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Highly sensitive and specific analysis of sterol profiles in biological samples by HPLC–ESI–MS/MS☆

Akira Honda[a,b], Teruo Miyazaki[a,b], Tadashi Ikegami[c], Junichi Iwamoto[c], Kouwa Yamashita[d], Mitsuteru Numazawa[d], Yasushi Matsuzaki[b,c,*]

[a] Center for Collaborative Research, Tokyo Medical University Ibaraki Medical Center, Ami, Ibaraki 300-0395, Japan
[b] Department of Development for Community Medicine, Tokyo Medical University Ibaraki Medical Center, Ami, Ibaraki 300-0395, Japan
[c] Department of Gastroenterology, Tokyo Medical University Ibaraki Medical Center, Ami, Ibaraki 300-0395, Japan
[d] Faculty of Pharmaceutical Science, Tohoku Pharmaceutical University, Sendai, Miyagi 981-8558, Japan

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ABSTRACT

High-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) is a powerful method for the microanalysis of compounds in biological samples. Compared with gas chromatography–mass spectrometry (GC–MS), this method is more broadly applicable to various compounds and usually does not require a derivatization step before analysis. However, when neutral sterols are analyzed, the sensitivities of usual HPLC–MS/MS method are not superior to those of GC–MS because the sterols are relatively resistant to ionization. In this review, we introduce the recent development of HPLC–MS/MS analysis for the quantification of non-cholesterol sterols. By adding an effective derivatization step to the conventional procedure, sterol analysis by HPLC–MS/MS surpassed that obtained by GC–MS in sensitivity. In addition, sufficient specificity of this method was achieved by selected reaction monitoring (SRM) and thorough chromatographic separation of each sterol.

1. Introduction

Some cholesterol precursors and oxidized cholesterol (oxysterols) are important molecules in the regulation of lipid homeostasis in the body [1]. In addition, they have been used as serum biomarkers for whole body cholesterol synthesis [2,3], intestinal cholesterol absorption [4], hepatic bile acid synthesis [5,6] and the diagnosis of inherited disorders in cholesterol metabolism [7–12]. Therefore, quantification of non-cholesterol sterols in biological samples is a very important technique in studies of lipid metabolism.

Gas chromatography (GC) with flame ionization detection [2,13], high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [14–16] or HPLC with refractive index (RI) detection [16] are the most generally used methods for the analyses of sterols. However, these methods cannot quantify minor components of endogenous sterols with sufficient sensitivity and specificity.

Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are powerful detection methods, which are suitable for GC and HPLC systems. These detectors are not only superior in terms of sensitivity but are also highly specific compared with flame ionization, UV and RI detectors. GC–MS has been widely accepted as a reliable analytical method for the determination of sterols in biological samples [17–19]. However, during the last decade, HPLC–MS or HPLC–MS/MS has also come to be used conveniently because these methods do not always require deconjugation and derivatization steps before analysis [20,21]. In addition, while HPLC methods do not cause decomposition of some labile sterols, such as 24S,25-epoxycholesterol, the high temperatures achieved during GC methods can cause degradation of unstable sterols [22,23].

In this review, we introduce the recent development of HPLC–MS/MS methods for the quantification of sterols in biological samples. An effective derivatization step, thorough chromatographic separation and selected reaction monitoring (SRM) by MS/MS have achieved excellent sensitivity and specificity for this method. The method has become a central approach for the simultaneous quantification of sterols in small amounts of biological samples.

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* Corresponding author at: Department of Gastroenterology, Tokyo Medical University Ibaraki Medical Center, 3-20-1 Chuoh, Ami, Inashiki, Ibaraki 300-0395, Japan. Tel.: +81 29 887 1161; fax: +81 29 887 9113.
E-mail address: ymatsuzaki-gi@umin.ac.jp (Y. Matsuzaki).

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2. Methods to increase the sensitivity of sterols

2.1. Ionization

Advances in ionization techniques have greatly contributed to the development of LC–MS. Electron impact ionization (EI) is the most commonly used approach for GC–MS analysis of sterols. This ionization method was applied to HPLC–MS by using a particle beam (PB) interface. In 1995 Sattler et al. [24] analyzed plasma 7-dehydrocholesterol and in 1998 Careri et al. [25] quantified oxysterols by HPLC–PB-EI–MS, with detection limits of 10 ng (about 26 pmol) and 2–3 ng (about 5–7.5 pmol), respectively.

While EI is not applicable to polar or high molecular weight compounds, electrospray ionization (ESI) is broadly applicable method for polar compounds in a wide range of molecular weights (Fig. 1). In addition, this ionization source is generally exchangeable in the same mass spectrometer with atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) sources that are applicable to less polar and low molecular weight compounds. Thus, ESI and the complementary use of APCI or APPI have recently become the standard ionization methods for HPLC–MS.

Since sterols are less polar and relatively low molecular weight compounds, APCI [26–33] or APPI [20,34] have been preferentially used for analysis by HPLC–MS. The detection limits of cholesterol precursors and sitosterol by HPLC-APCI–MS were well below 1 pmol [31], that of cholesterol by HPLC-APCI–MS/MS was 2.2 pmol [32], and those of oxysterols by HPLC-APCI–MS were in the range of 0.2–0.8 ng (about 0.5–2.0 pmol) [26] or 0.1–0.75 ng (about 0.25–1.9 pmol) [27].

While it had previously been considered that ESI was not suitable for the analysis of neutral sterols, in 2007 McDonald et al. reported that sterols were sufficiently ionized when HPLC–ESI–MS/MS was employed using the Applied Biosystems 4000 QTrap triple quadrupole system [21]. According to this report, detection limits of dihydroxy- or epoxysterols were 5–60 fmol while those of monohydroxysterols were 175–2000 fmol on-column. These sensitivities are not inferior to those of APCI, but one weak point is that the sensitivity depends greatly on the instruments.

2.2. Derivatization

As shown in Table 1, conventional HPLC-APCI–MS for the detection of one of the representative oxysterols, 7α-hydroxycholesterol (1.2 pmol) [27], is not as sensitive as GC–MS (4–120 fmol) [35,36] because sterols are relatively resistant to ionization. On the other hand, this oxysterol may be quantified by HPLC–ESI–MS/MS [21] with sensitivity (60 fmol) equivalent to GC–MS, but not all HPLC–ESI–MS/MS instruments are applicable to this sensitive analysis of sterols.

To overcome these problems, sterols have been derivatized to more polar structures. The charged moieties were introduced into the hydroxyl group of the sterols as an *N*-methylpyridyl ether [37], a ferrocenecarbamate ester [38], a sulfate [39], a mono-(dimethylaminoethyl) succinyl ester [40], a dimethylglycine ester [41], and a picolinyl ester [42–44]. Furthermore, the native carbonyl group of oxysterols or the 3-oxo structure, converted from 3β-hydroxysterols by cholesterol oxidase, was derivatized to Girard P hydrazone [45–47]. Each of these derivatizations enhanced the ionization efficiency of the sterols in the ESI process and markedly increased the sensitivity.

As for ionization polarity, the sulfate derivatives are easily deprotonated and exhibit a high ionization efficiency in the negative ESI mode [39]. In contrast, the other derivatives are positively charged permanently or easily protonated, so that they are suitable for the positive ESI mode. Generally speaking, the negative mode exhibits lower background noise compared with that in positive mode. However, the positive mode provides much abundant ions than negative one [48].

It may be noted here that derivatizations are useful to increase the ionization of steroids, not only in ESI, but also in the APCI processes [49,50]. However, derivatized sterols have been preferably analyzed by ESI because ESI is broadly applicable to various deriva-

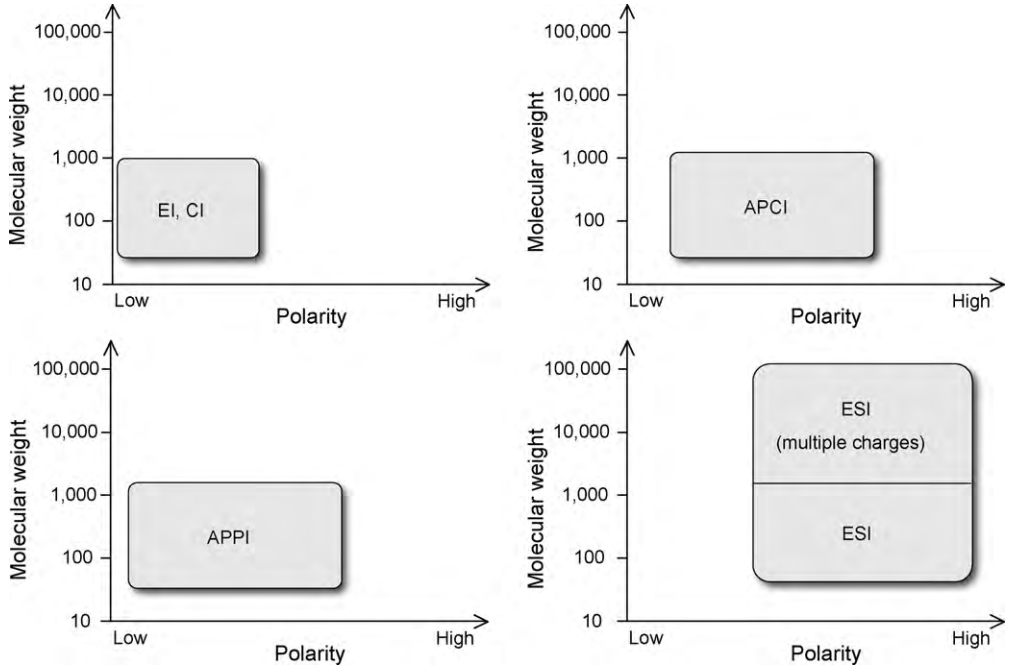

Fig. 1. Applications of various ionization methods to LC–MS. EI, electron impact ionization; CI, chemical ionization; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; ESI, electrospray ionization.

Table 1
Detection limits of cholesterol and representative oxysterols by different analytical methods.

Author	Year	Reference	Method (ionization mode)	Derivatization	Lower limit of detection	
					Cholesterol	Oxysterol[a]
Sanghvi et al.	1981	[36]	GC–MS (P-EI)	TMS ether	NA	120 fmol (7αOH)
Hylemon et al.	1989	[14]	HPLC-UV	C4	NA	20 pmol (7αOH)
Honda et al.	1991	[35]	GC–HR-MS (P-EI)	DMES ether	NA	4 fmol (7αOH)
Careri et al.	1998	[25]	HPLC–PB-MS (P-EI)	–[b]	5 pmol	5 pmol (7βOH)
Manini et al.	1998	[26]	HPLC–MS (P-APCI)	–	NA	500 fmol (7βOH)
Van Berkel et al.	1998	[38]	HPLC–MS/MS (P-ESI)	FC ester	41 amol	NA
Razzazi-Fazeli et al.	2000	[27]	HPLC–MS (P-APCI)	–	NA	1.2 pmol (7αOH)
Nagy et al.	2006	[31]	HPLC–MS (P-APCI)	–	<1 pmol	NA
Tian et al.	2006	[32]	HPLC–MS/MS (P-APCI)	–	2.2 pmol	NA
Griffiths et al.	2006	[46]	HPLC–MS/MS (P-ESI)	Girard P hydrazone	NA	<2.5 fmol
McDonald et al.	2007	[21]	HPLC–MS/MS (P-ESI)	–	1 pmol	60 fmol (7αOH)
Honda et al.	2008	[43]	HPLC–MS/MS (P-ESI)	picolinyl ester	260 amol	NA
Honda et al.	2009	[44]	HPLC–MS/MS (P-ESI)	picolinyl ester	NA	10 amol (7αOH)

Abbreviations: P-EI, positive electron impact ionization; TMS, trimethylsilyl; NA, not available; 7αOH, 7α-hydroxycholesterol; HPLC-UV, HPLC equipped with an ultraviolet detector; C4, 7α-hydroxy-4-cholesten-3-one; GC–HR-MS, high-resolution GC–MS; DMES, dimethylethylsilyl; HPLC–PB-MS, HPLC–MS with particle beam interface; 7βOH, 7β-hydroxycholesterol; P-APCI, positive atmospheric pressure chemical ionization; P-ESI, positive electrospray ionization; FC, ferrocenecarbamate.
[a] Data of 7α-hydroxycholesterol or other oxysterols with the structures similar to 7α-hydroxycholesterol.
[b] Without derivatization.

tives. In the positive APCI mode, the introduction of moieties with proton affinity increases ionization, while those with highly polar functional groups inhibit ionization and decrease the sensitivity [50]. Thus, the selection of effective derivatives for positive APCI is not as easy as that for ESI. Negative APCI is also used after the addition of electron affinity moieties to sterols. This electron-capturing derivatization in negative APCI mode was first reported by Singh et al. [51] and has been applied to the determination of tissue cholesterol by Kuo et al. [52].

3. Methods to increase the selectivity of each sterol

3.1. Use of appropriate internal standards

Deuterium-labeled sterols are ideal internal standards for quantification by HPLC–MS. The addition of internal standards compensates for the loss of target sterols during clean-up procedures and for the variation in injection volume onto the HPLC column. Thus, internal standards are necessary for accurate quantification by chromatographic methods. In addition, internal standards are used to determine the variation in the retention time of each sterol among samples. When peaks of target sterols are very small or they are not completely separated from interfering peaks, the retention time of the internal standard gives additional information to identify the target peaks. Several deuterated standards are commercially available, as reported by McDonald et al. [21]. Although deuterated analogs are not available for all sterols, deuterated sterol with a structure similar to the target sterol can be used as a surrogate [21,44]. Alternatively, we can use coprostanol as a convenient internal standard for monohydroxysterols in human serum [43]. Coprostanol is synthesized from cholesterol by intestinal bacteria but is not absorbed from the intestine and is not detected in human serum.

3.2. Sample clean-up

The structures of non-cholesterol sterols are similar to native cholesterol, which usually exists at least 100–10,000 times greater than the target sterols in bulk-lipid extracts from biological samples. Therefore, good separation from cholesterol is necessary for reliable quantification of the non-cholesterol sterols. Because oxysterols and epoxysterols are more polar than cholesterol, most of them can be separated from cholesterol by a solid-phase extraction cartridge [18,53]. However, the complete separation of some less polar oxysterols and non-cholesterol monohydroxysterols from cholesterol by using such a cartridge is difficult. Thus, for the analysis of whole sterol profiles in biological samples, the role of solid-phase extraction is limited to the elimination of nonpolar compounds, such as fatty acyl esters of cholesterol [53], that are strongly retained on reversed-phase HPLC columns.

3.3. Separation by HPLC

Since we have not achieved selective elimination of cholesterol from bulk-lipid extracts using solid-phase extraction cartridges, non-cholesterol sterols must be separated from cholesterol by the final HPLC–MS or HPLC–MS/MS analyses. In addition, the separation between non-cholesterol sterols is also important to quantify each sterol. However, isobaric sterol isomers, e.g. cholesterol and lathosterol [43] or 24S-hydroxycholesterol and 25-hydroxycholesterol [21,44], often exhibit similar precursor to product ion fragmentations, so that even SRM cannot always differentiate these sterols. Therefore, careful HPLC separation of each sterol is crucially important to quantify these isomers by selected ion monitoring (SIM) or SRM [33,43].

Although normal phase columns can be used for the separation of sterols by HPLC-PB–MS [24,25] and HPLC–APCI-MS [16], reversed-phase columns are preferably used in HPLC–PB-MS [25], most of the HPLC–APCI-MS [26–31,33], HPLC–APPI-MS [20,34], and virtually all HPLC–ESI–MS methods with [42–44,47] and without derivatization [21]. Normal phase HPLC sometimes achieves better separation of each sterol compared with reversed-phase HPLC [16], but the latter is preferred for HPLC–MS because it displays higher reproducibility than normal phase and the polar mobile phase favors ionization.

Our experiences show that there are many minor unidentified sterols in biological samples and complete chromatographic or mass spectrometric separation of all sterols by a single analysis is impossible at present. We need to select the best column and mobile phase according to the target sterols in which we are interested.

3.4. Selection by MS/MS

Although MS/MS is not an almighty method for the differentiation of each sterol, it is much more specific and sensitive than UV and RI detectors [16]. The triple quadrupole mass spectrometer is the most suitable instrument for the highly sensitive

quantification of sterols. SRM obtained by MS/MS can eliminate interfering peaks with different precursor to product ion fragmentations at specific collision energies. In addition, the monitoring of multiple SRM pairs for a single sterol adds confidence to the identification of the compound and provides further information regarding compound identification based on their relative intensities [21].

Another way to improve the selectivity of SRM is to increase the resolution of the triple quadrupole mass spectrometer. Although the resolution depends on the capacity of the mass spectrometer, analysis with higher resolution reduces interfering peaks and improves S/N ratio of the chromatogram. Furthermore, Griffiths et al. have reported high-resolution MS by a hybrid quadrupole/time of flight (TOF) mass spectrometer [46] or high-resolution MS^n by a hybrid linear ion-trap/Fourier transform mass spectrometer [54]. These mass spectrometers exhibit excellent selectivity, but sensitivity and dynamic range for quantification do not reach those achieved by the triple quadrupole mass spectrometer.

4. Characteristics of picolinyl ester derivative of sterols

4.1. Sensitivity

We have successfully introduced a picolinyl moiety into the hydroxyl group of various sterols and have demonstrated that the picolinyl ester derivatization is a simple and versatile method for sensitive and specific quantification using positive HPLC–ESI–MS/MS [42–44]. The idea originated from a report by Yamashita et al. [55] in which they compared HPLC–ESI–MS/MS behaviors among the picolinyl, 6-methylpicolinyl, nicotinyl, 2-methyoxynicotinyl and isonicotinyl derivatives of estrone, estradiol, dehydroepiandrosterone and testosterone. The picolinyl derivatives showed the best HPLC–ESI–MS/MS behavior and 100-fold higher detection response by SRM compared with underivatized steroid molecules [55,56]. In addition, they have successfully applied the picolinyl derivatization to corticosteroids [57,58] and aldosterone [59,60].

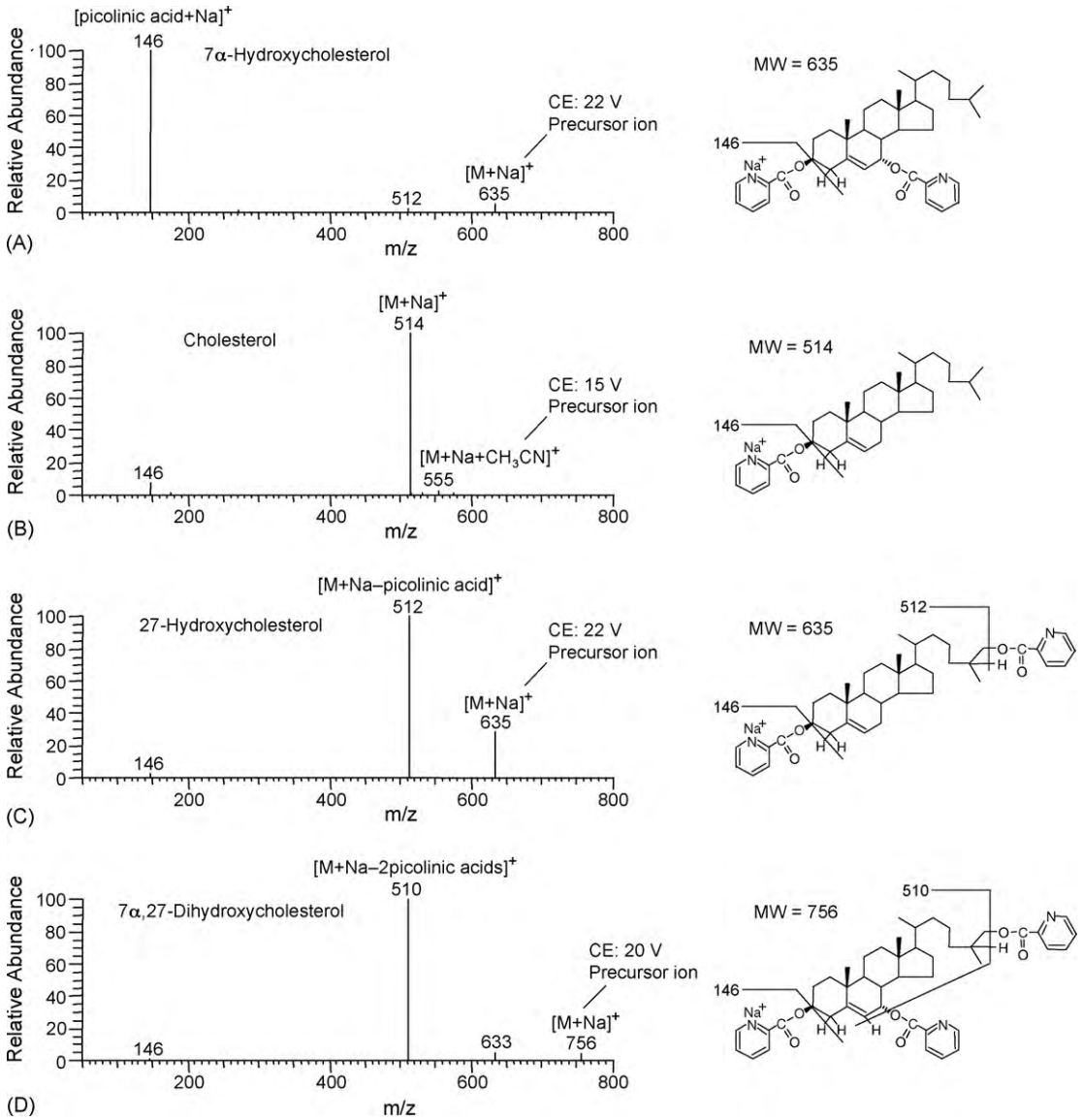

Fig. 2. Representative positive ESI–MS/MS fragmentation patterns of the picolinyl ester derivatives of sterols. (A) 7α-hydroxycholesterol, (B) cholesterol, (C) 27-hydroxycholesterol, (D) 7α,27-dihydroxycholesterol. $[M+Na]^+$ was used as precursor ions for A, C and D, while $[M+Na+CH_3CN]^+$ was used as a precursor ion for B. Fragmentation patterns of A, B, C and D correspond to those in Table 2. The general LC–MS/MS conditions were as follows: introducing solvent, acetonitrile–methanol–water (45:45:10, v/v/v) containing 0.1% acetic acid; flow rate, 300 μl/min; spray voltage, 1000 V. CE, collision energy. In the case of oxysterols with multiple hydroxyl groups (A, C and D), the position of sodium in the picolinyl derivatives has not been determined. In structural formulae, sodium ion was tentatively added to picolinyl group at the C-3β position.

As for sterols, the detection limits (S/N = 3) of cholesterol picolinate and oxysterol dipicolinates by HPLC–ESI–MS/MS (SRM) analysis were about 260 amol and 5–25 amol on-column, respectively [43,44], which was about 3860-fold and 1000-fold, respectively, more sensitive than those with underivatized HPLC–ESI–MS/MS analysis [21]. On the other hand, the detection limits of native cholesterol and oxysterols by HPLC-APCI–MS/MS analysis were about 100 fmol and 10 fmol, respectively [43,44].

4.2. Mass spectra

All picolinyl ester derivatives of 3β-monohydroxysterols exhibited adduct ions of $[M + Na + CH_3CN]^+$ as the base peaks [43], while those of di-, tri- and tetra-hydroxysterols and 3-ketosterols showed $[M + Na]^+$ ions as the base peaks under our HPLC–ESI–MS conditions [44]. However, it should be noted here that the base peaks would change depending on the composition of mobile phase. In contrast to other derived moieties, the picolinyl group is not permanently charged, so that even in the case of oxysterols with multiple hydroxyl groups, a single charged ion was predominant in the positive ESI mass spectra.

Collision of $[M + Na + CH_3CN]^+$ of the picolinyl derivatives of 3β-monohydroxysterols at a relatively low collision energy (10–15 V) resulted in the predominant formation of $[M + Na]^+$ as product ions, while the use of higher collision energies (25–30 V) resulted in the [picolinic acid + Na]$^+$ (m/z = 146) ion as the most abundant product ion. In contrast, collision of $[M + Na]^+$ of the picolinyl derivatives of di-, tri- and tetra-hydroxysterols and 3-ketosterols resulted in the formation of [M + Na–picolinic acid]$^+$ or [picolinic acid + Na]$^+$ ions at any specific collision energy depending on the sterols (10–30 V). Representative MS/MS fragmentation patterns of the picolinyl derivatives are shown in Fig. 2, and the most suitable collision energies and precursor to product ions of each sterol for SRM are listed in Table 2.

4.3. Synthesis of derivatives

The derivatization and purification steps are very simple [44]. As shown in Fig. 3, the reagent mixture, consisting of 2-methyl-6-nitrobenzoic anhydride, 4-dimethylaminopyridine, picolinic acid, pyridine and triethylamine, is added to the sterol extract, and incubated at 80 °C for 60 min. Excess reagents are then precipitated by the addition of *n*-hexane, and the clear supernatant containing picolinyl ester derivatives is collected and evaporated at 80 °C under nitrogen. The residue is redissolved in 50 μl of acetonitrile and an aliquot is used for HPLC–ESI–MS/MS analysis. The

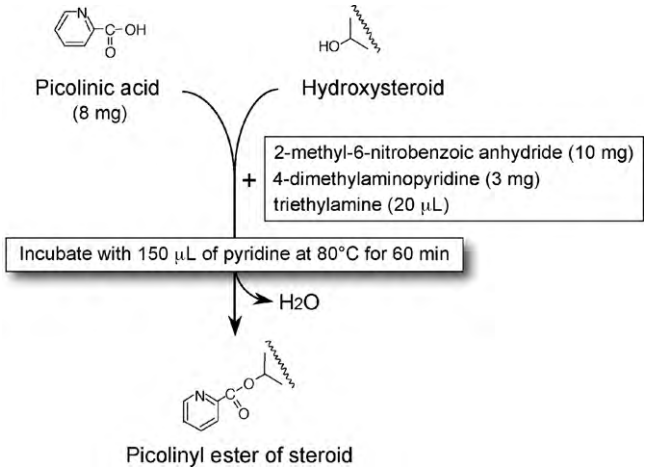

Fig. 3. The formation of picolinyl ester derivative and the conditions of the reaction.

derivatives are stable for at least 6 months in the acetonitrile solution.

In general, this esterification progresses easily at room temperature, but the hydroxyl groups at the C-5α, C-20α and C-25 positions of oxysterols are resistant to picolinyl ester formation at room temperature. In these resistant positions, C-25 is completely esterified by heating at 80 °C for 60 min, but the C-5α and C-20α positions are not esterified at all even if the reaction mixture is heated at 80 °C.

It has been pointed out that cholesterol can be autoxidized during sample preparation [61]. However, to analyze whole sterol profiles in biological samples, it is difficult to remove cholesterol selectively before derivatization. Therefore, we determined the formation of oxysterols from pure cholesterol in the derivatizing conditions, and no significant amounts of oxysterols were detected. The results suggest that the autoxidation of cholesterol during the derivatization step is negligible.

Transesterification of fatty acyl esters during the formation of picolinyl esters is another possibility for the overestimation of sterols. However, the incubation of pure cholesteryl stearate in the reaction mixture showed that the transesterification was not probable.

4.4. Chromatographic separation

HPLC is performed using a reversed-phase Hypersil GOLD column (150 mm × 2.1 mm I.D., 3 μm, Thermo Fisher Scientific, San Jose, CA, USA). In our previous reports, monohydroxysterols [43] and oxysterols [44] were measured separately, but both sterols can be analyzed simultaneously because the HPLC column and gradient conditions are the same. Initially, the mobile phase is comprised of acetonitrile–methanol–water (40:40:20, v/v/v) containing 0.1% acetic acid, and it is then programmed in a linear manner to acetonitrile–methanol–water (45:45:10, v/v/v) containing 0.1% acetic acid over 20 min. The final mobile phase is maintained constant for an additional 20 min. The flow rate is 300 μl/min, and the column is maintained at 40 °C using a column oven.

Relative retention times (RRTs), expressed relative to the retention time of cholesterol, are listed in Table 2. The RRTs show that the separation of sterols by the Hypersil GOLD column is excellent, but several weak points are also indicated. First, 7β-hydroxycholesterol gives a peak just before 7α-hydroxycholesterol, and reliable quantification of each hydroxycholesterol can occasionally be difficult. Second, the retention times of 7α-hydroxy-4-cholesten-3-one and 24S,25-epoxycholesterol are very close to each other, and these sterols show similar MS and MS/MS spectra. However, because 7α-hydroxy-4-cholesten-3-one does not survive alkaline hydrolysis, the peak detected after alkaline hydrolysis is 24S,25-epoxycholesterol alone. Third, lanosterol gives a peak just after cholesterol. Although the monitoring ion for lanosterol is different from that for cholesterol, a huge cholesterol peak in biological samples can sometimes interfere with the lanosterol peak.

These problems are resolved by using another reversed-phase column, Hypersil GOLD aQ (150 mm × 2.1 mm I.D., 3 μm, Thermo Fisher Scientific). This column is usually used for separations employing highly aqueous mobile phases, but we use it as follows: initially, the mobile phase is comprised of acetonitrile–methanol–water (40:40:20, v/v/v) containing 0.1% acetic acid; it is then programmed in a linear manner to acetonitrile–methanol (50:50, v/v) containing 0.1% acetic acid over 40 min. The final mobile phase is maintained constant for an additional 2 min. The flow rate is 300 μl/min, and the column is maintained at 40 °C using a column oven.

The RRTs by the Hypersil GOLD aQ column are also shown in Table 2. Compared with the Hypersil GOLD column, the width of each peak tends to be wide, and the order of elution from the column is very different. Good chromato-

Table 2
Positive ESI-SRM and HPLC data of the picolinyl ester derivative of each sterol[a].

Picolinyl ester derivatives	SRM condition			HPLC data (RRT[c])	
	Precursor to product (*m/z*)	Collision energy (*V*)	Pattern[b]	C18[d]	C18 aQ[e]
24S-Hydroxy-4-cholesten-3-one	528 → 146	24	A	0.34	0.30
25-Hydroxy-4-cholesten-3-one	528 → 146	24	A	0.36	0.37
27-Hydroxy-4-cholesten-3-one	528 → 146	24	A	0.40	0.42
7α-Hydroxy-4-cholesten-3-one	528 → 146	24	A	0.42	0.33
7β-Hydroxy-4-cholesten-3-one	528 → 146	24	A	0.44	0.36
5α-Cholesta-8(9),14,24-trien-3β-ol	551 → 510	12	B	0.71	0.71
Cholesta-5,7,24-trien-3β-ol	551 → 510	12	B	0.73	0.79
Cholesta-5,8,24-trien-3β-ol	551 → 510	12	B	0.75	0.78
5α-Cholesta-7,24-dien-3β-ol	553 → 512	12	B	0.81	0.87
Zymosterol	553 → 512	12	B	0.82	0.86
Desmosterol	553 → 512	12	B	0.83	0.88
5α-Cholesta-8(9),14-dien-3β-ol	553 → 512	12	B	0.84	0.87
5α-Cholesta-6,8(9)-dien-3β-ol	553 → 512	12	B	0.85	0.83
7-Dehydrocholesterol	553 → 512	12	B	0.87	0.92
8-Dehydrocholesterol	553 → 512	12	B	0.89	0.91
Lathosterol	555 → 514	15	B	0.97	0.98
8-Lathosterol	555 → 514	15	B	0.98	0.98
Cholesterol	555 → 514	15	B	1.00	1.00
Coprostanol	557 → 516	14	B	1.05	0.91
Cholestanol	557 → 516	14	B	1.10	1.04
4-Methyl-5α-cholesta-8(9),24-dien-3β-ol	567 → 526	12	B	0.89	0.89
4-Methyl-5α-cholesta-8(9),14-dien-3β-ol	567 → 526	12	B	0.90	0.92
24S,25-Epoxycholesterol	569 → 528	12	B	0.42	0.53
7-Ketocholesterol	569 → 528	12	B	0.53	0.48
4-Methyl-5α-cholest-8(9)-en-3β-ol	569 → 528	12	B	1.07	1.01
Campesterol	569 → 528	12	B	1.10	1.03
20α-Hydroxycholesterol[f]	571 → 530	14	B	0.40	0.43
5β,6β-Epoxycholestanol	571 → 530	14	B	0.68	0.64
5α,6α-Epoxycholestanol	571 → 530	14	B	0.70	0.68
4,4′-Dimethyl-5α-cholesta-8(9),14,24-trien-3β-ol	579 → 538	14	B	0.84	0.78
4,4′-Dimethyl-5α-cholesta-8(9),24-dien-3β-ol	581 → 540	14	B	0.97	0.93
4,4′-Dimethyl-5α-cholesta-8(9),14-dien-3β-ol	581 → 540	14	B	1.01	0.99
4,4′-Dimethyl-5α-cholest-8(9)-en-3β-ol	583 → 542	14	B	1.19	1.04
Sitosterol	583 → 542	14	B	1.22	1.07
Sitostanol	585 → 544	14	B	1.36	1.11
Lanosterol	595 → 554	12	B	1.01	0.90
Dihydrolanosterol	597 → 556	15	B	1.24	1.01
27-Hydroxy-7-dehydrocholesterol	633 → 510	22	C	0.49	0.62
7β-Hydroxycholesterol	635 → 146	22	A	0.61	0.53
7α-Hydroxycholesterol	635 → 146	22	A	0.62	0.51
6-Hydroxycholesterol	635 → 146	22	A	0.69	0.63
4β-Hydroxycholesterol	635 → 146	22	A	0.78	0.76
22R-Hydroxycholesterol	635 → 512	22	C	0.47	0.55
22S-Hydroxycholesterol	635 → 512	22	C	0.50	0.48
24R-Hydroxycholesterol	635 → 512	22	C	0.50	0.56
24S-Hydroxycholesterol	635 → 512	22	C	0.50	0.57
25-Hydroxycholesterol	635 → 512	22	C	0.53	0.66
27-Hydroxycholesterol	635 → 512	22	C	0.58	0.71
5β-Cholestane-3α,7α-diol	637 → 514	22	C	0.64	0.49
7α,27-Dihydroxy-4-cholesten-3-one	649 → 146	28	A	0.18	0.17
7α,12α-Dihydroxy-4-cholesten-3-one	649 → 146	28	A	0.19	0.14
Cholestan-3β,5α,6β-triol[f]	653 → 146	28	A	0.60	0.50
7α,27-Dihydroxycholesterol	756 → 510	20	D	0.33	0.31
5β-Cholestane-3α,7α,12α-triol	758 → 635	28	C	0.32	0.24
5β-Cholestane-3α,7α,12α,25-tetrol	879 → 756	20	C	0.15	0.12

Abbreviations: ESI, electrospray ionization; SRM, selected reaction monitoring; RRT, relative retention time.

[a] Some data in this table have been reported in our previous paper [43,44].

[b] Patterns of precursor to product ions. A: $[M+Na]^+ \rightarrow$ [picolinic acid + Na]$^+$; B: $[M+Na+CH_3CN]^+ \rightarrow [M+Na]^+$; C: $[M+Na]^+ \rightarrow$ [M + Na–picolinic acid]$^+$; D: $[M+Na]^+ \rightarrow$ [M + Na–2 picolinic acids]$^+$. A, B, C and D correspond to those in Fig. 2.

[c] RRTs are expressed relative to the retention time of cholesterol 3β-picolinate.

[d] A reversed-phase C18 column, Hypersil GOLD (150 mm × 2.1 mm I.D., 3 μm, Thermo Fisher Scientific) was employed. Initially, the mobile phase was comprised of acetonitrile–methanol–water (40:40:20, v/v/v) containing 0.1% acetic acid, then it was programmed in a linear manner to acetonitrile–methanol–water (45:45:10, v/v/v) containing 0.1% acetic acid over 20 min. The final mobile phase was kept constant for an additional 20 min. The flow rate was 300 μl/min, and the column was maintained at 40 °C using a column oven. The retention time of cholesterol 3β-picolinate by this condition was around 28.5 min.

[e] Polar endcapped C18 column, Hypersil GOLD aQ (150 mm × 2.1 mm I.D., 3 μm, Thermo Fisher Scientific) was used. Initially, the mobile phase was comprised of acetonitrile–methanol–water (40:40:20, v/v/v) containing 0.1% acetic acid; then it is programmed in a linear manner to acetonitrile–methanol (50:50, v/v) containing 0.1% acetic acid over 40 min. The final mobile phase was kept constant for an additional 2 min. The flow rate was 300 μl/min, and the column was maintained at 40 °C. The retention time of cholesterol 3β-picolinate by this condition was around 36.5 min.

[f] Hydroxyl groups at the C-5α and C-20α positions of oxysterols are not derivatized.

graphic separations are achieved between 7α-hydroxycholesterol and 7β-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one and 24S,25-epoxycholesterol, and cholesterol and lanosterol. However, the lathosterol and 8-lathosterol peaks are not differentiated.

The separations of picolinylated sterols by these reversed-phase columns are not at all inferior to the separation of free sterols by reversed-phase HPLC [15,21]. For example, the separation of 24-hydroxycholesterol and 25-hydroxycholesterol was difficult, but DeBarber et al. achieved the chromatographic separation and quantification by using a mobile phase consisted of acetonitrile–methanol–water and APCI-MS/MS detector [33]. In contrast, McDonald et al. failed to quantify these sterol isomers separately by using an eluent of methanol–water and ESI–MS/MS detector [21]. They did not use acetonitrile because the presence of acetonitrile significantly reduced signal intensity of sterols analyzed by this detector. As for picolinylated 24- and 25-hydroxycholesterols, they were well separated each other by using acetonitrile–methanol–water as a mobile phase, and excellent sensitivities were achieved by ESI–MS/MS detector.

4.5. Sample preparation

Long term storage or repeated freeze and thaw of biological samples should be avoided because it stimulates cholesterol autoxidation [61]. Addition of the antioxidant, butylated hydroxytoluene, to the sample before sample preparation produced only a modest decrease in oxidation. Therefore, minimizing oxidation by using good lab practices is important [21].

To analyze the unesterified fraction of sterols, serum (1–5 μl), subcellular fraction of tissue (0.1–1.0 mg protein), or cell homogenate (1×10^4–1×10^5 cells) is dried with the added internal standards, and directly derivatized to the picolinyl esters [43]. To analyze the total (unesterified + esterified) fraction, saponification is carried out in 1 N ethanolic KOH at 37 °C for 1 h, and sterols are extracted with *n*-hexane before derivatization [44]. It may be mentioned here that some sterols occur as conjugates with sulfuric or glucuronic acid [62–64]. Negative ESI mode without derivatization is suitable for the analyses of these conjugated sterols, and the conjugated sterols are much more polar than picolinyl esters of unconjugated sterols.

Because this assay method is very sensitive, we can minimize the loading of derivatized sample on the HPLC column. Although the solid-phase extraction/purification step is omitted, target sterols are successfully separated by the HPLC–MS/MS step. In case of human serum analysis, less than 1 ng of picolinyl esters of non-cholesterol sterols are injected onto the column with approximately 200 ng of cholesterol picolinate. Under our HPLC conditions, this amount of cholesterol picolinate is easily trapped in the Hypersil GOLD and Hypersil GOLD aQ columns and eluted at around 28.5 min and 36.5 min, respectively, which is well separated from the picolinyl esters of most non-cholesterol sterols.

While picolinyl esters of sterols are very soluble in acetonitrile, nonpolar compounds, such as fatty acyl esters of cholesterol, remain underivatized and do not dissolve in the final acetonitrile solution. Nonpolar compounds are strongly retained on reversed-phase HPLC columns, but in this method, loading of the nonpolar compounds on the column is minimized.

4.6. Precision and accuracy

The linearity of the standard curves, as determined by simple linear regression, was excellent, as reported in our previous papers [43,44]. Reproducibilities and recoveries of some sterols were validated according to a one-way layout and polynomial equation, respectively [43,44]. The variances between sample preparations and between measurements by this method were calculated to be 1.6–12.7% and 2.5–16.5%, respectively. In these results, higher values of the variances (over 10%) were obtained by the quantification of sterols that showed extremely low concentrations in the samples. To test matrix effects, the recovery experiments were performed using human serum or rat liver microsomes spiked with 0.05–12 ng of sterols. Recoveries of the sterols ranged from 86.7% to 107.3% with a mean recovery of 99.3%, which suggests that matrix effects are not significant in this assay. This method provides reproducible and reliable results for the quantification of sterols in small amounts of biological samples.

5. Perspectives

HPLC–MS or HPLC–MS/MS does not require a derivatization step before the analysis of sterols, which is advantageous for a high-throughput assay. However, the addition of the derivatization step has markedly improved the sensitivities of the neutral sterols. Thus, simple and rapid procedures do not always produce good results for the microanalysis of biological samples. In addition, since many sterols have the same molecular weight and similar structures, a thorough chromatographic separation is essential to maintain the selectivity even if the latest model of mass spectrometer is operated in a high-resolution mode.

The recent development of steroid biochemistry has demonstrated that there are considerable bioactive or biomarker sterols among intermediates and their derivatives in the biosynthetic pathways of cholesterol, bile acids and steroid hormones. Moreover, there are still many unidentified sterols in biological samples. Therefore, not only sensitive and specific quantification of targeted sterols but also metabolomic analysis of whole sterol profiles will become an important methodology for steroid biochemistry and its clinical applications.

References

[1] S. Gill, R. Chow, A.J. Brown, Sterol regulators of cholesterol homeostasis and beyond: the oxysterol hypothesis revisited and revised, Prog. Lipid Res. 47 (2008) 391–404.
[2] I. Björkhem, T. Miettinen, E. Reihner, S. Ewerth, B. Angelin, K. Einarsson, Correlation between serum levels of some cholesterol precursors and activity of HMG-CoA reductase in human liver, J. Lipid Res. 28 (1987) 1137–1143.
[3] H.J. Kempen, J.F. Glatz, J.A. Gevers Leuven, H.A. van der Voort, M.B. Katan, Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans, J. Lipid Res. 29 (1988) 1149–1155.
[4] T.A. Miettinen, R.S. Tilvis, Y.A. Kesäniemi, Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population, Am. J. Epidemiol. 131 (1990) 20–31.
[5] I. Björkhem, E. Reihnér, B. Angelin, S. Ewerth, J.-E. Åkerlund, K. Einarsson, On the possible use of the serum level of 7α-hydroxycholesterol as a marker for increased activity of the cholesterol 7α-hydroxylase in humans, J. Lipid Res. 28 (1987) 889–894.
[6] T. Yoshida, A. Honda, N. Tanaka, Y. Matsuzaki, B. He, T. Osuga, N. Kobayashi, K. Ozawa, H. Miyazaki, Simultaneous determination of mevalonate and 7α-hydroxycholesterol in human plasma by gas chromatography–mass spectrometry as indices of cholesterol and bile acid biosynthesis, J. Chromatogr. 613 (1993) 185–193.
[7] G.S. Tint, M. Irons, E.R. Elias, A.K. Batta, R. Frieden, T.S. Chen, G. Salen, Defective cholesterol biosynthesis associated with the Smith–Lemli–Opitz syndrome, N. Engl. J. Med. 330 (1994) 107–113.
[8] D.R. FitzPatrick, J.W. Keeling, M.J. Evans, A.E. Kan, J.E. Bell, M.E. Porteous, K. Mills, R.M. Winter, P.T. Clayton, Clinical phenotype of desmosterolosis, Am. J. Med. Genet. 75 (1998) 145–152.
[9] R.I. Kelley, W.G. Wilcox, M. Smith, L.E. Kratz, A. Moser, D.S. Rimoin, Abnormal sterol metabolism in patients with Conradi–Hunermann–Happle syndrome and sporadic lethal chondrodysplasia punctata, Am. J. Med. Genet. 83 (1999) 213–219.
[10] P.A. Krakowiak, C.A. Wassif, L. Kratz, D. Cozma, M. Kovarova, G. Harris, A. Grinberg, Y. Yang, A.G. Hunter, M. Tsokos, R.I. Kelley, F.D. Porter, Lathosterolosis: an inborn error of human and murine cholesterol synthesis due to lathosterol 5-desaturase deficiency, Hum. Mol. Genet. 12 (2003) 1631–1641.
[11] G. Salen, S. Shefer, L. Nguyen, G.C. Ness, G.S. Tint, V. Shore, Sitosterolemia, J. Lipid Res. 33 (1992) 945–955.
[12] G. Halperin, M. Elisaf, E. Leitersdorf, D. Harats, A new method for determination of serum cholestanol by high-performance liquid chromatography with ultraviolet detection, J. Chromatogr. B Biomed. Sci. Appl. 742 (2000) 345–352.

[13] B.J. Koopman, J.C. van der Molen, B.G. Wolthers, J.B. Vanderpas, Determination of some hydroxycholesterols in human serum samples, J. Chromatogr. 416 (1987) 1–13.
[14] P.B. Hylemon, E.J. Studer, W.M. Pandak, D.M. Heuman, Z.R. Vlahcevic, Y.L. Chiang, Simultaneous measurement of cholesterol 7α-hydroxylase activity by reverse-phase high-performance liquid chromatography using both endogenous and exogenous [4-^{14}C]cholesterol as substrate, Anal. Biochem. 182 (1989) 212–216.
[15] B. Ruan, N. Gerst, G.T. Emmons, J. Shey, G.J. Schroepfer Jr., Sterol synthesis. A timely look at the capabilities of conventional and silver ion high performance liquid chromatography for the separation of C27 sterols related to cholesterol biosynthesis, J. Lipid Res. 38 (1997) 2615–2626.
[16] T. Saldanha, A.C. Sawaya, M.N. Eberlin, N. Bragagnolo, HPLC separation and determination of 12 cholesterol oxidation products in fish: comparative study of RI, UV, and APCI-MS detectors, J. Agric. Food Chem. 54 (2006) 4107–4113.
[17] A. Cohen, H.S. Hertz, J. Mandel, R.C. Paule, R. Schaffer, L.T. Sniegoski, T. Sun, M.J. Welch, V.E. White, Total serum cholesterol by isotope dilution/mass spectrometry: a candidate definitive method, Clin. Chem. 26 (1980) 854–860.
[18] S. Dzeletovic, O. Breuer, E. Lund, U. Diczfalusy, Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry, Anal. Biochem. 225 (1995) 73–80.
[19] H.S. Ahmida, P. Bertucci, L. Franzo, R. Massoud, C. Cortese, A. Lala, G. Federici, Simultaneous determination of plasmatic phytosterols and cholesterol precursors using gas chromatography–mass spectrometry (GC–MS) with selective ion monitoring (SIM), J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 842 (2006) 43–47.
[20] J. Lembcke, U. Ceglarek, G.M. Fiedler, S. Baumann, A. Leichtle, J. Thiery, Rapid quantification of free and esterified phytosterols in human serum using APPI-LC–MS/MS, J. Lipid Res. 46 (2005) 21–26.
[21] J.G. McDonald, B.M. Thompson, E.C. McCrum, D.W. Russell, Extraction and analysis of sterols in biological matrices by high performance liquid chromatography electrospray ionization mass spectrometry, Methods Enzymol. 432 (2007) 145–170.
[22] Z. Zhang, D. Li, D.E. Blanchard, S.R. Lear, S.K. Erickson, T.A. Spencer, Key regulatory oxysterols in liver: analysis as Δ^4-3-ketone derivatives by HPLC and response to physiological perturbations, J. Lipid Res. 42 (2001) 649–658.
[23] B. Ruan, W.K. Wilson, J. Pang, N. Gerst, F.D. Pinkerton, J. Tsai, R.I. Kelley, F.G. Whitby, D.M. Milewicz, J. Garbern, G.J. Schroepfer Jr., Sterols in blood of normal and Smith–Lemli–Opitz subjects, J. Lipid Res. 42 (2001) 799–812.
[24] W. Sattler, H.J. Leis, G.M. Kostner, E. Malle, Quantification of 7-dehydrocholesterol in plasma and amniotic fluid by liquid chromatography/particle beam-mass spectrometry as a biochemical diagnostic marker for the Smith–Lemli–Opitz syndrome, Rapid Commun. Mass Spectrom. 9 (1995) 1288–1292.
[25] M. Careri, P. Ferretti, P. Manini, M. Musci, Evaluation of particle beam high-performance liquid chromatography–mass spectrometry for analysis of cholesterol oxides, J. Chromatogr. A 794 (1998) 253–262.
[26] P. Manini, R. Andreoli, M. Careri, L. Elviri, M. Musci, Atmospheric pressure chemical ionization liquid chromatography/mass spectrometry in cholesterol oxide determination and characterization, Rapid Commun. Mass Spectrom. 12 (1998) 883–889.
[27] E. Razzazi-Fazeli, S. Kleineisen, W. Luf, Determination of cholesterol oxides in processed food using high-performance liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization, J. Chromatogr. A 896 (2000) 321–334.
[28] I. Burkard, K.M. Rentsch, A. von Eckardstein, Determination of 24S- and 27-hydroxycholesterol in plasma by high-performance liquid chromatography–mass spectrometry, J. Lipid Res. 45 (2004) 776–781.
[29] J.J. Palmgren, A. Toyras, T. Mauriala, J. Monkkonen, S. Auriola, Quantitative determination of cholesterol, sitosterol, and sitostanol in cultured Caco-2 cells by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 821 (2005) 144–152.
[30] K. Raith, C. Brenner, H. Farwanah, G. Müller, K. Eder, R.H.H. Neubert, A new LC/APCI-MS method for the determination of cholesterol oxidation products in food, J. Chromatogr. A 1067 (2005) 207–211.
[31] K. Nagy, A. Jakab, F. Pollreisz, D. Bongiorno, L. Ceraulo, M.R. Averna, D. Noto, K. Vekey, Analysis of sterols by high-performance liquid chromatography/mass spectrometry combined with chemometrics, Rapid Commun. Mass Spectrom. 20 (2006) 2433–2440.
[32] Q. Tian, M.L. Failla, T. Bohn, S.J. Schwartz, High-performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry determination of cholesterol uptake by Caco-2 cells, Rapid Commun. Mass Spectrom. 20 (2006) 3056–3060.
[33] A.E. DeBarber, D. Lutjohann, L. Merkens, R.D. Steiner, Liquid chromatography-tandem mass spectrometry determination of plasma 24S-hydroxycholesterol with chromatographic separation of 25-hydroxycholesterol, Anal. Biochem. 381 (2008) 151–153.
[34] R. Karuna, A. von Eckardstein, K.M. Rentsch, Dopant assisted-atmospheric pressure photoionization (DA-APPI) liquid chromatography–mass spectrometry for the quantification of 27-hydroxycholesterol in plasma, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 261–268.
[35] A. Honda, J. Shoda, N. Tanaka, Y. Matsuzaki, T. Osuga, N. Shigematsu, M. Tohma, H. Miyazaki, Simultaneous assay of the activities of two key enzymes in cholesterol metabolism by gas chromatography–mass spectrometry, J. Chromatogr. 565 (1991) 53–66.
[36] A. Sanghvi, E. Grassi, C. Bartman, R. Lester, M. Galli Kienle, G. Galli, Measurement of cholesterol 7α-hydroxylase activity with selected ion monitoring, J. Lipid Res. 22 (1981) 720–724.
[37] J.M.E. Quirke, C.L. Adams, G.J. Van Berkel, Chemical derivatization for electrospray ionization mass spectrometry. 1. Alkyl halides, alcohols, phenols, thiols, and amines, Anal. Chem. 66 (1994) 1302–1315.
[38] G.J. Van Berkel, J.M. Quirke, R.A. Tigani, A.S. Dilley, T.R. Covey, Derivatization for electrospray ionization mass spectrometry. 3. Electrochemically ionizable derivatives, Anal. Chem. 70 (1998) 1544–1554.
[39] R. Sandhoff, B. Brugger, D. Jeckel, W.D. Lehmann, F.T. Wieland, Determination of cholesterol at the low picomole level by nano-electrospray ionization tandem mass spectrometry, J. Lipid Res. 40 (1999) 126–132.
[40] D.W. Johnson, H.J. ten Brink, C. Jakobs, A rapid screening procedure for cholesterol and dehydrocholesterol by electrospray ionization tandem mass spectrometry, J. Lipid Res. 42 (2001) 1699–1705.
[41] X. Jiang, D.S. Ory, X. Han, Characterization of oxysterols by electrospray ionization tandem mass spectrometry after one-step derivatization with dimethylglycine, Rapid Commun. Mass Spectrom. 21 (2007) 141–152.
[42] A. Honda, K. Yamashita, M. Numazawa, T. Ikegami, M. Doy, Y. Matsuzaki, H. Miyazaki, Highly sensitive quantification of 7α-hydroxy-4-cholesten-3-one in human serum by LC–ESI–MS/MS, J. Lipid Res. 48 (2007) 458–464.
[43] A. Honda, K. Yamashita, H. Miyazaki, M. Shirai, T. Ikegami, G. Xu, M. Numazawa, T. Hara, Y. Matsuzaki, Highly sensitive analysis of sterol profiles in human serum by LC–ESI–MS/MS, J. Lipid Res. 49 (2008) 2063–2073.
[44] A. Honda, K. Yamashita, T. Hara, T. Ikegami, T. Miyazaki, M. Shirai, G. Xu, M. Numazawa, Y. Matsuzaki, Highly sensitive quantification of key regulatory oxysterols in biological samples by LC–ESI–MS/MS, J. Lipid Res. 50 (2009) 350–357.
[45] W.J. Griffiths, S. Liu, G. Alvelius, J. Sjövall, Derivatisation for the characterisation of neutral oxosteroids by electrospray and matrix-assisted laser desorption/ionisation tandem mass spectrometry: the Girard P derivative, Rapid Commun. Mass Spectrom. 17 (2003) 924–935.
[46] W.J. Griffiths, Y. Wang, G. Alvelius, S. Liu, K. Bodin, J. Sjövall, Analysis of oxysterols by electrospray tandem mass spectrometry, J. Am. Soc. Mass Spectrom. 17 (2006) 341–362.
[47] K. Karu, M. Hornshaw, G. Woffendin, K. Bodin, M. Hamberg, G. Alvelius, J. Sjovall, J. Turton, Y. Wang, W.J. Griffiths, Liquid chromatography-mass spectrometry utilizing multi-stage fragmentation for the identification of oxysterols, J. Lipid Res. 48 (2007) 976–987.
[48] K. Hiraoka, I. Kudaka, Negative-mode electrospray-mass spectrometry using nonaqueous solvents, Rapid Commun. Mass Spectrom. 6 (1992) 265–268.
[49] T. Higashi, K. Shimada, Derivatization of neutral steroids to enhance their detection characteristics in liquid chromatography–mass spectrometry, Anal. Bioanal. Chem. 378 (2004) 875–882.
[50] T. Higashi, N. Takayama, T. Nishio, E. Taniguchi, K. Shimada, Procedure for increasing the detection responses of estrogens in LC–MS based on introduction of a nitrobenzene moiety followed by electron capture atmospheric pressure chemical ionization, Anal. Bioanal. Chem. 386 (2006) 658–665.
[51] G. Singh, A. Gutierrez, K. Xu, I.A. Blair, Liquid chromatography/electron capture atmospheric pressure chemical ionization/mass spectrometry: analysis of pentafluorobenzyl derivatives of biomolecules and drugs in the attomole range, Anal. Chem. 72 (2000) 3007–3013.
[52] M.S. Kuo, J.M. Kalbfleisch, P. Rutherford, D. Gifford-Moore, X.D. Huang, R. Christie, K. Hui, K. Gould, M. Rekhter, Chemical analysis of atherosclerotic plaque cholesterol combined with histology of the same tissue, J. Lipid Res. 49 (2008) 1353–1363.
[53] E.G. Lund, U. Diczfalusy, Quantitation of receptor ligands by mass spectrometry, Methods Enzymol. 364 (2003) 24–37.
[54] W.J. Griffiths, M. Hornshaw, G. Woffendin, S.F. Baker, A. Lockhart, S. Heidelberger, M. Gustafsson, J. Sjövall, Y. Wang, Discovering oxysterols in plasma: a window on the metabolome, J. Proteome Res. 7 (2008) 3602–3612.
[55] K. Yamashita, S. Kobayashi, S. Tsukamoto, M. Numazawa, Synthesis of pyridine-carboxylate derivatives of hydroxysteroids for liquid chromatography–electrospray ionization–mass spectrometry, Steroids 72 (2007) 50–59.
[56] K. Yamashita, M. Okuyama, Y. Watanabe, S. Honma, S. Kobayashi, M. Numazawa, Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography–electrospray ionization tandem mass spectrometry, Steroids 72 (2007) 819–827.
[57] K. Yamashita, M. Takahashi, S. Tsukamoto, M. Numazawa, M. Okuyama, S. Honma, Use of novel picolinoyl derivatization for simultaneous quantification of six corticosteroids by liquid chromatography–electrospray ionization tandem mass spectrometry, J. Chromatogr. A 1173 (2007) 120–128.
[58] K. Yamashita, R. Nakagawa, M. Okuyama, S. Honma, M. Takahashi, M. Numazawa, Simultaneous determination of tetrahydrocortisol, allotetrahydrocortisol and tetrahydrocortisone in human urine by liquid chromatography–electrospray ionization tandem mass spectrometry, Steroids 73 (2008) 727–737.
[59] K. Yamashita, Y. Tadokoro, M. Takahashi, M. Numazawa, Preparation and structural elucidation of the picolinyl ester of aldosterone for liquid chromatography–electrospray ionization tandem mass spectrometry, Chem. Pharm. Bull. 56 (2008) 873–877.
[60] K. Yamashita, M. Okuyama, R. Nakagawa, S. Honma, F. Satoh, R. Morimoto, S. Ito, M. Takahashi, M. Numazawa, Development of sensitive derivatization method for aldosterone in liquid chromatography–electrospray ionization

tandem mass spectrometry of corticosteroids, J. Chromatogr. A 1200 (2008) 114–121.
[61] L.L. Smith, Cholesterol Autoxidation, Plenum Press, New York, 1981.
[62] A.K. Batta, G. Salen, S. Shefer, G.S. Tint, M. Batta, Increased plasma bile alcohol glucuronides in patients with cerebrotendinous xanthomatosis: effect of chenodeoxycholic acid, J. Lipid Res. 28 (1987) 1006–1012.
[63] L.J. Meng, W.J. Griffiths, H. Nazer, Y. Yang, J. Sjövall, High levels of (24S)-24-hydroxycholesterol 3-sulfate, 24-glucuronide in the serum and urine of children with severe cholestatic liver disease, J. Lipid Res. 38 (1997) 926–934.
[64] X. Li, W.M. Pandak, S.K. Erickson, Y. Ma, L. Yin, P. Hylemon, S. Ren, Biosynthesis of the regulatory oxysterol, 5-cholesten-3β,25-diol 3-sulfate, in hepatocytes, J. Lipid Res. 48 (2007) 2587–2596.