined it with a microwave-enhanced process. We were able to shorten the reaction time to just 20 min and present, therefore, an extremely fast way to obtain a deuterated IS.

After evaporation, the labelled substances were reconstituted in mobile phase and directly injected into the MS. A chromatographic LC–MS run at higher concentrations revealed that less than 1% of all glycosides remained unlabelled. Nevertheless, this amount was not detectable in samples, which were spiked with the IS (2.5 ng d3-digoxin, 25 ng d3-digitoxin).

^{1}H-NMR data were used to determine the exact position of the deuterium. To allow a re-exchange of unstable deuterium bonds, the substance was reconstituted and evaporated to dryness in methanol. NMR spectra of labelled and unlabelled digoxin were measured in DMSO-d6 and compared to literature data [18]. The recording showed that a double substitution at position 21 (H21= 4.89, H21′=4.80) and a single substitution at position 22 (H22=5.79) in the unsaturated lactone ring (Fig. 1) had taken place.

Stability of labelled glycosides

The labelled analytes were stored as dry substances and also dissolved in methanol at −18°C and showed no measurable deuterium re-exchange under these conditions for 6 months. It was, however, noticed that under thermic stress (such as evaporation at 40°C) in combination with a protic solvent (such as methanol) a measurable re-exchange was possible. We were also able to observe a re-exchange during extraction studies at pH>9. This can be explained

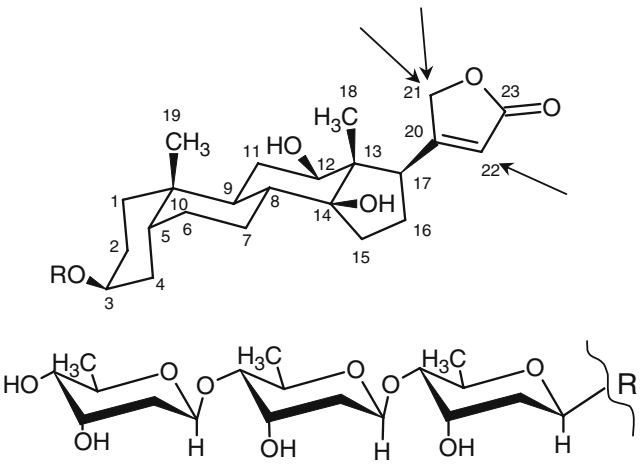

Fig. 1 Structure of digoxin. The arrows point out the positions where a deuterium exchange has taken place

by the chemical properties of the labelled cardenolid ring of the analytes [19].

For these reasons, we chose an extraction procedure at pH=7 and used acetone for the SPE elution.

Specificity

The blank blood samples and the blank samples spiked with IS did not show any interference in the signals of the analytes. Blanks with IS were also tested before each run as part of a calibration and to assure the stability of the IS.

Calibration curve and linearity

Calibration curves were constructed by plotting peak area ratio (y) of the cardio glycosides to the respective IS vs the glycoside concentration (x). An eight-point calibration was measured in duplicates for all analytes. The concentrations were chosen to cover the therapeutic as well as the toxic concentration range. The digoxin calibration curve was found to be linear over a range of 0.1–10 ng/g as well as digitoxin in a range from 0.5–50 ng/g. Correlation coefficients were R^2=0.999 for both substances.

The deuterated glycosides were mainly triply labelled. Therefore, initially, the masses M+3 amu (in that case 806, 790, and 820 m/z) were consequently measured. Due to the isotope distribution of the cardio glycosides [20] (Fig. 2), the standard substance was able to simulate IS at those masses (spectral overlap), and no linear correlation was found. For that reason, we eventually chose to measure the M+4 amu isotope of the substances.

Accuracy and precision

To determine the intra-assay accuracy and precision, five replicate analyses were performed with spiked blood samples at 0.2, 2.0, and 6 ng/g for digoxin and at 2.0, 20, and 40 ng/g for digitoxin. IS was added, and the samples were extracted and analyzed within 1 day. Inter-assay accuracy and precision were determined by repeating this procedure at the same concentrations on three different days. Data thus obtained were evaluated in reference to the IS (Table 2). Acceptable precisions were coefficients of variation (% CV) below 15% at every concentration except for the lower limit of quantitation (LLQ) where 20% CV was acceptable [21, 22]. The method was found to be precise with a CV<9% for all substances as well as accurate with less than 9% deviation from the nominal concentration.

LLQ and LOD

According to Food and Drug Administration guidelines [22], the signal at the LOQ should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%. As seen in the intra- and inter-assay precision experiments, the LLQ with 0.2 ng/g digoxin (Table 2, Fig. 3) and 2 ng/g digitoxin were acceptable.

The LOD of digoxin was determined empirically at a concentration of 0.1 ng/ml (S/N=15), meeting the International Conference on Harmonisation [13] recommendation for a S/N Ratio of at least 2–3. Lower concentrations have been measured and have not yielded in an acceptable S/N

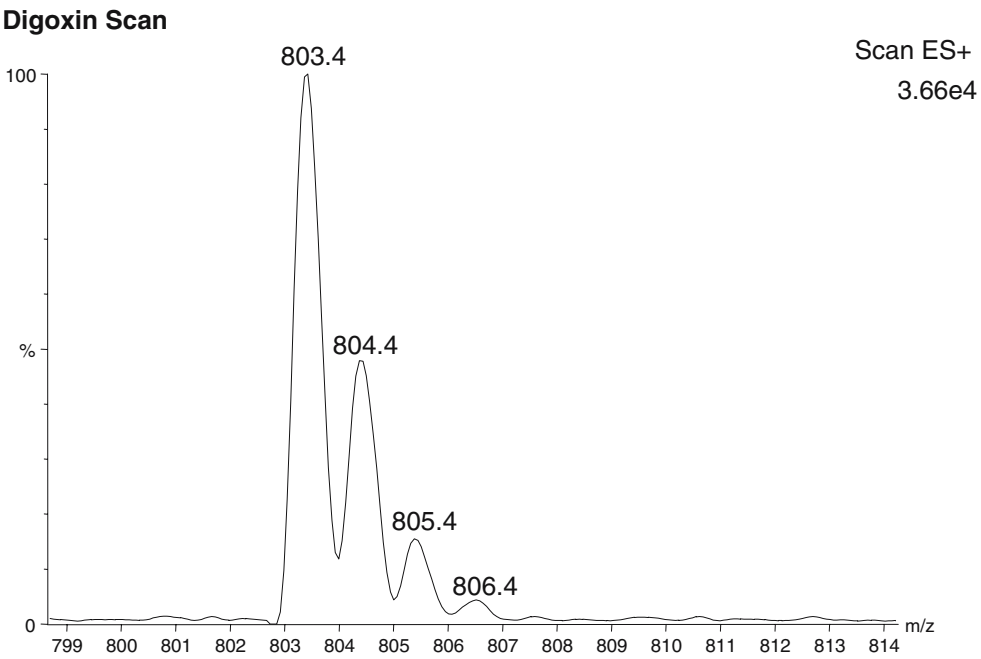

Fig. 2 Isotope distribution of digoxin. Even though the signal intensity of m/z 806 is small compared to the main isotope, it was able to interfere with a linear response

Table 2 Precision and accuracy for the determination of digoxin and digitoxin

	Concentration added (ng/g blood)					
	Digoxin			Digitoxin		
	0.2	2.0	6.0	2	20	40
Determined concentration (ng/g blood)						
Series 1 (n=5)	0.19	2.0	6.0	1.9	20.5	39.4
Series 2 (n=5)	0.21	2.0	6.0	2.0	20.1	39.9
Series 3 (n=5)	0.20	1.9	6.0	2.0	20.1	40.0
Mean value	0.2	2.0	6.0	2.0	20.2	39.7
Intra-assay precision (CV %)						
Series 1	8.4	1.9	1.1	2.8	0.9	1.1
Series 2	5.2	1.6	0.7	1.4	1.0	0.7
Series 3	5.0	1.3	1.6	1.3	1.2	0.8
Inter-assay precision (CV %)	2.7	3.7	0.4	1.7	1.5	2.1
Intra-assay accuracy (bias)						
Series 1	−3.0	2.3	0.7	−2.3	2.4	−1.5
Series 2	5.0	0.1	0.3	−1.4	0.7	−0.2
Series 3	2.0	−3.1	0.8	−1.8	0.4	0
Inter-assay accuracy Bias	8.3%	0.8%	−0.1%	−2.8%	2.0	−1.2

ratio. The empirical determination of the LOD was chosen because, in this case, it led to more realistic LOD values than extrapolation.

Ion suppression and recovery

Due to the thorough extraction procedure, the MEs observed were no more than 10%. Nevertheless, ion suppression and enhancement were still present. These MEs have to be taken into account for the evaluation of the recovery. For this reason, the approach of Matuszewski et al. [14] was chosen to determine both parameters (Table 3).

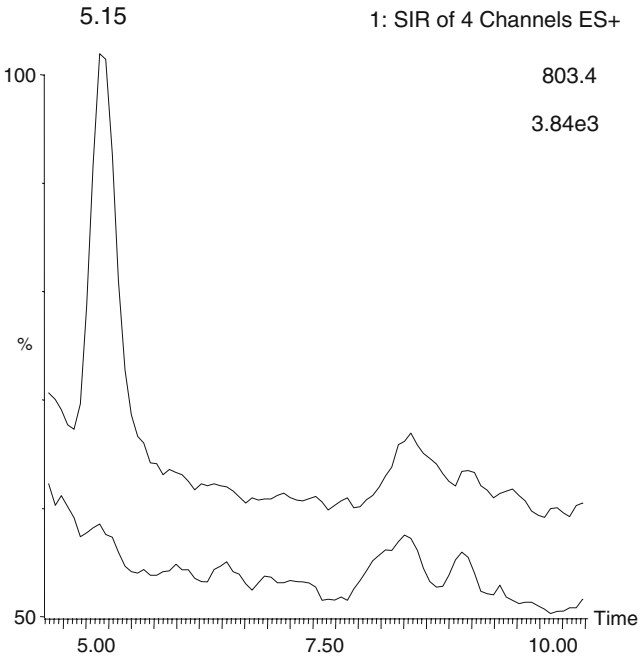

Fig. 3 SIM Chromatograms of a spiked sample concentration of 0.2 ng/g Digoxin (RT 5.15 min) and a blank sample (superimposed, 15%)

Forensic samples

The developed method was applied in two forensic cases with known digoxin intoxications and in one case with digitoxin intoxication. The measurements were compared with formerly made immunoassays and showed a good correlation.

Discussion

A simple LC–MS method for the determination of cardio glycosides was developed for the forensic use. Only small sample amounts are needed, and results are obtained within one working day. A complete validation was done with whole blood samples. Good results were also obtained with serum. A comparison with immunoassay data, good

Table 3 SPE recovery and matrix effects

Compound	Recovery % n=5			ME % n=5
Digoxin	1 ng/g	2 ng/g	3 ng/g	2 ng/g
	89	89	82	96
Digitoxin	10 ng/g	20 ng/g	40 ng/g	20 ng/g
	87	94	84	110

precision, and accuracy as well as high sensitivity also demonstrate the applicability of this method for the clinical field.

We chose the selected ion monitoring mode (SIM), although the instrumental setup provided the possibility to perform multiple reaction monitoring (MRM) experiments [7] and could, therefore, achieve a higher selectivity. In our experiments, sensitivity obtained in the MRM mode was not sufficient. This was obviously due to the formation of the stable sodium adduct of the analytes. Any attempts to suppress this formation were not successful. Even if a mobile phase containing ammonium ions was applied, the main fraction of the analytes remained as sodium adduct. For this reason, the sodium adducts were chosen for the detection in the SIM mode. Due to the stability of these adducts, the detection of further fragment ions or transitions as recommended, e.g., by the European commission [21] was not possible. This disadvantage was accepted in favor of an enhanced sensitivity.

We were able to enhance a deuteration process for the cardio glycosides. The use of a microwave provides quick and satisfactory results. The IS can be prepared directly before use in just 20 min. With ^{1}H-NMR data, we were able to identify the deuterium position in these labelled substances.

Despite a sophisticated extraction method and chromatography, MEs could not completely be avoided. For this reason, the use of a suitable IS that can compensate these effects to a certain degree is of utmost importance.

References

1. Adamowicz P, Chudzikiewicz E, Kala M (2002) Digoxin in a forensic laboratory. Z Zag Nauk Sadow 49:114–127
2. Arnold W, Puschel K. (1979) Forensic aspects of digoxin-poisoning. J Leg Med 83:265–272
3. Valdes R, Jr., Jortani SA (2002) Unexpected suppression of immunoassay results by cross-reactivity: now a demonstrated cause for concern. Clin Chem 48:405–406
4. Steimer W, Muller C, Eber B, Emmanuilidis K (1999) Intoxication due to negative canrenone interference in digoxin drug monitoring. Lancet 354:1176–1177
5. Van Landeghem AA, De Letter EA, Lambert WE, Van Peteghem CH, Piette MHA (2007) Aconitine involvement in an unusual homicide case. Int J Legal Med 121:214–219. DOI 10.1007/s00414-006-0119-5
6. Pietsch J, Oertel R, Trautmann S, Schulz K, Kopp B, Dreßler J (2005) A non-fatal oleander poisoning. Int J Legal Med 119:236–240
7. Yao M, Zhang H, Chong S, Zhu M, Morrison RA (2003) A rapid and sensitive LC/MS/MS assay for quantitative determination of digoxin in rat plasma. J Pharm Biomed Anal 32:1189–1197
8. Lacassie E, Marquet P, Martin-Dupont S, Gaulier JM, Lachatre G (2000) A non-fatal case of intoxication with foxglove, documented by means of liquid chromatography–electrospray–mass spectrometry. J Forensic Sci 45:1154–1158
9. Tracqui A, Kintz P, Ludes B, Mangin P (1997) High-performance liquid chromatography–ionspray mass spectrometry for the specific determination of digoxin and some related cardiac glycosides in human plasma. J Chromatogr B Biomed Sci Appl 692:101–109
10. Guan F, Ishii A, Seno H, Watanabe-Suzuki K, Kumazawa T, Suzuki O (1999) Identification and quantification of cardiac glycosides in blood and urine samples by HPLC/MS/MS. Anal Chem 71:4034–4043
11. Plum J, Daldrup T (1986) Detection of digoxin, digitoxin, their cardioactive metabolites and derivatives by high-performance liquid chromatography and high-performance liquid chromatography-radioimmunoassay. J Chromatogr 377:221–231
12. Kaiser P, Akerboom T, Wood WG, Reinauer H (2006) A novel LC–IDMS/MS method for the determination of the cardiac glycosides digoxin and digitoxin using caesium adducts. Clin Lab 52:37–42
13. ICH (1995) Harmonised tripartite guideline Q2 (R1): validation of analytical procedures: text and methodology. Fed Regist 60:11260
14. Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. Anal Chem 75:3019–3030
15. Hayes BL (2004) Recent advances in microwave-assisted synthesis. Aldrichimica Acta 37:66–77
16. Anto S, Getvoldsen GS, Harding JR, Jones JR, Lu S-Y, Russell JC (2000) Microwave-enhanced hydrogen isotope exchange studies of heterocyclic compounds. J Chem Soc Perkin Trans 2:2208–2211
17. Keßler A (2001) Massenspektrometrische Isotopenverdünnungsanalyse von Digoxin und Digitoxin in menschlichem Serum-Referenzmethoden in der Klinischen Chemie, Dissertation zur Erlangung des Doktorgrades, Rheinische Friedrich-Wilhelms-Universität Bonn
18. Aulabaugh AE, Crouch RC, Martin GE et al (1992) The conformational behavior of the cardiac glycoside digoxin as indicated by NMR spectroscopy and molecular dynamics calculations. Carbohydr Res 230:201–212
19. El Nemr A, Tsuchiya T (1998) A convenient method for deuteration at the alpha position of an oxo group in carbohydrates. Tetrahedron Lett 39:3543–3546
20. Pickup JF, McPherson K (1976) Theoretical considerations in stable isotope dilution mass spectrometry for organic analysis. Anal Chem 48:1885–1890
21. Kommission EU (2002) 2002/657/EC. Off J Eur Communities L Legis 221:8–36
22. U.S. Department of Health and Human Services FDA, Center for Drug Evaluation and Research, Center of Veterinary Medicine (2001) Guidance for industry: bioanalytical method validation. The United States Pharmacopeia Convention, Rockville, MD