Development of a Rapid Normal-Phase LC–Positive Ion APCI–MS Method for Simultaneous Detection and Quantitation of Cholesterol, Androst-4-ene-3,17-dione, and Androsta-1,4-diene-3,17-dione

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Abstract

This paper describes the development of a normal-phase liquid chromatograph–UV–diode array detection–positive ion atmospheric pressure chemical ionization–mass spectrometry method for the simultaneous identification and quantitation of cholesterol, androst-4-ene-3,17-dione (AD), and androsta-1,4-diene-3,17-dione (ADD) in fermentation broths. The compounds detected under positive ion atmospheric pressure chemical ionization on a mass spectrometer by selected ion monitoring are separated by normal-phase high-performance liquid chromatography. $[M+H]^+$ ions are taken into consideration for quantitation of AD and ADD, and $[M–H_2O+H]^+$ ions are considered for quantitation of cholesterol. The compounds are analyzed on a Si_{60} silica (5 µm, 125 × 4-mm i.d.) Merck column using a 2:3 isocratic mixture of isopropyl alcohol and hexane. The calibration curves resulting from the reference compounds in the concentration range of 100–5000 pg on column exhibit a good linear correlation ($r^2 \geq 0.996$). The method is validated by analyzing six replicates of broth samples fortified with three compounds, namely, cholesterol, AD, and ADD, at 0.050 and 0.5 µg/g levels. The mean recoveries for the fortifications range from 90% to 98% with relative standard deviations in the range of 3.36% to 9.78%. The method is developed to study the qualitative as well as quantitative conversion of cholesterol to AD and ADD by a microorganism identified as *Nocardia sp*. These studies helped the investigation of the reaction kinetics, which showed that the molar biotransformation of cholesterol into AD and ADD was 21%, even when the reaction was prolonged for 96 h.

Introduction

One of the latest market buzz words is androsta-1,4-diene-3,17-dione, a direct hormone precursor of testosterone, estrone, and estradiol. Androst-4-ene-3, 17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD) (Figure 1) are recognized as economically viable intermediates for the production of androgenic and estrogenic hormones (1,2). Cholesterol side-chain degradation ability is widespread among the microorganisms, and the number of microorganisms capable of selectively degrading the sterol side chain has been explored in a number of ways, such as free/immobilized cells (3,4) or as and enzyme source (5) in the steroid industry. The biotransformations involving microorganisms are important ecofriendly tools for the large-scale production of natural or modified steroid analogues (6,7). Chemical conversions of sterols are not economically feasible (8,9) as these conversions involve a number of repetitive steps as well as costly and hazardous reagents (10,11). Selective cleavage of sterols (12), such as cholesterol, soyabean sterols, β-sitosterol (13–15), campesterol, and stigmasterol, is of basic importance in steroid chemistry and biotechnology (16–19). Today, an increasing number of pharmacologically active steroids are being produced in the industry, involving transformations catalyzed by the action of microbes or enzymes (20).

Our studies on the degradation of sterol side chains led to the identification of an organism capable of selectively degrading the cholesterol side chain. This organism, which was identified by 16S RNA as *Nocardia sp*., degrades cholesterol into two main products, ADD and AD (Figure 1). To study these biotransformations, an analytical method was developed which could provide information on the qualitative as well as quantitative conversion of cholesterol to AD and ADD. In this

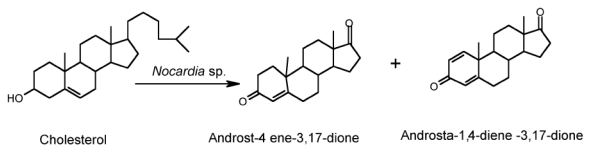

Figure 1. Conversion of cholesterol to AD and ADD by nocardia sp

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direction, a normal-phase liquid chromatography (LC)–positive ion atmospheric pressure chemical ionization (APCI)–mass spectrometry (MS) method proved to be an ideal approach, contributing immensely to the monitoring of the bio-conversion of cholesterol to AD and ADD. A number of methods for separate analysis of cholesterol and simultaneous analysis of AD and ADD by high-performance liquid chromatography (HPLC) and HPLC–MS (21) are available, but to our knowledge, there is no single method in the literature for the simultaneous analysis of cholesterol, AD, and ADD, including normal-phase LC–MS.

Experimental

Material

All the chemicals used in the study were of analytical grade. Yeast extract, tryptone, sodium chloride, and tween 80 were purchased from Himedia (Mumbai, India). Cholesterol was obtained from Sisco Laboratories (Mumbai, India). Kieselgel 60 mit and F_{254} fluorescent TLC plates were obtained from Machery-Nagel (Merck, Darmstadt, Germany). All the solvents used for analysis were of HPLC grade and were purchased from Ranbaxy Laboratories Limited (Gurgaon, India). The low-resistivity (LR)-grade ethylacetate (Ranbaxy Laboratories Limited) was used for extraction of the samples. Standard ADD and AD were purchased from Sigma-Aldrich (Bangalore, India).

Organism, downstreaming, and sample preparation

The microorganism (Actinomycete) used in the study was isolated from Manikaran soil of Himachal (Pradesh, India). The isolate was obtained by an enrichment technique and was identified as *Nocardia sp*. by 16S RNA gene analysis. The 16S rRNA gene (500 bp) was amplified by polymerase chain reaction (PCR) with a set of primers. The PCR products obtained were loaded on an ABI Prism 310 Genetic Analyzer (PerkinElmer, Waltham, MA) for sequencing. Identification was achieved by comparing the sequence data obtained with the sequences in the gene bank by BlastN program (22). This culture is now a part of the culture collection section in the biotechnology department of our institute. The culture has been assigned the accession number RRL450.

Nocardiae are widely distributed and are abundant in soil. The strains can be isolated from complex mixed communities, such as those found in soil, by plating dilution on suitable media. This actinomycete strain survived on cholesterol as its sole carbon source and was found to have the capability of degrading the sterol side chain selectively in the presence of the enzyme inhibitor 2,2'-dipyridyl. The organism was grown aerobically at 30°C in yeast extract tryptone medium, which contained 5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl, 1 g/L cholesterol, and tween 80, and the pH of the medium was 7.2. Flasks were inoculated with 1 mL of 24 h-old culture grown in the induced medium and incubated at 30°C (220 rpm). A 0.3mM solution of 2,2'-dipyridyl in ethanol was added aseptically to the growing culture after every 24 h to inhibit 9α-hydroxylase, an enzyme responsible for ring fission. The fermentation broths were taken out at different times and were extracted thrice with equal volumes of ethyl acetate. The extracts were dried over anhydrous sodium sulfate and distilled under reduced pressure to yield residue.

Preparation of samples

Accurately weighed and well-dried residues obtained after the extraction of fermentation broths were dissolved in a 2:1 solvent mixture of isopropyl alcohol and hexane followed by filtration through 0.2-µm filters before injection into the LC–MS system.

Preparation of stock and standard solutions

Stock solutions of the reference compounds (cholesterol, AD, and ADD) were prepared in a concentration of 3 mg/mL in a solvent mixture of isopropyl alcohol–hexane (2:1). These stock solutions were diluted and mixed in equal volumes to give concentrations in the range 100–5000 pg on the column. A 10-µL volume of each concentration (six injections of each concentration) was injected into the LC–MS for LC–APCI–MS analysis.

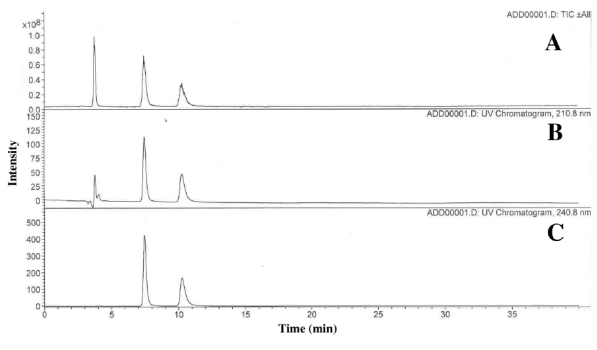

Figure 2. Total ion current trace of three reference compounds, cholesterol, AD, and ADD, under positive mode APCI (A), and the LC–UV–DAD chromatogram of the reference compounds at 210 nm (B) and 240 nm (C).

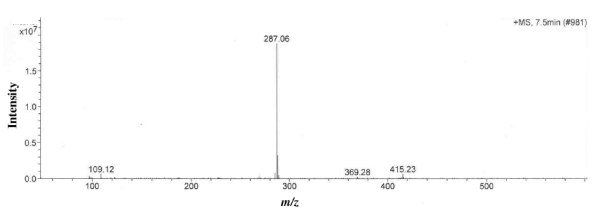

Figure 3. LC–APCI–MS of AD.

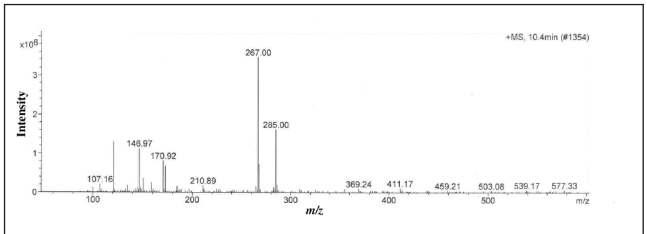

Figure 4. LC–APCI–MS of ADD.

Instrumentation

The HPLC system consisted of an Agilent 1100 instrument equipped with a binary pump, an autosampler, an automatic electronic degasser, an automatic thermostatic column oven, a diode array detector, and a computer with HP ChemStation software for analysis (Agilent, Palo Alto, CA). HPLC was used in combination with a quadrupole ion trap MS (Esquire 3000) from Bruker (Bremen, Germany). The MS was equipped with an APCI interface, a heated nebulizer, and Esquire control (version 5.0) software. High purity nitrogen from a nitrogen generator was used as the carrier gas.

LC–APCI–MS analysis

The LC separations were achieved using a silica Si_{60} (5 µm, 125 × 4-mm i.d.) Merck column (Merck, Darmstadt, Germany). The mobile phase consisted of isopropyl alcohol–hexane (2:3), which was delivered at a flow rate of 0.5 mL/min, and the entire LC flow was delivered into the mass detector. The column temperature was optimized at 30°C to achieve efficient separations. The UV chromatograms were recorded at 210 and 240 nm. All the interface parameters were optimized by injecting standard solutions of cholesterol, AD, and ADD during LC–APCI–MS experiments. The conditions for mass spectrum analysis during the LC–MS studies were optimized at a dry gas flow of 11 L/min with nebulizer pressure at 35 psi and drying gas temperature at 320°C, whereas the APCI heater temperature was 450°C. The mass range was from 50–500 *m/z*, the ion charge control target value was 8000, and the maximum accumulation time was 200 milliseconds. For flow injection (FI)–APCI–MS analysis, the values for dry gas flow and nebulizer were 6 L/min and 12 psi, respectively. During FI–APCI–MS analysis, only the values for these two parameters were changed, whereas values for all other parameters, such as drying gas temperature, APCI heater temperature, etc., were the same as in the case of the LC–APCI–MS experiments.

LC–UV-DAD–MS analysis

Using the previously mentioned conditions, reproducible results were obtained in positive mode LC–APCI–MS. Figure 2 shows the total ion chromatogram (TIC) (Figure 2A) and the LC–UV-DAD chromatogram at 210 (Figure 2B) and 240 nm (Figure 2C) of three reference compounds, namely, cholesterol, AD, and ADD, which were eluted at retention times of 3.8, 7.5 and, 10.4 min, respectively. During the LC–APCI–MS analysis, UV chromatograms were acquired at 210 and 240 nm. Cholesterol did not exhibit any absorbance at 240 nm. Figures 3–5 show LC–APCI–MS spectra of three reference compounds. The compounds AD and ADD exhibited positive ion $[M+H]^+$ mass spectra at *m/z* 285 and 287, respectively, whereas cholesterol exhibited protonated molecular ions at *m/z* 369 after the loss of water moiety from the molecule. Figure 6 shows the chromatograms of the sample wherein the presence of AD, ADD, and cholesterol has been observed.

Method development

Full-scan mass spectra were acquired under FI–APCI–MS analysis in the positive mode from *m/z* 50–500. The protonated ions $[M+H]^+$ at *m/z* 287 and 285 (Figures 7 and 8) for AD and ADD, and, similarly, the protonated ions after loss of water $[M–H_2O+H]^+$ at *m/z* 369 (Figure 9) for cholesterol were identified and considered for qualitative as well as quantitative LC–APCI–MS analysis by selected ion monitoring (SIM) detection.

Quantitation

All the three reference compounds were quantitated by means of an external standard calibration curve constructed at six calibration levels ranging from 100 to 5000 pg. Calibration curves were established by injecting six replicates of each concentration level of all the three reference compounds. Quantitation of the two reference compounds, namely, AD and ADD, was carried out using SIM detection of the $[M+H]^+$ ions, whereas $[M–H_2O+H]^+$ ions were used in SIM mode of detection for quantitation of cholesterol. Excellent calibration curves were obtained for all the reference compounds where R^2 = curve coefficients of 0.99660, 0.99979, and 0.99863 were obtained for cholesterol, AD and ADD, respectively.

Limit of quantitation (LOQ) values for the three reference compounds (i.e., cholesterol, ADD, and AD) were 38, 42, and 64 pg/µL, respectively, whereas limit of detection (LOD) values for these compounds were 12, 15, and 24 pg/µL, respectively. The method was validated by analyzing fermentation samples (six injections of each sample) fortified with cholesterol, AD, and ADD at two concentration levels of 0.05 and 0.5 µg/g. The mean recoveries ranged from 90% to 94% [coefficient of variation (CV) = 4.56% to 9.78%] for the samples fortified at concentration level 0.05 µg/g and 92% to 98% for samples fortified at concentration level 0.5 µg/g (CV = 3.36% to 6.57%).

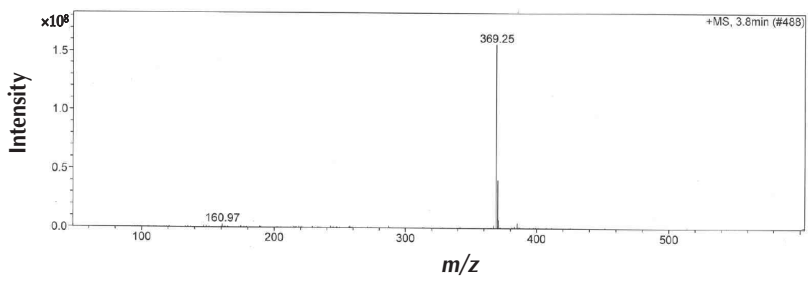

Figure 5. LC–APCI–MS of cholesterol.

Results and Discussion

Application of LC using MS was applied for the determination of cholesterol, AD, and ADD in the fermentation broths. This study also led to the possibility of using LC–MS via a heated nebulizer interface for simultaneous detection and quantitation of the three mentioned compounds. The optimization of the LC–APCI–MS method required a thorough evaluation of cholesterol, AD, and ADD ionization. A robust normal-phase LC separation method was devel-

oped which was compatible with MS conditions. The efficiency of the method was on expected lines as these compounds exhibited better ionization as well as compatibility under normal-phase LC conditions. It was observed that positive mode APCI is the most suitable ionization for the developed LC conditions. In order to evaluate the separation of cholesterol, AD, and ADD, a mixture of three reference compounds was prepared from the individual stock solutions and was diluted serially to obtain the required concentrations. The separation was achieved on a silica Si_{60} column using isopropyl alcohol–hexane (2:3) at a flow rate of 0.5 mL/min. The reasonable differences in the retention times of the three marker compounds facilitated their quantitation in the samples. It was necessary to use MS detection, as the method provides improved limits of detection for trace mixture analysis. The inherent selectivity of MS detection was also expected to be beneficial in developing a selective and sensitive method.

Fermentation of cholesterol by *Nocardia sp*. isolated from soil resulted in the production of two compounds (Figure 1), which were identified as AD and ADD on the basis of LC–MS data. The kinetics of the biotransformation of cholesterol to AD and ADD by *Nocardia sp*. under defined conditions has been studied. The yield of ADD ranged from 13.0% to 21.7% at an optimized substrate concentration of 0.5 g/L. LC–APCI–MS monitoring of the progress of the fermentations indicated that AD was formed at a rapid rate in the early stages of fermentation, but in later stages, it is converted to ADD by the dehydrogenating ability of organism at C1 of AD. It was observed that the *Nocardia sp*. produced good yields of AD in the yeast extract tryptone medium, although the yields were somewhat lower than those of ADD. The results have been detailed in Table I.

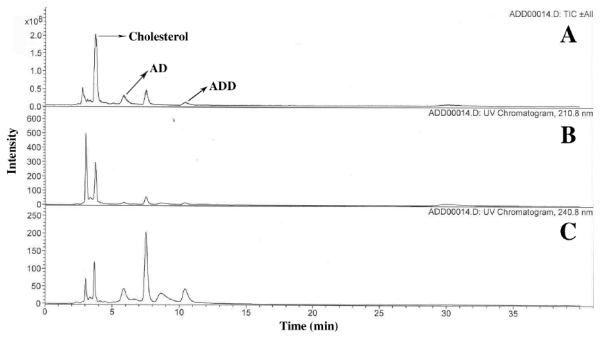

Figure 6. Total ion current trace of a fermentation broth sample (A) and LC–UV–DAD chromatogram of the same at 210 nm (B) and 240 nm (C) where the presence of cholesterol, AD, and ADD has been observed.

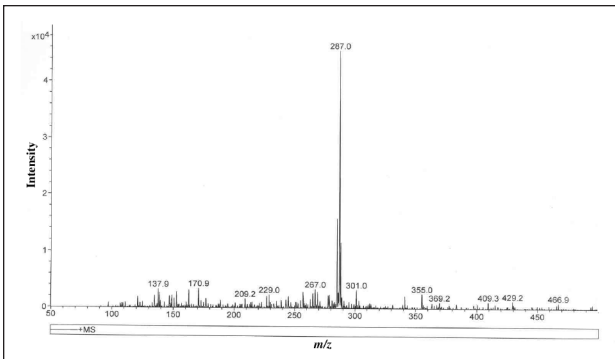

Figure 7. APCI–MS of AD.

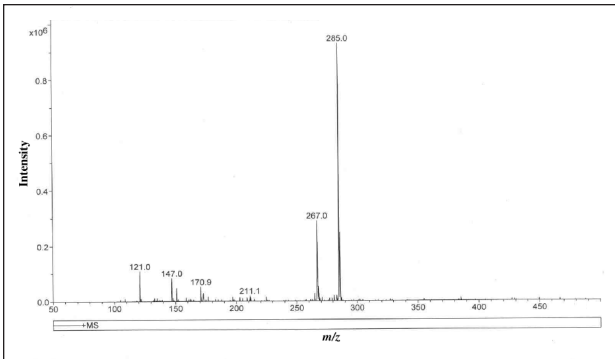

Figure 8. APCI–MS of ADD.

Conclusion

The aim of this study was to develop a specific, sensitive, and reliable LC–APCI–MS method for the simultaneous confirmatory analysis of cholesterol, AD, and ADD in fermentation broths without the need of a sample cleanup procedure. The unambiguous confirmation of these compounds in fermentation broth samples in the proposed method resulted in reten-

Table I. Kinetics of Cholesterol to AD and ADD

Time Duration	Residual Cholesterol	AD	ADD
24 h	0.2% (259.689 ng)	6.4% (5920.0 ng)	13.0% (12574.567 ng)
48 h	1.36% (1772.18 ng)	6.5% (6271.0 ng)	17.0% (16854.49 ng)
72 h	0.67% (824.84 ng)	8.0% (742.16 ng)	16.3% (15541.779 ng)
96 h	0.43% (547.42 ng)	0.6% (649.824 ng)	21.7% (20175.010 ng)

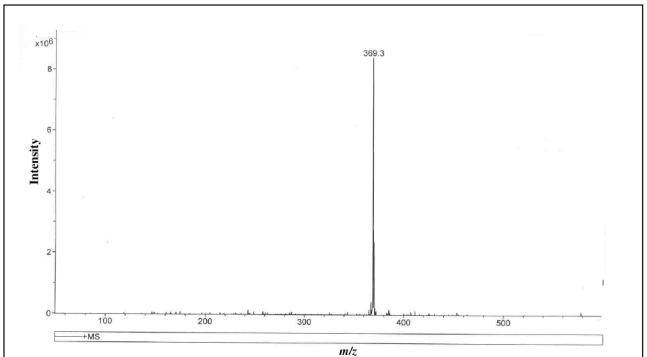

Figure 9. APCI–MS of cholesterol.

tion time information with the presence of protonated molecular ions for AD and ADD and also the protonated ions for cholesterol after the loss of water fragments. The LC–APCI–MS method was successfully used by our laboratory to analyze a large number of samples over a wide range of concentration, and it was found that the simplified sample preparation and sensitivity of the developed analytical method made it particularly suitable for routine analysis. Moreover, the technology of biotransformation of cholesterol under the influence of microorganisms like *Nocardia sp*. can provide us valuable intermediates, such as ADD and AD, for the production of steroidal hormones. These types of biotransformation technologies are attracting much attention, leading to the development of several useful processes. Further, the isocratic normal-phase LC applied in this method at a flow rate of 0.5 mL/min minimizes the efforts of sample cleanup as compared to HPLC, gas chromatography (GC), and GC–MS. The retention times were 3.8 min (cholesterol), 7.5 min (AD), and 10.4 min (ADD). Intense $[M+H]^+$ for AD and ADD and $[M-H_2O+H]^+$ ions for cholesterol were recorded. An ion trap mass spectrometer fixed with an orthogonal spray ionization source and a heated nebulizer was used as the detector under positive mode APCI. For all the quantitation purposes, the mass spectrometer was operated in SIM detection mode.

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