methods

A rapid screening procedure for cholesterol and dehydrocholesterol by electrospray ionization tandem mass spectrometry

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Abstract The mono-(dimethylaminoethyl) succinyl (MDMAES) ester is a new derivative for rapid, mild, and sensitive electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of cholesterol and dehydrocholesterol. It is an order of magnitude more sensitive than the previous most practical alternative, the N-methylpyridyl ether derivative. The MDMAES derivative was used to develop a rapid screening procedure for the biochemical diagnosis of Smith-Lemli-Opitz syndrome (SLOS) by measuring the dehydrocholesterol/cholesterol ratio in plasma (5 μl) and plasma spotted onto filter paper. Details of the synthesis of [25,26,26,26,27,27,27-$^2H_7$]-7-dehydrocholesterol, used as a standard for quantitation, are included. The measurement of total sterols as MDMAES esters, after base hydrolysis of plasma, afforded a dehydrocholesterol/cholesterol ratio of 0.05–2.95 for SLOS patient samples (n = 5) compared with 0.001–0.003 for normal adult controls (n = 20). Direct hexane extraction of plasma without base hydrolysis enabled the measurement of free sterols with a total sample analysis time of <1 h. The free dehydrocholesterol/cholesterol ratio was 0.10–4.47 for SLOS patient samples (n = 5) and 0.003–0.011 for normal adult controls (n = 20).—Johnson, D. W., H. J. ten Brink, and C. Jakobs. **A rapid screening procedure for cholesterol and dehydrocholesterol by electrospray ionization tandem mass spectrometry.** ***J. Lipid Res.*** **2001.** 42: **1699–1705.**

Supplementary key words ESI-MS/MS • Smith-Lemli-Opitz syndrome • plasma

Smith-Lemli-Opitz syndrome (SLOS) is an inherited disorder of cholesterol biosynthesis (1). Reduced activity of 7-dehydrocholesterol reductase leads to the accumulation of 7- and 8-dehydrocholesterol and reduced total cholesterol in blood (2, 3). Biochemical diagnosis of the disorder is from a high dehydrocholesterol/cholesterol ratio. Diagnosis from stored blood spots collected at birth (4, 5) and the beneficial effects of dietary treatment of affected children have been demonstrated (6). The currently employed gas chromatography and gas chromatography-mass spectrometry (GC-MS) (2, 4) methods require a minimum of 12 μl of blood or plasma and incorporate a slow separation step that makes them unsuitable for rapid screening for SLOS. A method using direct time-of-flight secondary ion mass spectrometry analysis of 3-mm blood spots has been described (5). It is not a quantitative method; rather a ratio of ions deriving from dehydrocholesterol and cholesterol is measured. The instrumentation is uncommon in screening laboratories and sample throughput is limited.

Electrospray ionization tandem mass spectrometry (ESI-MS/MS) has solved the dual problems of speed and small sample size for other classes of compounds. Cholesterol, however, is poorly ionized by electrospray, and use of a liquid chromatography-mass spectrometry technique (7) for its measurement requires a minimum of 40 μl of plasma. More sensitive assays for cholesterol have been developed that include its derivatization as an N-methylpyridyl ether (8), a ferrocene carbamate (9), or a sulfate (10).

This article describes the development of a new derivative, a mono-(dimethylaminoethyl) succinyl (MDMAES) ester 3 (**Scheme 1**) for rapid and sensitive ESI-MS/MS analysis of cholesterol and dehydrocholesterol. It is formed by reacting the imidazolide 2 of mono-(dimethylaminoethyl) succinate 1 with the sterol (ROH in Scheme 1). Because no stable isotope-labeled standard was commercially available for the measurement of dehydrocholesterol, a heptadeuterated 7-dehydrocholesterol was first synthesized.

The new ESI-MS/MS method for the analysis of cholesterol and dehydrocholesterol was developed for plasma

Abbreviations: DMG, dimethylglycine; ESI-MS/MS, electrospray ionization tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; MDMAEG, mono-(dimethylaminoethyl) glutaryl; MDMAES, mono-(dimethylaminoethyl) succinyl; MRM, multiple reaction monitoring; PTAD, 4-phenyl-1,2,4-triazolone-3,5-dione; SLOS, Smith-Lemli-Opitz syndrome; TMG, trimethylglycine.

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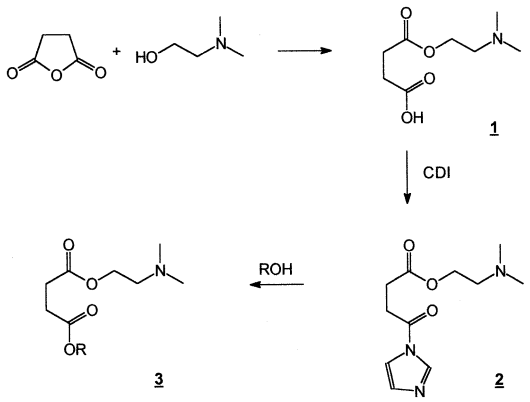


Scheme 1. Preparation of mono-(dimethylaminoethyl) succinate 1 and mono-(dimethylaminoethyl) succinyl esters 3.

and plasma spotted onto filter paper. The dehydrocholesterol/cholesterol ratios of five patients with SLOS were measured by this method and compared with those from controls for both total and free sterols.

MATERIALS AND METHODS

Patient samples

Blood samples from healthy adults were obtained from the Red Cross Blood Bank in Adelaide, South Australia, and the plasma was separated and stored at −20°C. Plasma samples from SLOS patients were referred to the Amsterdam laboratory for analysis and were stored frozen. Five-microliter portions were spotted onto methanol-washed filter paper, dried under nitrogen, sealed in an airtight plastic bag, and sent by post to the North Adelaide laboratory. Permission was obtained to use these samples for this study.

Materials

[25,26,26,26,27,27,27-$^2H_7$]cholesterol was purchased from C/D/N Isotopes (Montreal, Canada). [25,26,26,26,27,27,27-$^2H_7$]-7-dehydrocholesterol and mono-(dimethylaminoethyl) succinate were prepared by the methods described below. All other reagents were purchased from Sigma-Aldrich (Castle Hill, Australia) in the highest purity available. All solvents were HPLC grade.

GC-MS analysis

GC-MS analysis was performed on a Perkin Elmer Turbomass gas chromatograph/mass spectrometer equipped with a 20-m-long, 0.18-mm i.d., 0.18 μm phase thickness Perkin Elmer PE-5MS chromatography column. The instrument was operated in positive ion electron impact mode.

Synthesis of [25,26,26,26,27,27,27-$^2H_7$]-7-dehydrocholesterol (see Scheme 2)

A mixture of [25,26,26,26,27,27,27-$^2H_7$]cholesterol 4 (80 mg, 0.2 mmol), 4-dimethylaminopyridine (5 mg), acetic anhydride (2 ml), and pyridine (1.5 ml) was heated at 60°C for 20 h. Ice (5 g) and chloroform (5 ml) were added. The chloroform solution was separated and the aqueous solution reextracted with chloroform (5 ml). The combined chloroform solution was washed twice with hydrochloric acid (5 ml, 1 mol/l) and with aqueous potassium carbonate (5 ml, 10%). The chloroform solution was evaporated to afford [25,26,26,26,27,27,27-$^2H_7$]cholesteryl acetate as a white solid (91 mg, 100%).

To a boiling solution of the acetate in carbon tetrachloride (12 ml) was added N-bromosuccinamide (40 mg, 0.25 mmol) and benzoyl peroxide (5 mg, 0.02 mmol) in one portion. The mixture was boiled under reflux for 20 min, cooled to 0°C, and filtered, and the solvent was evaporated in a stream of nitrogen. The bromide 5, a pale yellow semi-solid, was used without purification in the next step.

The crude bromide 5 was dissolved in a mixture of xylene (11 ml) and collidine (2.5 ml) and heated under reflux for 90 min. The mixture was cooled, diluted with ethyl acetate, and washed several times with water. The organic phase was evaporated in a stream of nitrogen, and the last traces of xylene were removed by azeotrope evaporation with dichloromethane. This gave a mixture of dienes, which was dissolved in dichloromethane (5 ml) and titrated with a solution of 4-phenyl-1,2,4-triazolone-3,5-dione (PTAD, 0.1 mol/l in dichloromethane) until the red color persisted. The solution was allowed to stand at room temperature for 1 h with occasional swirling. The solution was evaporated and chromatographed on Florisil (5 g). Elution with diethyl ether/hexane (1:9) removed nonpolar products. Elution with ethyl acetate/hexane (1:1) and evaporation of the solvent afforded the PTAD adduct 6. Analysis by silica TLC, with diethyl ether/hexane (1:1) as elution solvent and staining with iodine, showed one major component with rf 0.15 identical to that of the unlabeled PTAD adduct of cholesteryl acetate, which had been prepared independently.

To the crude PTAD adduct **6** in dry tetrahydrofuran (7 ml) was added lithium aluminium hydride (200 mg). The mixture was boiled under reflux for 3 h. It was cooled in ice, quenched with water, acidified with dilute hydrochloric acid, and extracted with ethyl acetate. The ethyl acetate solution was evaporated, and the residue was chromatographed on Florisil with hexane and eluted with a gradient of ethyl acetate in hexane. The fractions containing 7-dehydrocholesterol, as determined by TLC analysis, were pooled. The solvent was evaporated to afford [25,26,26,26,27,27,27-$^2H_7$]-7-dehydrocholesterol **7** (12.1 mg, 15% overall yield) as a pale yellow solid, m.p. 115–117°C. GC-MS analysis of a small portion derivatized as the trimethylsilyl derivative showed a major component (93%) with m/z 463 (M^+ for [$^2H_7$]-7-dehydrocholesterol trimethylsilyl ether) and a faster eluting minor component (7%) with m/z 461.

Synthesis of mono-(dimethylaminoethyl) succinate (see Scheme 1)

Succinic anhydride (1.00 g, 10 mmol) was added portionwise to dimethylaminoethanol (2.67 g, 30 mmol) over 10 min with vortex mixing (exothermic reaction). The mixture was heated at 65°C for 4 h. The excess dimethylaminoethanol was removed by evaporation in a stream of nitrogen and finally by azeotrope evaporation with dichloromethane. The residue was suspended in hexane, filtered, and dried in air to give succinate 1 (1.89 g, 100%) as a white solid. It was recrystallized from diethyl ether/acetone as a white powder m.p. 57–58°C. ESI-MS analysis in acidic solution gave ions at m/z 190 (MH^+, 100%), 145 (27%), 99 (38%), 72 (27%), and 55 (9%), consistent with the expected product.

Preparation of MDMAES imidazolide reagent

Mono-(dimethylaminoethyl) succinate 1 (18.9 mg, 0.1 mmol) and 1,1′-carbonyldiimidazole (16.5 mg, 0.1 mmol) were suspended in dichloromethane (1.0 ml) and shaken periodically. When the solid had dissolved (~15 min), additional dichloromethane (9.0 ml) was added. The reagent was stored in a sealed container in a dry place.

Extraction of total sterols from plasma and plasma on filter paper

To a 75 × 10-mm glass tube was added [25,26,26,26,27,27,27-$^2H_7$]cholesterol 4 (25 μl, 1 mmol/l in methanol), [25,26,26,26,27,-27,27-$^2H_7$]-7-dehydrocholesterol **7** (5 μl, 1 mmol/l in methanol), plasma (5 μl) or plasma on filter paper, ethanol (300 μl), and aqueous sodium hydroxide solution (60 μl, 7 mol/l). The mixture was left at room temperature for 1 h with occasional shaking. Water (500 μl) and hexane (1 ml) were added, and the mixture was vortex mixed for 1 min. The hexane layer was added to a 100 × 13-mm glass screw cap tube and evaporated in a stream of nitrogen.

Extraction of free sterols from plasma on filter paper

To a 75 × 10-mm glass tube was added [25,26,26,26,27,27,27-$^2H_7$]cholesterol 4 (25 μl, 1 mmol/l in methanol), [25,26,26,26,27,-27,27-$^2H_7$]-7-dehydrocholesterol **7** (5 μl, 1 mmol/l in methanol), plasma (5 μl) on filter paper, and hexane (1 ml). The mixture was vortex mixed for 1 min and left to stand for 15 min. The hexane solution was removed, added to a 100 × 13-mm glass screw cap tube, and evaporated in a stream of nitrogen.

Preparation of mono-(dimethylaminoethyl) succinyl esters of sterols

To the sterol containing residue was added MDMAES imidazolide 2 reagent (100 μl, 10 mmol/l in dichloromethane) and triethylamine (1 μl). The glass tube was loosely capped (to allow slow evaporation of the solvent) and heated at 70°C for 10 min. The residual solvent was evaporated in a stream of nitrogen, and the residue was dissolved in 2 ml of acetonitrile–water–formic acid 50/50/0.25 (v/v/v) for mass spectral analysis. The solutions are stable at room temperature for at least 2 weeks.

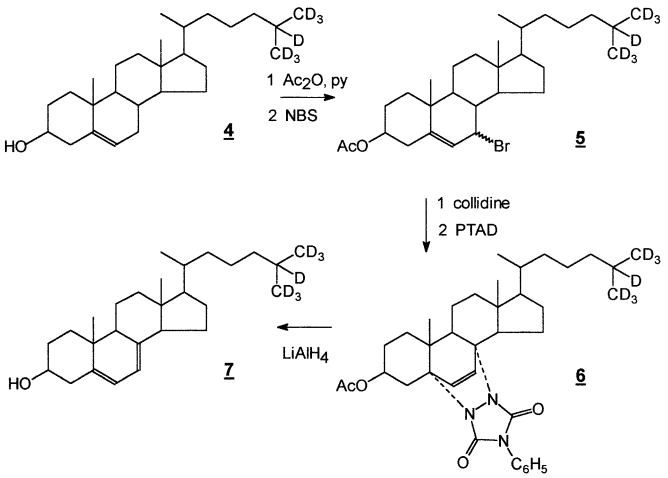
ESI-MS/MS analysis

ESI-MS/MS analysis was performed on a Perkin-Elmer SCIEX API365 instrument equipped with an Ionspray assembly, Hewlett Packard 1100 HPLC, and a Gilson 215 autosampler. The HPLC flow rate was 35 μl/min, and the autosampler injection volume was 20 μl. The mobile phase was acetonitrile/water/formic acid [50/50/0.25 (v/v/v)]. The API365 was operated in MRM (multiple reaction monitoring) mode and monitored the following ion pairs: 558.5/369.4 (cholesterol), 565.5/376.4 ([25,26,26,26,27,27,-27-$^2H_7$]cholesterol), 556.5/367.4 (dehydrocholesterol), and 563.5/374.4 ([25,26,26,26,27,27,27-$^2H_7$]-7-dehydrocholesterol). Response ratios for 1:1 mixtures of labeled/unlabeled cholesterol and dehydrocholesterol were determined and used to correct raw data.

RESULTS

Synthesis of [25,26,26,26,27,27,27-$^2H_7$]-7-dehydrocholesterol

The preparation of deuterium-labeled 7-dehydrocholesterol **7**, outlined in **Scheme 2**, is based on a synthesis of Vitamin D_3 analogs (11). It involved the preparation of a 4-phenyl-1,2,4-triazolone-3,5-dione derivative, which has also been used in the synthesis of [3α-^{3}H]-7-dehydrocholesterol (12). A preliminary synthesis with unlabeled cholesterol provided samples of intermediates for TLC comparison at each step. The 3-hydroxyl group of deuterium-labeled cholesterol 4 was protected as the acetate and allylically brominated to bromide 5. This was dehydrobrominated to a mixture of 4,6- and 5,7-dienes. The 5,7-diene was separated from the mixture by forming the



Scheme 2. Synthesis of [25,26,26,26,27,27,27-$^2H_7$]-7-dehydrocholesterol 7 from [25,26,26,26,27,27,27-$^2H_7$]cholesterol 4.

4-phenyl-1,2,4-triazoline-3,5-dione adduct 6, which was purified by chromatography. Treatment of adduct 6 with excess lithium aluminium hydride afforded [25,26,26,26,27,-27,27-$^2H_7$]-7-dehydrocholesterol **7** in 15% overall yield. GC-MS analysis indicated that it was 93% chemically pure and 98% isotopically pure. The minor contaminant is the deuterated equivalent of an oxidation product also found in commercial 7-dehydrocholesterol. The standard must be stored in a cold, dark place under nitrogen to minimize oxidation.

Development of the MDMAES derivative for ESI-MS/MS analysis of cholesterol and dehydrocholesterol

Dimethylglycine (DMG) esters were recently developed as derivatives for rapid and sensitive ESI-MS/MS analysis of alcohols (13). Esterification with DMG proved superior to other published derivatization methods displaying limitations in speed, sensitivity, or practicality. For example, ferrocene carbamate derivatives (9), with very low detection limits, are prepared at >90°C in toluene. These are not ideal conditions for the oxidation-prone dehydrocholesterol.

As the exception to the rule, however, the DMG ester of cholesterol was the least sensitive with ESI-MS/MS analysis of any class of alcohols studied. The quaternary ammonium analog, trimethylglycine (TMG) ester, was 5-fold more sensitive, similar to N-methylpyridyl ether (13). This was still considered less than the sensitivity required for analysis of dehydrocholesterol in 3-mm blood spots or small plasma (<5 μl) samples.

For fatty acids, sensitive ESI-MS/MS analysis has been achieved using dimethylaminoethyl (DMAE) esters prepared from acid chlorides and dimethylaminoethanol (14, 15). Was there a way of coupling dimethylaminoethanol to an alcohol? The solution was to react dimethylaminoethanol with an anhydride (succinic) to form a hemi-ester and convert its free carboxylic acid group to an imidazolide for reaction with the alcohol (Scheme 1). In essence, the derived MDMAES ester 3 is a hybrid of the DMAE and DMG derivatives. The conditions for the preparation of

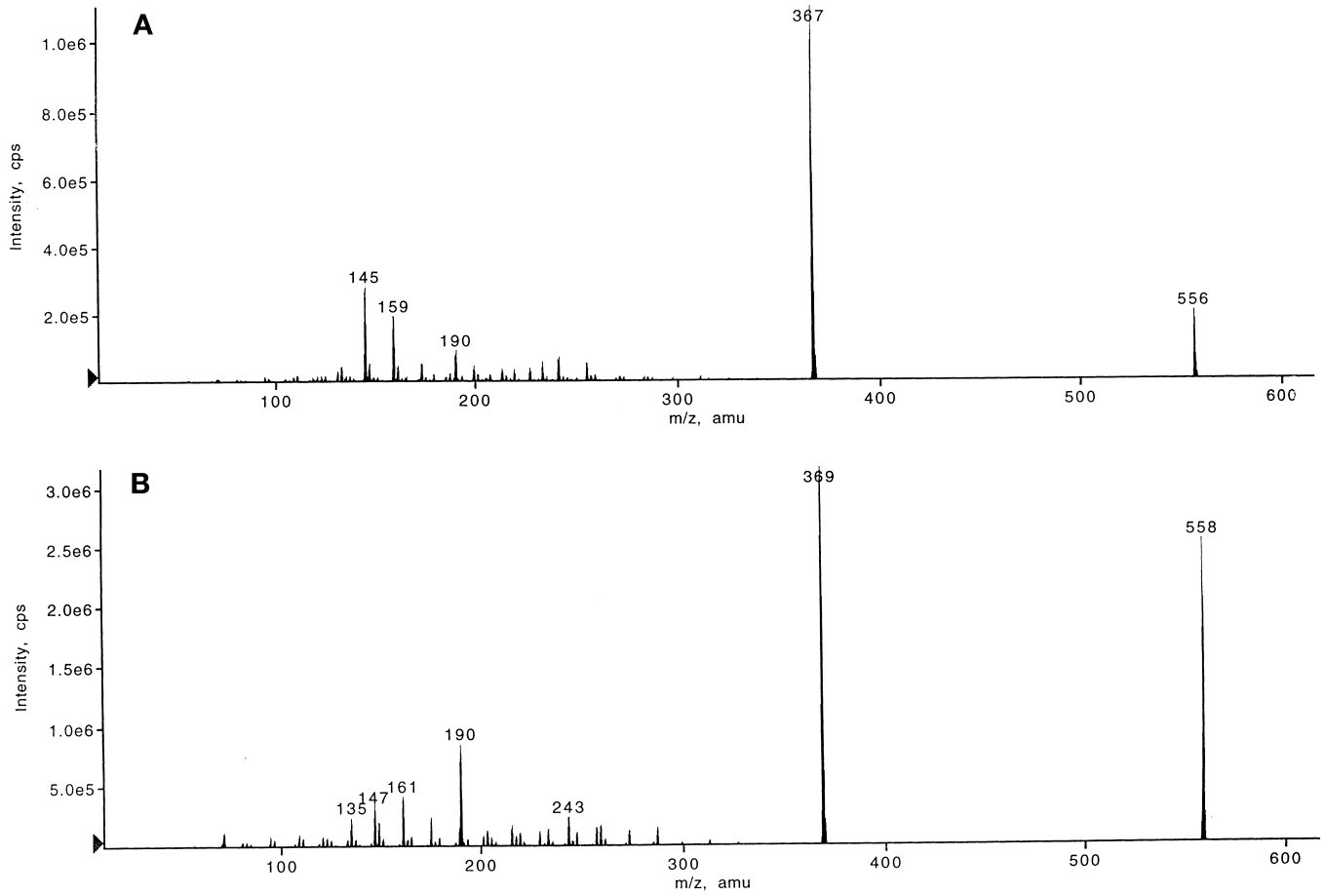


Fig. 1. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis, showing the product ion spectra of the protonated molecular ions, of the mono-(dimethylaminoethyl) succinyl (MDMAES) derivatives of (A) dehydrocholesterol and (B) cholesterol.

this derivative are identical to those for the DMG derivative (13) and are found in Materials and Methods. Attempts to purify the intermediate imidazolide 2 by crystallization from solvent mixtures were both unsuccessful and unnecessary. The imidazolide reagent is stable for at least 2 weeks provided it is sealed from moisture. ESI-MS/MS analysis of the MDMAES derivatives of 7-dehydrocholesterol (**Fig. 1A**) and cholesterol (Fig. 1B) both showed a dominant product ion from neutral loss of 189 Da [mono-(dimethylaminoethyl) succinate] from their strong protonated molecular ion.

To determine the comparative sensitivity of the MDMAES derivative for cholesterol analysis, 10 μmol/l solutions of different derivatives of cholesterol were sequentially infused into the tandem mass spectrometer. The ion intensities (average of 10 scans) of the major product ion of the protonated molecular ion or molecular cation were optimized using Autotune software and are shown in **Table 1** relative to the MDMAES derivative. The MDMAES derivative of cholesterol afforded the greatest signal intensity and proved to be an order of magnitude more sensitive for ESI-MS/MS analysis than the previously most practical and sensitive derivative, N-methylpyridyl ether. The mono-(dimethylaminoethyl) glutaryl (MDMAEG) derivative, prepared in an analogous way from glutaric anhydride instead of succinic anhydride (details not shown), afforded intermediate sensitivity.

Mixtures of labeled and unlabeled cholesterol and 7-dehydrocholesterol with molar ratios (unlabeled/labeled) between 0 and 2, as MDMAES derivatives, were analyzed by ESI-MS/MS using the set of MRM experiments detailed in Materials and Methods. A five-point standard curve afforded an R^2 of 0.999 for both sterols. This indicated that the MDMAES derivatives were suitable for quantitation of cholesterol and dehydrocholesterol.

TABLE 1. Relative ion intensities of the major product ion generated from the protonated molecular ion or molecular cation during ESI-MS/MS analysis of cholesterol derivatives

Derivative	Product Ion	Relative Intensity
	m/z	
Dimethylglycine (DMG)	369	0.014
Trimethylglycine (TMG)	118	0.07
N-methylpyridyl ether (MPE)	369	0.10
Mono-(dimethylaminoethyl) glutaryl (MDMAEG)	369	0.19
Mono-(dimethylaminoethyl) succinyl (MDMAES)	369	1.00

Abbreviation: ESI-MS/MS, electrospray ionization tandem mass spectrometry.

Analysis of total cholesterol and dehydrocholesterol in plasma

The ESI-MS/MS method for measurement of total cholesterol and dehydrocholesterol in plasma, outlined in Materials and Methods, was based on published GC-MS methods (2, 4). The MDMAES derivative was prepared instead of the trimethylsilyl derivative, and deuterated cholesterol and dehydrocholesterol were used as internal standards. The cholesterol level of a control plasma sample, measured in replicate (n = 8), after 1 h base hydrolysis, was 102 ± 5% that after 2 h hydrolysis. This indicated that hydrolysis for 1 h was sufficient.

Analysis of a large batch of control plasma samples (n = 50) showed a cholesterol plasma concentration of 2.86–7.42 (mean = 4.44) mmol/l, consistent with published reference ranges.

Replicate analysis (n = 8) of 5 μl of a normal plasma sample afforded intraassay variations of 4.1% (cholesterol) and 20.2% (dehydrocholesterol) and interassay variations of 4.3% (cholesterol) and 24.5% (dehydrocholesterol). The concentration of dehydrocholesterol in normal plasma is negligible in comparison with cholesterol, and the high variation for dehydrocholesterol is due to the background noise. Replicate analysis (n = 6) of 5 μl of SLOS patient (5 in **Table 2**) plasma sample afforded intraassay variations of 2.7% (cholesterol) and 1.2% (dehydrocholesterol) and interassay variations of 8.1% (cholesterol) and 4.4% (dehydrocholesterol).

A method was also sought for the analysis of cholesterol in plasma spotted onto filter paper. This medium facilitates inexpensive transport of patient samples. Initially, an ethanol extraction of the dried plasma on filter paper for 15 min followed by base hydrolysis was tried. This afforded only 82 ± 5% of the cholesterol obtained by direct analysis of the same volume of plasma. Hydrolysis with the filter paper present afforded a more acceptable 91 ± 3%. To minimize signal suppression during ESI-MS/MS analysis, the filter paper was methanol washed and dried before spotting the plasma. Replicate analysis (n = 8) of 5 μl normal plasma on filter paper afforded intraassay variations of 5.1% (cholesterol) and 21.3% (dehydrocholesterol) and interassay variations of 5.5% (cholesterol) and 19.9% (dehydrocholesterol). For SLOS patient plasma, the intraassay variations (n = 6) were 9.0% (cholesterol) and 4.0% (dehydrocholesterol), and the interassay variations (n = 6) were 10.2% (cholesterol) and 4.2% (dehydrocholesterol). By way of illustration, **Fig. 2** shows a comparison of ESI-MS/MS analyses for control plasma on filter paper (Fig. 2A) and SLOS patient (1 on Table 2) plasma on filter paper (Fig. 2B).

Plasma from five SLOS patients spotted onto filter paper was analyzed. The results displayed in Table 2 show a dehydrocholesterol/cholesterol ratio at least 16 times the highest control. Measurement of free sterols in the same patient and control plasma samples was also performed. The dehydrocholesterol/cholesterol ratio (Table 2) was at least nine times the highest control. The SLOS patient plasma samples were collected at various patient ages outside of the newborn period. There is no published evidence that the dehydrocholesterol/cholesterol ratio is age dependent, so adult controls were used for comparison with all SLOS patient samples.

DISCUSSION

The strategy of coupling dimethylaminoethanol to cholesterol produced the MDMAES derivative with unique application to the sensitive ESI-MS/MS analysis of unsaturated sterols. Functional group modifications to the derivative including the preparation of glutaryl (MDMAEG) and trimethylamino (MTMAES) analogs failed to improve

TABLE 2. Analyses for cholesterol and dehydrocholesterol in plasma

	Cholesterol	Dehydrocholesterol	Dehydrocholesterol Cholesterol
	mmol/l	*mmol/l*	
Total sterols in plasma			
Controls (n = 50, mean ± SD)	4.44 ± 1.07	0.0167 ± 0.0062	0.0039 ± 0.0015
Blanks (n = 8, mean ± SD)[a]	0.034 ± 0.015	0.0021 ± 0.0004	
Total sterols in plasma spotted on filter paper			
Controls (n = 20, mean ± SD)	4.88 ± 0.78	0.0084 ± 0.0019	0.0021 ± 0.0006
SLOS patient 1	0.43	1.26	2.95
SLOS patient 2	1.28	0.33	0.26
SLOS patient 3	1.18	0.20	0.17
SLOS patient 4	1.33	0.064	0.048
SLOS patient 5	1.38	0.94	0.68
Free sterols in plasma spotted on filter paper			
Controls (n = 20, mean ± SD)	1.14 ± 0.23	0.005 ± 0.0022	0.005 ± 0.0024
SLOS patient 1	0.27	1.22	4.47
SLOS patient 2	0.23	0.15	0.64
SLOS patient 3	0.27	0.12	0.45
SLOS patient 4	0.47	0.046	0.098
SLOS patient 5	0.18	0.31	1.75

SLOS, Smith-Lemli-Optiz syndrome.
[a] Distilled water substituted for plasma.

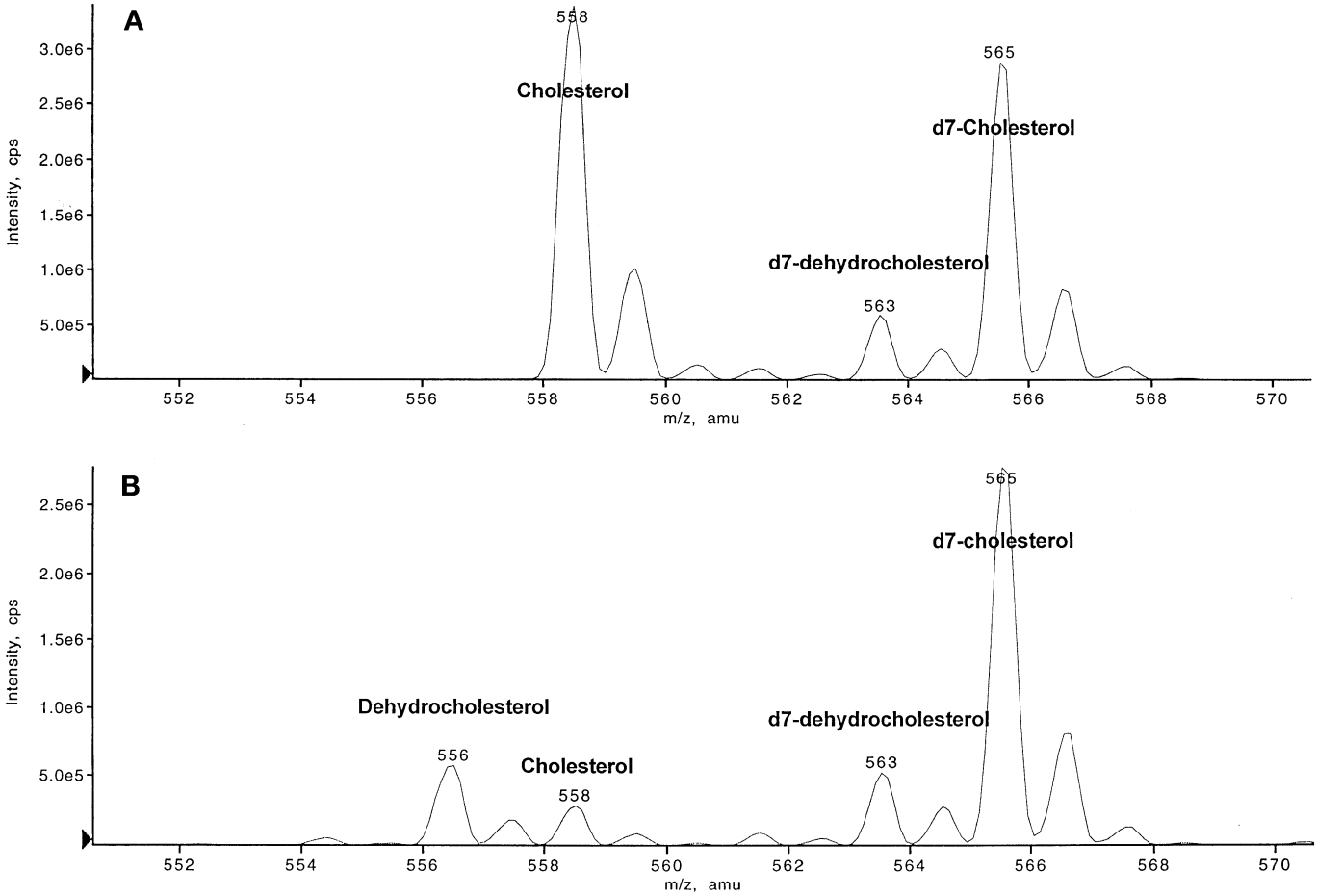


Fig. 2. ESI-MS/MS analysis, showing a neutral loss of 189 Da scan, for the MDMAES derivatives of sterols in hydrolyzed extracts of (A) normal plasma and (B) plasma from a Smith-Lemli-Opitz syndrome patient.

the sensitivity. The ESI-MS/MS analysis of the MDMAES derivatives of saturated alcohols gave weak neutral loss of 189-Da product ions. For example, dihydrocholesterol (cholestanol) afforded a neutral loss of 189-Da product ions with an intensity of <1% of that for cholesterol, when analyzed in an equimolar mixture. MDMAES esters of other alcohols present in plasma thus produced few interfering ions in the measurement of cholesterol and dehydrocholesterol. Disappointingly, with the exception of unsaturated sterols, the MDMAES derivative was inferior to the DMG derivative (13) for all other classes of alcohols compared. During ESI-MS/MS analysis for both protonated derivatives (DMG and MDMAES), there are two major competing fragmentation pathways. Cleavage of the C-O alcohol bond to give a cation, stabilized by double bonds, is favored with the larger MDMAES derivative. Loss of the protonated acid after a hydrogen abstraction is favored by the smaller DMG derivative (13).

The major criticism of electrospray ionization analysis without preceding liquid chromatography separation is its inability to distinguish isomeric species. The only sterol with identical molecular weight to cholesterol is lathosterol, which is present in <0.3% of cholesterol in plasma (7). Both 7- and 8-dehydrocholesterol are found in the plasma of SLOS patients (3). There appears to be no disadvantage, however, in measuring the summation of these isomeric forms for the diagnosis of SLOS except in samples stored on paper for long periods (4). 7-Dehydrocholesterol is more easily oxidized, and measurement of 8-dehydrocholesterol is therefore the more accurate in old samples. There are no sterols with molecular weights that might interfere with the measurement of the deuterated dehydrocholesterol and cholesterol standards.

Diagnosis of SLOS in blood spots has previously been shown to be successful by determination of free dehydrocholesterol/cholesterol ratios with the use of a nonstandard time-of-flight instrument (5). The method described above is applicable to high-throughput ESI-MS/MS instruments currently in routine use in diagnostic centers. Additionally, the method has application to the measurement of very small amounts of cholesterol. It provides an alternative to the nano-ion spray technique for the sulfate ester (10). To avoid errors of sample measurement, 5 μl plasma samples were analyzed in this study, and the derivative was dissolved in 2 ml of solvent. As a consequence, <1% of the sample was consumed. In addition, further ESI-MS/MS signal intensity can be obtained by removing the excess derivatizing reagent. The MDMAES esters are soluble

in hydrocarbon solvents whereas mono-(dimethylaminoethyl) succinate is water soluble.

This screening procedure may not, however, solve the major clinical problem of detecting mildly affected SLOS patients, who usually benefit the most from current treatment. Plasma from a single mildly affected patient was analyzed. The dehydrocholesterol/cholesterol ratios for potentially milder patients may overlap with the normal range, particularly if plasma is exposed to air, spread on filter paper, for extended periods before analysis.

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REFERENCES

1. Smith, D., L. Lemli, and J. M. Opitz. 1964. A newly recognised syndrome of multiple congenital abnormalities. *J. Pediatr.* **64:** 210–217.
2. Tint, G. S., M. Irons, E. R. Elias, A. K. Batta, R. Freiden, T. S. Chen, and G. Salen. 1994. Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N. Engl. J. Med.* **330:** 107–113.
3. Batta, A. K., G. S. Tint, S. Shefer, D. Abuelo, and G. Salen. 1995. Identification of 8-dehydrocholesterol (cholesta-5,8-dien-3β-ol) in patients with Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **36:** 705–711.
4. Starck, L., and A. Lovgren. 2000. Diagnosis of Smith-Lemli-Opitz syndrome from stored filter paper blood specimens. *Arch. Dis. Child.* **82:** 490–492.
5. Zimmerman, P. A., D. M. Hercules, and E. W. Naylor. 1997. Direct analyses of filter paper blood specimens for identification of Smith-Lemli-Opitz syndrome using time-of-flight secondary ion mass spectrometry. *Am. J. Med. Genet.* **68:** 300–304.
6. Irons, M., E. R. Elias, D. Abuelo, M. J. Bull, C. L. Greene, V. P. Johnson, L. Keppen, C. Schanen, G. S. Tint, and G. Salen. 1997. Treatment of Smith-Lemli-Opitz syndrome; results of a multicenter trial. *Am. J. Med. Genet.* **68:** 311–314.
7. Kock, R., B. Delvoux, and H. Greiling. 1997. Determination of total cholesterol in serum by liquid chromatography–isotope dilution mass spectrometry. *Clin. Chem.* **43:** 1896–1903.
8. Quirke, J. M. E., C. L. Adams, and G. J. Van Berkel. 1994. Chemical derivatization for electrospray ionization mass spectrometry. 1. Alkyl halides, alcohols, phenols, thiols and amines. *Anal. Chem.* **66:** 1302–1315.
9. Van Berkel, G. J., J. M. E. Quirke, R. A. Tigani, A. S. Dilley, and T. R. Covey. 1998. Derivatization for electrospray ionization mass spectrometry. 3. Electrochemically ionizable derivatives. *Anal. Chem.* **70:** 1544–1554.
10. Sandhoff, R., B. Brugger, D. Jeckel, W. D. Lehmann, and F. T. Wieland. 1999. Determination of cholesterol at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *J. Lipid Res.* **40:** 126–132.
11. Gill, H. S., J. M. Londowski, R. A. Corradino, A. R. Zinsmeister, and R. Kumar. 1988. The synthesis and biological activity of 25-hydroxy-26,27-dimethylvitamin D_3 and 1,25-dihydroxy-26,27-dimethylvitamin D_3: highly potent novel analogs of vitamin D_3. *J. Steroid Biochem.* **31:** 147–160.
12. Batta, A. K., G. Salen, G. S. Tint, A. Honda, and S. Shefer. 1997. Synthesis of [3 alpha-3H]7-dehydrocholesterol via stable tritiated 4-phenyl-1,2,4-triazoline-3,5-dione derivative. *Steroids.* **62:** 700–702.
13. Johnson, D. W. 2001. Analysis of alcohols, as dimethylglycine esters, by electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **36:** 277–283.
14. Johnson, D. W. 1999. Dimethylaminoethyl esters for trace, rapid analysis of fatty acids by electrospray tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **13:** 2388–2393.
15. Johnson, D. W., H. J. ten Brink, R. C. Schuit, and C. Jakobs. 2001. Rapid and quantitative analysis of unconjugated C_{27} bile acids in plasma and blood samples by tandem mass spectrometry. *J. Lipid Res.* **42:** 9–16.