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A validated liquid chromatography–tandem mass spectrometry method for the quantitative determination of 4β-hydroxycholesterol in human plasma

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ABSTRACT

A novel liquid chromatography–tandem mass spectrometry method is described for the quantitative determination of the endogenous CYP 3A4/5 marker 4β-hydroxycholesterol in human K_2-EDTA plasma. It is based on alkaline hydrolysis to convert esterified to free 4β-hydroxycholesterol, followed by analyte extraction from plasma by hexane and purification of the hexane extract by normal-phase solid-phase extraction. The analyte is chromatographically separated from endogenous isobaric plasma oxysterols and excess cholesterol by a 16-min reversed-phase gradient on a C18 column; detection is performed by atmospheric pressure photoionization tandem mass spectrometry in the positive ion mode, using toluene as a dopant.

Using 400 μl of plasma, 4β-hydroxycholesterol can be quantified in the concentration range 10.0–250 nM. Validation results show that the method is sufficiently selective towards endogenous plasma sterols and capable of quantifying the analyte with good precision and accuracy. The analyte is sufficiently stable in all relevant matrices and solvents; the addition of the anti-oxidant butylated hydroxytoluene to prevent *in vitro* formation of 4β-hydroxycholesterol from cholesterol during storage or analysis is not necessary, provided that long-term frozen storage of plasma occurs at −70 °C.

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1. Introduction

Biological samples contain a wide variety of cholesterol oxidation products, or oxysterols, most of which are formed from cholesterol, either by enzymatic action or as a result of autoxidation [1,2]. One of the most abundant oxysterols in the human circulation is 4β-hydroxycholesterol (Fig. 1A). Identified in human plasma in the mid 1990s as part of the LDL and HDL fractions [3], this compound has been the subject of increasing interest. It was shown to be formed solely by the drug-metabolizing enzyme cytochrome P450 (CYP) 3A4/5 [4] and highly elevated concentrations were found in patients treated with drugs known to induce the activity of this enzyme [5–8]. Despite some recent criticism [9], there is increasing evidence that 4β-hydroxycholesterol is a useful endogenous marker of CYP 3A4/5 activity, since a good correlation was found between 4β-hydroxycholesterol plasma levels and accepted markers for CYP 3A4/5 activity [10–12]. For this reason, the assessment of CYP 3A4/5 activity by measurement of 4β-hydroxycholesterol plasma levels has become increasingly popular in drug development programmes, as it does not require the administration of exogenous compounds to the participating subjects.

Over the years, a variety of liquid chromatographic (LC) and gas chromatographic (GC) methods has been applied for the determination of oxysterols in biological samples [13], but GC with mass spectrometric detection (GC–MS) has been most commonly used, generally in combination with deuterium-labelled internal standards [14,15]. Most of the studies with 4β-hydroxycholesterol have been performed using this approach: the analyte as well as other oxysterols are extracted from the sample and typically converted into their trimethylsilyl derivatives, which are amenable to further GC–MS analysis [3]. The high resolving power of GC and the good sensitivity and selectivity of MS detection have been important drivers for the dominance of this methodology, but the long (>25 min) run times and need for an overnight derivatization step are disadvantages for the high-throughput determination of 4β-hydroxycholesterol, which is typically required for the support of clinical trials.

In the past decade, LC with tandem mass spectrometric detection (LC–MS/MS) has replaced GC–MS for many bioanalytical applications, because of its better compatibility with non-volatile

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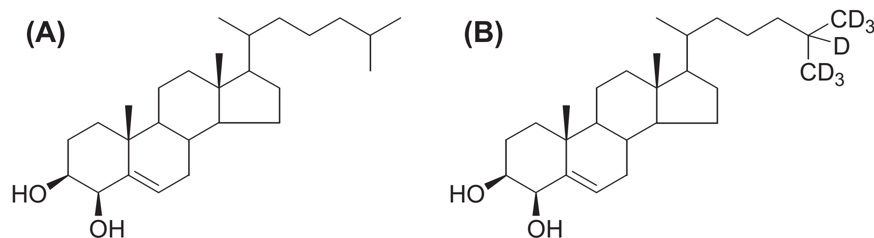

Fig. 1. Molecular structures of 4β-hydroxycholesterol (A) and 4β-hydroxycholesterol-d_7, the internal standard (B).

analytes, higher speed and better robustness. LC–MS/MS with electrospray ionization (ESI) has been described for the determination of underivatized sterols, among which 4β-hydroxycholesterol, in cell cultures and brain tissue [16], but because of the absence of acidic and basic functional groups, it is relatively difficult to ionize oxysterols in this way and for sufficient sensitivity a relatively unselective mass transition (loss of water) has to be selected. Other approaches are, therefore, generally preferred. The only LC–MS/MS method described for 4β-hydroxycholesterol in plasma so far applies a derivatization into a picolinyl ester to enhance the ionization efficiency in the ESI process [17], but with this approach there is no clear gain in sample throughput compared to GC–MS, because of the required 1-h derivatization step and the unfavourable total run time of 40 min. The use of atmospheric pressure chemical ionization (APCI) is an alternative for the determination of underivatized oxysterols, as has been demonstrated for 24S- and 27-hydroxycholesterol [18], but probably the most suitable ionization technique is atmospheric pressure photoionization (APPI), which shows an efficient ionization, especially for relatively non-polar compounds such as steroids [19,20]. This technique has been used for the quantitation of 27-hydroxycholesterol in plasma and was reported to be more than two-fold more sensitive than APCI [21], being able to quantify concentrations down to 10 ng/ml in a 15-min chromatographic run.

In this paper, we describe a method for the rapid determination of underivatized 4β-hydroxycholesterol using APPI-LC–MS/MS. It addresses the challenges related to the determination of this compound: the presence of a variety of isobaric endogenous oxysterols, among which 4α-hydroxycholesterol, which might interfere with analyte quantification and the possible autoxidation of the excess of plasma cholesterol during storage and analysis, which might cause *in vitro* formation of the analyte and lead to the overestimation of 4β-hydroxycholesterol concentrations. Results of an extensive method validation and the application to clinical samples are shown as well.

2. Materials and methods

2.1. Chemicals

4β-Hydroxycholesterol was purchased from Steraloids (Newport, RI, USA) and 4β-hydroxycholesterol-d_7, the internal standard (Fig. 1B), from Avanti Polar Lipids (Alabaster, AL, USA). 4α-Hydroxycholesterol was obtained from Syncom (Groningen, The Netherlands), cholesterol, 5,6α- and 5,6β-epoxycholesterol from Sigma (St. Louis, MO, USA), 7α-, 7β-, and 25-hydroxcholesterol from Steraloids, 24(S)-hydroxycholesterol from Avanti Polar Lipids and 27-hydroxycholesterol from Medical Isotopes (Pelham, NH, USA). Acetonitrile, methanol, ethanol, hexane, ethyl acetate, 2-propanol and toluene were obtained from Merck (Darmstadt, Germany) and sodium methoxide and butylated hydroxytoluene from Sigma. HPLC grade water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Human plasma and whole blood on K_2-EDTA were provided by PRA International (Zuidlaren, The Netherlands).

2.2. Standard solutions, calibration and validation samples

Stock solutions were prepared in 2-propanol at 500 μM for both 4β-hydroxycholesterol and internal standard. Standard dilutions were prepared at 50.0 and 5.00 μM in 2-propanol for 4β-hydroxycholesterol. An internal standard working solution was prepared in 2-propanol at 1000 nM. All solutions were stored in glass tubes at +4 °C.

Calibration samples were prepared at 10.0, 20.0, 40.0, 80.0, 120, 160, 200 and 250 nM in 2-propanol (corresponding to a range of ca 4–100 ng/ml and 0.8–20 pmol injected on-column). For method validation, spiked and unspiked human plasma samples were used as well as spiked 2-propanol samples; plasma was spiked with small volumes of standard dilutions in 2-propanol, not exceeding 5% of the total plasma volume. For determination of precision and accuracy, a validation sample at the lower limit of quantitation (LLOQ) was prepared in 2-propanol. Validation samples in plasma were prepared at four concentrations: unspiked plasma was used at a relatively low endogenous concentration and this batch of plasma was also spiked with an additional 50.0 nM, 150 nM or 350 nM 4β-hydroxycholesterol to obtain medium, high and over-curve concentrations, respectively. To test the effect of matrix variability, six individual plasma batches were analysed unspiked and after spiking with an additional 50.0 nM.

For stability assessment, one unspiked human whole blood sample, two unspiked human plasma samples (at a relatively low and a relatively high endogenous concentration) and two spiked 2-propanol samples (at 30.0 and 200 nM) were used. Calibration and validation samples were prepared from separate stock solutions and all samples were stored in polypropylene tubes at −70 °C.

2.3. Sample preparation

An aliquot of 400 μl of well-homogenized plasma was mixed with 50 μl of internal standard working solution and 800 μl of sodium methoxide solution (2 M in ethanol, prepared freshly every day) and the mixture was left at ambient temperature for 20 min to convert esterified to free 4β-hydroxycholesterol. Subsequently, 1 ml of water and 2 ml of hexane were added and the sample was extracted for 1 min at ambient temperature. After centrifugation at 2500 × *g* for 5 min at 20 °C, the aqueous layer was frozen in a mixture of acetone and dry-ice and the upper organic layer was transferred completely to an Isolute diol (100 mg, 1 ml) solid-phase extraction (SPE) cartridge obtained from Sopachem (Eke, Belgium), which had been conditioned with 1 ml of hexane. The cartridge was washed with 1 ml of hexane and eluted with two 750-μl aliquots of a mixture of ethyl acetate and hexane (80:20, v/v). The eluate was evaporated to dryness under nitrogen at 40 °C and redissolved in 100 μl of methanol. Calibration samples in 2-propanol (400 μl) were mixed with 50 μl of internal standard working solution and evaporated and redissolved in the same way. The extracts were stored at 10 °C in the autosampler until injection.

2.4. Separation and detection

The chromatographic system consisted of a Shimadzu (Kyoto, Japan) LC-10 ADvp high-pressure pump, a HTC PAL (CTC Analytics, Zwingen, Switzerland) injector, a Shimadzu Shim-pack ODS column (100 mm × 3 mm, 2.2-μm particle size), conditioned at 50 °C in a Shimadzu CTO10ACvp column heater, and an AB Sciex (Foster City, CA, USA) API 3000 mass spectrometer, equipped with an APPI probe. Aliquots of 20 μl of the sample extracts were injected. The mobile phase, a mixture of water (solvent A) and acetonitrile (solvent B) was delivered at 1.0 ml/min and completely directed to the mass spectrometer. A gradient was applied, starting at 20% A and 80% B, changing linearly to 10% A and 90% B at 8.0 min after injection. This composition was maintained until 9.0 min, after which it was changed to 100% B. At 14.0 min, the mobile phase was changed back to the original composition of 20% A and 80% B until 15.0 min after injection. Between 11.0 and 13.5 min, the LC flow was diverted to waste to prevent excess cholesterol from entering the mass spectrometer.

Quantitation was achieved by MS/MS detection in the positive ion mode. Toluene was used as the dopant in the APPI source at 60 μl/min; a source temperature of 480 °C and an ionspray voltage of 1500 V were applied. Detection of the ions was performed by multiple reaction monitoring (MRM) of the transitions m/z 385.4–109.1 for 4β-hydroxycholesterol and m/z 392.5–109.1 for the internal standard.

2.5. Validation

Method validation was based on the general principles laid down in the FDA guidance for industry [22]; specific issues for the validation of methods for endogenous compounds were addressed as described in [23]. A linear model with weighting factor $1/x$ was used to describe the relation between analyte concentration and instrument response (peak area ratio of analyte over internal standard). The precision and accuracy of the method were assessed at five concentrations (one in 2-propanol and four in plasma) by analysis of the validation samples in six-fold in three analytical runs. For the plasma sample spiked at the concentration outside the calibration range, half of the sample volume (i.e. 200 μl) was used for analysis. As a measure for the precision, within-run, between-run and total CV were calculated by analysis of variance. As a measure for the accuracy, the (overall) bias for spiked samples was determined by calculating the difference between the mean concentration of the spiked samples and the mean concentration of the corresponding unspiked samples and comparing this difference to the theoretical value. The effect of matrix variability was investigated by analysis of six individual plasma samples before and after addition of 50.0 nM 4β-hydroxycholesterol.

The extraction recovery was evaluated for the internal standard as surrogate analyte at three plasma concentrations (30.0, 100 and 200 nM) by comparing the peak areas obtained after regular plasma analysis to the peak areas, obtained from samples, spiked after extraction. The stability of the analyte was evaluated under various conditions by analysis in triplicate by comparison of the mean concentrations found after storage to those found before storage. In plasma, freeze/thaw stability, stability at ambient temperature and stability at −70 °C were determined (the latter both in absence and in presence of 50 μg/ml butylated hydroxytoluene, BHT). In calibration solvent (2-propanol) the stability at ambient temperature and at 4 °C were determined, in whole blood the stability after storage at ambient temperature and at 0 °C (measured by analysis of plasma, prepared from whole blood after the stability experiments) and in processed plasma and processed calibration solvent the stability at 10 °C. Analyte stability in stock solution was assessed after storage at ambient temperature and at 4 °C, by comparing peak areas found after injection of the stored stock solutions to the peak areas found for freshly prepared stock solutions.

3. Results and discussion

3.1. Sample preparation

For endogenous compounds, there are several ways to prepare calibrators in an analyte-free matrix [23]. Charcoal stripping was no option, since this hardly removed 4β-hydroxycholesterol from plasma, probably because of its presence in lipoproteins. Due to the limited solubility in water even in the presence of proteins, the use of a synthetic aqueous matrix was not considered. Instead, calibrators were prepared in 2-propanol, which is a usual approach for steroids.

Just as in the case of many other oxysterols, part of the 4β-hydroxycholesterol in plasma occurs in the form of long-chain fatty acid esters [13]. For the determination of the total concentration, esterified 4β-hydroxycholesterol has, therefore, to be converted to the free form prior to extraction. Alkaline hydrolysis using sodium methoxide in ethanol was used rather than the more usual approach with ethanolic potassium hydroxide, because of its speed and simplicity. As judged from the maximum analyte responses that were obtained and which did not increase upon further storage, hydrolysis with sodium methoxide was found to be complete after just 20 min at ambient temperature, while typical procedures with potassium hydroxide may take as long as several hours and require heating [4,17].

A simple extraction of the analyte from plasma (400 μl) could be achieved with hexane (2000 μl). The addition of water (1000 μl) was necessary to effect a good phase separation between the aqueous and organic layers after the extraction and properly remove plasma from the hexane phase. An additional clean-up step of the hexane extract by normal-phase SPE was found to improve method selectivity and robustness. Comparison of chromatograms obtained with and without SPE showed that about 80% of co-extracted cholesterol was removed in this way, as well as some minor unidentified oxysterols, which eluted closely to the analyte peak. Without the additional SPE step, the extracts of more lipidaemic plasma samples were visually turbid and a steady increase in column back-pressure was found upon injection of larger series of samples, indicating the build-up of plasma components on the LC column. With the inclusion of the additional SPE step, clear extracts were obtained and an excessive increase in column back-pressure could be prevented.

The recovery of the two-step extraction procedure was not complete, ranging between 45 and 69%, as judged from the results for the internal standard. Still, it was similar for the analyte and the internal standard, as the peak area ratio of analyte and internal standard after extraction of plasma (after correction for endogenous 4β-hydroxycholesterol) was identical to the ratio in unextracted calibration samples. This illustrates the crucial importance of the availability of an isotopically labelled internal standard to normalize the responses and correct for incomplete extraction recovery.

The *in vitro* formation of oxysterols during storage and/or analysis, by autoxidation of the large (typically 50,000-fold) excess of plasma cholesterol, is an important phenomenon to consider. To minimize this effect, blood is usually collected in EDTA tubes, as this anti-coagulant complexates metal ions, which are thought to catalyse cholesterol autoxidation. An anti-oxidant such as butylated hydroxytoluene (BHT) may be added as an extra precaution [13]. In our study, blood and plasma on EDTA were selected *a priori* and other anti-coagulants were not further investigated. The addition of 50 μg BHT per ml of sample prior to analysis did not significantly change the 4β-hydroxycholesterol plasma concen-

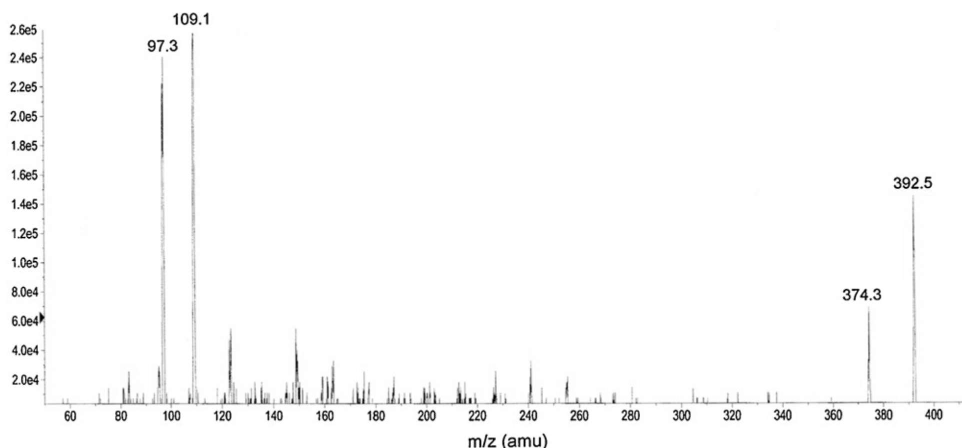

Fig. 2. MS/MS spectrum for 4β-hydroxycholesterol-d_7, precursor ion *m*/*z* 392.5.

trations found, whence it was concluded that no *in vitro* analyte formation occurs during the analytical procedure. The use of a short hydrolysis step at ambient temperature is a helpful method characteristic in this respect. The findings are in line with the general observation, that – compared to some other oxysterols – relatively little 4β-hydroxycholesterol is formed by cholesterol autoxidation [4].

3.2. Separation and detection

Both 4β-hydroxycholesterol and its seven-fold deuterated internal standard lost water in the APPI process, which is the usual case for underivatized oxysterols [24], and formed $[M\text{-}H_2O+H]^+$ precursor ions at *m*/*z* 385.4 and *m*/*z* 392.5, respectively. The precursor ions showed characteristic fragmentation patterns. Both lost an additional molecule of water, yielding product ions at *m*/*z* 367.2 and 374.3, respectively. The most intense product ion for both compounds was found at *m*/*z* 109.1 (Fig. 2). The exact structure of this ion is difficult to elucidate because of the complicated fragmentation mechanism of sterols, but it possibly is a protonated form of the sterol A ring, after loss of water and dehydrogenation. In any case, with MRM transitions of the $[M\text{-}H_2O+H]^+$ precursor ions to the *m*/*z* 109.1 product ions and by using toluene as a dopant in the APPI process at 60 μl/min, sufficiently high responses were obtained to determine 4β-hydroxycholesterol concentrations down to 10 nM.

Human plasma contains a variety of oxysterols. As many of these have the same molecular mass as 4β-hydroxycholesterol, chromatographic separation of interfering oxysterols from the analyte or the selection of a unique analyte product ion is required to obtain a sufficiently selective analytical method. In addition, separation of 4β-hydroxycholesterol from the large amount of cholesterol is important to prevent excessive suppression of analyte ionization. Analysis of standard solutions of cholesterol and eight of the most abundant plasma oxysterols (4α-, 7α-, 7β-, 24(S)-, 25- and 27-hydroxycholesterol and 5,6α- and 5,6β-epoxycholesterol) revealed that they all show responses at the selected MRM transition of 4β-hydroxycholesterol. For the isobaric oxysterols, this is understandable. For cholesterol, which contains one oxygen atom less, it could mean that oxidation takes place under the conditions in the ion source, resulting in one or more species with the same *m*/*z* value as 4β-hydroxycholesterol itself, which subsequently follow the same ionization and fragmentation pattern. An alternative explanation could be that cholesterol is dehydrogenated in the ion source, thus forming an ion isobaric to the oxysterols after loss of water.

Because of the insufficient mass spectrometric selectivity, proper chromatographic resolution was necessary to separate 4β-hydroxycholesterol from interfering plasma sterols. The use of a column with a high plate number was essential for the combination of sufficient resolution and a reasonably short analytical run time. As is shown in Fig. 3, a column with 10 cm length and 2.2 μm particle size provided sufficient separation of 4β-hydroxycholesterol from cholesterol and the most abundant plasma oxysterols within a 16-min run. It appeared advantageous for method robustness to divert the LC flow to waste rather than to the mass spectrometer between 11 and 13.5 min. In this time window, cholesterol elutes off the column and its very high concentration caused contamination of the ion source, leading to a gradual increase of the background signal.

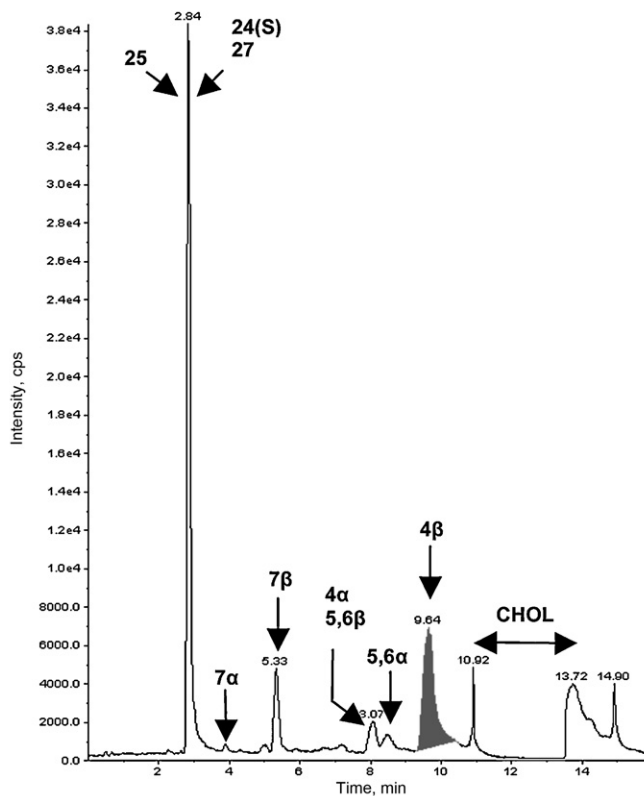

Fig. 3. LC–MS/MS chromatogram of fresh, unspiked human plasma, indicating the presence of cholesterol (CHOL, diverted to waste) and 24(S)-, 25-, 27-, 7α-, 7β-, 4α- and 4β-hydroxycholesterol and 5,6α- and 5,6β-epoxycholesterol.

Table 1
Summary of precision and accuracy results.

Matrix	Nominal concentration (nM)	Calculated concentration (nM)	Bias (%)	Within-run CV (%)	Between-run CV (%)	Total CV (%)	*n*
2-Propanol	10.0	10.5	+5.3	4.8	7.6	9.0	18
Plasma	Unknown	37.0	n.a.[a]	2.5	1.0	2.7	18
Plasma	87.0	82.8	−4.8	1.7	2.0	2.6	18
Plasma	187	173	−7.5	1.9	3.6	4.0	18
Plasma	387[b]	344	−11.0	3.7	4.2	5.6	18

[a] Not applicable, endogenous level.
[b] Partial volume (200 μl) analysis.

3.3. Validation

For all calibration curves recorded during method validation, the back-calculated concentrations of the calibrators were within 10% of the theoretical value with a CV of less than 5%, which indicates the suitability of the linear calibration model with weighting factor 1/*x*. Table 1 summarizes the accuracy and precision results, as obtained for the repeatedly ($n = 18$) analysed validation samples. Results for accuracy, expressed as the average bias from the theoretical value, were between −11.0% and +5.3% and method precision, expressed as within-run, between-run and total CV, was below 10% in all cases, which is well within the acceptance limits [22].

Upon spiking an additional 50.0 nM 4β-hydroxycholesterol to six different lots of plasma, the bias from the theoretical spiked concentration was below 11%, in all cases, which illustrates that variability in the matrix does not negatively affect method performance.

Under normal analytical and storage conditions, the analyte is sufficiently stable in the relevant matrices and solvents, as the bias of the concentrations found after storage compared to those in the corresponding unstored samples was well within 10% for all conditions tested (Table 2). In plasma, stability was demonstrated over three freeze/thaw cycles (between −70 °C and ambient temperature), for up to 24 h at ambient temperature and for up to 343 days at −70 °C (without BHT added). In processed plasma at 10 °C, the storage conditions in the autosampler, stability was sufficient for up to 98 h. In whole blood, no analyte degradation was observed for storage up to 4 h at ambient temperature and at 0 °C. Stability in the calibration solvent (2-propanol) was sufficient at ambient temperature for 24 h and at 4 °C for up to 11 days; in processed calibration solvent at 10 °C, stability was demonstrated for up to 98 h. In stock solution (2-propanol), the stability was sufficient both at ambient temperature for 24 h and at 4 °C for 183 days.

The effect of the presence of BHT in plasma on the stability of 4β-hydroxycholesterol during prolonged storage at −70 °C is shown in Fig. 4. After frozen storage for 61, 159, 265, 291 and 343 days without BHT and for 57, 155, 261, 287 and 339 days with 50 μg/ml BHT, the deviation from the $t = 0$ value was typically <15% in all cases. This illustrates that no *in vitro* formation of 4β-hydroxycholesterol occurs at −70 °C and that no addition of BHT is needed if plasma is stored at this temperature. However, as is shown in Fig. 5, the abundance of plasma oxysterols, including 4β-hydroxycholesterol itself, increases considerably when plasma is stored at −20 °C for (much) longer periods of time. Apparently, more *in vitro* autoxidation of cholesterol takes place under these storage conditions, which could be due to the higher storage temperature, but also to the fact that the −20 °C (walk-in) freezer used is continuously illuminated, while cholesterol oxidation is known to be sensitive to photoirradiation [25]. This phenomenon was not further investigated in the present study, as storage at −70 °C and in the dark was judged to be a workable alternative. Should storage at −20 °C be necessary, additional research is required to define optimal storage conditions.

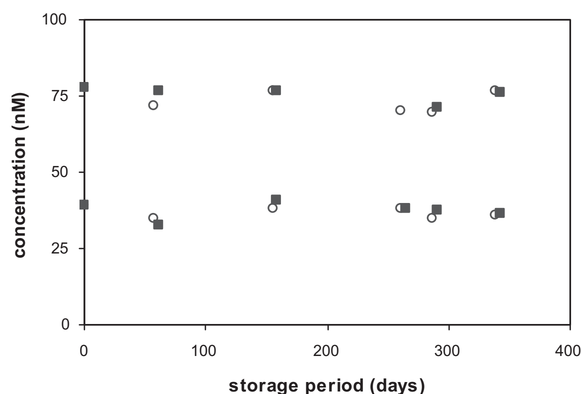

Fig. 4. Low and high endogenous 4β-hydroxycholesterol concentrations in human plasma after storage at −70 °C with (○) or without (■) butylated hydroxytoluene (50 μg/ml) added.

3.4. Application to clinical samples

To date, more than 1000 plasma samples from six different clinical trials have been analysed with the described method. The average accuracy (expressed as % bias from the nominal concentration) and precision (expressed as % CV), found for quality control samples, typically is in the order of 10% or below. A sample throughput of about 80 per day (ca 60 study samples plus calibrators and quality control samples) is routinely achieved. As an illustration,

Table 2
Summary of stability results.

Matrix	Analyte concentration (nM)	Temperature	Period	Mean deviation from $t = 0$ (%)
Plasma	37.0/76.4	−70 °C	343 days	−6.1/−2.0
	37.0/76.4	Ambient	24 h	−1.4/−0.3
	41.8	Freeze/thaw	3 cycles	+0.9
Processed plasma	37.0/76.4	10 °C	98 h	+0.4/+1.3
Whole blood	42.8	0 °C	4 h	−1.8
	42.8	Ambient	4 h	−1.0
Stock solvent	500 μM	4 °C	183 days	+2.1
	500 μM	Ambient	24 h	−1.5
Calibration solvent	30.0/200	4 °C	11 days	+3.8/−1.0
	30.0/200	Ambient	24 h	−2.4/+0.1
Processed calibration solvent	30.0/200	10 °C	98 h	+0.4/−1.0

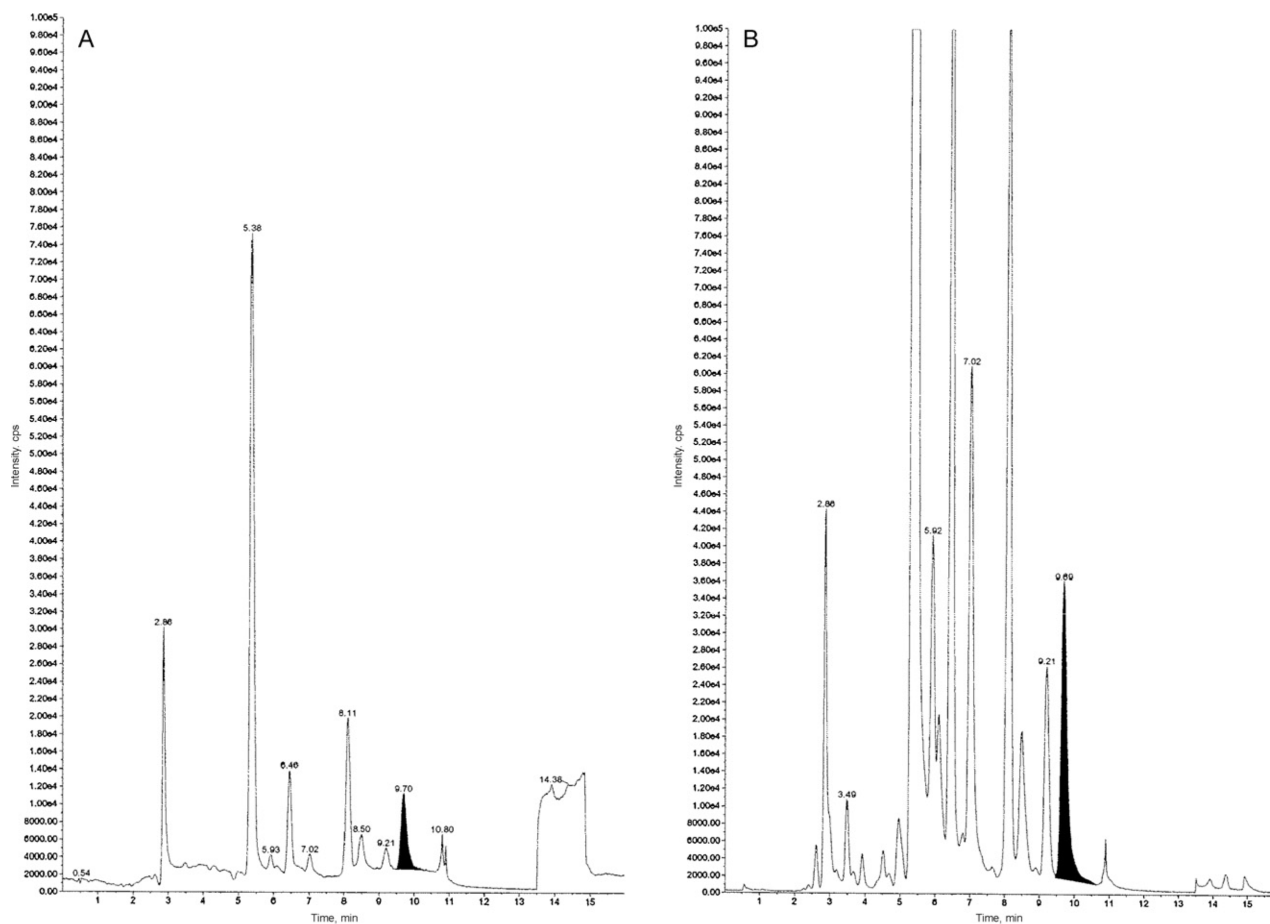

Fig. 5. LC–MS/MS chromatograms of two unspiked human plasma samples, stored at −20 °C in an illuminated freezer for 146 days, concentration 47.4 nM (A) or for 1874 days, concentration 206 nM (B).

Table 3
4β-Hydroxycholesterol concentrations in plasma from 129 untreated volunteers.

Mean (nM)	SD (nM)	Minimum (nM)	Maximum (nM)
55.9	23.7	20.2	148

the concentrations of 4β-hydroxycholesterol determined in plasma samples from 129 healthy, untreated volunteers, are summarized in Table 3. Published plasma 4β-hydroxycholesterol concentrations, obtained with GC–MS, vary somewhat from report to report, but typically range between 20 and 50 ng/ml (ca 50–125 nM) for untreated, adult subjects [3,4,7,10], which is comparable to our findings. The reproducibility of the described method for incurred study samples was tested by reanalysis of a selection of ca 10% of the plasma samples ($n = 14$). The deviation of the repeat results from the original ones was +3.2% on average and varied from −7.8% to +10.0%, which proves that the described methodology yields reproducible results.

4. Conclusions

The APPI-LC–MS/MS method described in this paper allows the quantification of 4β-hydroxycholesterol in human plasma at the relevant physiological levels (10.0–250 nM); if necessary, higher concentrations can be determined by partial volume analysis. Because of the good sensitivity and selectivity of the method, the favourable accuracy and precision, the good stability of the analyte and the relatively short analysis time, the method is very suitable for the support of clinical studies with 4β-hydroxycholesterol. The method has demonstrated its value in the analysis of over 1000 samples. Compared to previously described methods, it has a higher sample throughput and is more rigorously validated. In addition, it is more selective than direct ESI-LC–MS/MS and, compared to GC–MS and other ESI-LC–MS/MS methods, it avoids a critical derivatization step.

Ethical aspects of the research

The authors state that appropriate institutional review board approval and written informed consent has been obtained for the investigations involving human subjects.

References

[1] G.J. Schroepfer, Physiol. Rev. 80 (2000) 361–554.
[2] I. Björkhem, J. Clin. Invest. 110 (2002) 725–730.
[3] O. Breuer, J. Lipid Res. 36 (1995) 2275–2281.
[4] K. Bodin, L. Bretillon, Y. Aden, L. Bertilsson, U. Broomé, C. Einarsson, U. Diczfalusy, J. Biol. Chem. 276 (2001) 38685–38689.
[5] K. Wide, H. Larsson, L. Bertilsson, U. Diczfalusy, Br. J. Clin. Pharmacol. 65 (2008) 708–715.
[6] M. Niemi, K.T. Kivistö, U. Diczfalusy, K. Bodin, L. Bertilsson, M.F. Fromm, M. Eichelbaum, Pharmacogenet. Genomics 16 (2006) 565–568.
[7] U. Diczfalusy, K.P. Kanebratt, E. Bredberg, T.B. Andersson, Y. Böttiger, L. Bertilsson, Br. J. Clin. Pharmacol. 67 (2008) 38–43.
[8] F. Josephson, L. Bertilsson, Y. Böttiger, L. Flamholc, M. Gisslén, V. Ormaasen, A. Sönnerborg, U. Diczfalusy, Eur. J. Clin. Pharmacol. 64 (2008) 775–781.
[9] D. Tomalik-Scharte, D. Lütjohann, O. Doroshyenko, D. Frank, A. Jetter, U. Fuhr, Clin. Pharmacol. Ther. 86 (2009) 147–153.
[10] K.P. Kanebratt, U. Diczfalusy, T. Bäckström, E. Sparve, E. Bredberg, Y. Böttiger, T.B. Andersson, L. Bertilsson, Clin. Pharmacol. Ther. 84 (2008) 589–594.
[11] E. Gebeyehu, E. Engidawork, A. Bijnsdorp, A. Aminy, U. Diczfalusy, E. Aklillu, Pharmacogen. J. 11 (2011) 130–137.

[12] Z. Yang, A.D. Rodrigues, J. Clin. Pharmacol. 50 (2010) 1330–1338.
[13] U. Diczfalusy, J. AOAC Int. 87 (2004) 467–473.
[14] O. Breuer, I. Björkhem, Steroids 55 (1990) 185–192.
[15] S. Dzeletovic, O. Breuer, E. Lund, U. Diczfalusy, Anal. Biochem. 225 (1995) 73–80.
[16] J.G. McDonald, B.M. Thompson, E.C. McCrum, D.W. Russell, Meth. Enzymol. 432 (2007) 145–170.
[17] A. Honda, K. Yamashita, T. Hara, T. Ikegami, T. Miyazaki, M. Shirai, G. Xu, M. Numazawa, Y. Matsuzaki, J. Lipid Res. 50 (2009) 350–357.
[18] I. Burkard, K.M. Rentsch, A. von Eckardstein, J. Lipid Res. 45 (2004) 776–781.
[19] K.A. Hanold, S.M. Fischer, P.H. Cormia, C.E. Miller, J.A. Syage, Anal. Chem. 76 (2004) 2842–2851.
[20] Y. Cai, D. Kingery, O. McConnell, A.C. Bach II., Rapid Commun. Mass Spectrom. 19 (2005) 1717–1724.
[21] R. Karuna, A. von Eckardstein, K.M. Rentsch, J. Chromatogr. B 877 (2009) 261–268.
[22] US Food and Drug Administration, Guidance for Industry, Bioanalytical Method Validation, May, 2001.
[23] N.C. van de Merbel, Trends Anal. Chem. 27 (2008) 924–933.
[24] B. Rossmann, K. Thurner, W. Luf, Monatsh. Chem 138 (2007) 437–444.
[25] F. Guardiola, S. Garcia-Cruset, R. Bou, R. Codony, J. AOAC Int. 87 (2004) 493–498.