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Analysis of ergosteroids
VIII: Enhancement of signal response of neutral steroidal compounds in liquid chromatographic–electrospray ionization mass spectrometric analysis by mobile phase additives

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Abstract

The signal response of moderately polar to nonpolar neutral steroidal compounds in positive ion mode was significantly improved in electrospray ionization mode by addition of volatile organic acids (trifluoroacetic acid, acetic and formic) at concentrations much lower than those normally employed for high-performance liquid chromatographic separations of ionic compounds. Each of the three acids enhanced the sensitivity, the order being: formic acid (~50–200 ppm, v/v)>acetic acid (100–500 ppm)>trifluoroacetic acid (5–20 ppm). Higher concentrations caused decrease in the sensitivity. The extent of increase in the sensitivity was compound specific and also depended on the nature of organic modifier present in the mobile phase. Acetic acid was the acid of choice for the 'wrong-way-round' ionization of sulfate conjugates. The postcolumn addition of silver nitrate produced highly stable $(M+Ag)^+$ adducts with concomitant increase in signal response and reduction in baseline noise.

Keywords: Mobile phase composition; Ergosteroids; Steroids; Dehydroepiandrosterone; Trifluoroacetic acid; Formic acid; Acetic acid; Silver ions

1. Introduction

The advent of bench-top mass spectrometers incorporating soft ionization techniques, which can be coupled online to the high-performance liquid chromatography has opened new vistas in the study of highly polar and nonvolatile metabolites in intact form without prior derivatization. Atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are the most common techniques used to analyze small molecules ($M_r < 2000$). ESI-MS is increasingly becoming the method of choice for the analysis of a large variety of organic compounds in general, and polar, nonvolatile compounds in particular. However, the ESI technique is exclusively dependent on the ability of the molecule to be converted into an ion (positive or negative), and this phenomenon of ionization at high voltage and at the inter-phase of liquid and gas is a prisoner of mobile phase composition, pH, presence of additives, type of biomatrix etc.

Trifluoroacetic acid (TFA) has been used exten-

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sively to enhance high-performance liquid chromatographic performance. However TFA is not MS friendly and even low concentrations of TFA (0.1%; 1000 ppm, v/v; ~13 m*M*) are known to cause signal suppression [1–4]. It has been reported that regardless of the additive type, analyte responses decreased with increasing concentration (1–100 m*M*) of volatile organic acids (TFA, acetic acid and formic acid) with TFA being the worst [4]. The signal suppressing effect of TFA has been attributed to a combined effect of ion-pairing, high conductivity, and surface tension modifications caused by the presence of this strong acid in the spray chamber [1]. Apffel et al. [1] have discussed in detail the effect of TFA on ion generation including a method referred to as 'TFA fix' to improve the signal-to-noise ratio (10–100×). Current HPLC and LC–MS literature is replete with examples dealing with the use of TFA as the mobile phase additive for the LC–MS of peptides, proteins and polar (acidic and basic) compounds. Ma and Kim [5] observed a slight increase in the sensitivity of steroids in LC–APCI-MS when 1–2% acetic acid was added to the mobile phase. They observed that in the ESI mode the best sensitivity and lowest limit of detection for steroids having keto and hydroxy groups were obtained when pure methanol and water without a trace of acidity were used as mobile phase. To the best of our knowledge no study has been carried out on the influence of trace amounts of TFA and other volatile organic acids on the sensitivity of LC–ESI-MS for the analysis of nonpolar to moderately polar neutral compounds. We report here that addition of very low concentrations of commonly used volatile acids such as TFA (~10 ppm; 0.001% or ~0.1 m*M*), acetic acid (~250 ppm; 0.025% or 5 m*M*) and formic acid (~100 ppm; 0.01% or 2.7 m*M*) can result in a dramatic increase in the sensitivity in LC–MS; and even nonpolar fatty acid esters of steroids can be analyzed by LC–ESI-MS by adding a minuscule amount of acid to the mobile phase. We have also discovered that the postcolumn addition of metal salts such as silver can also increase the sensitivity several fold. Postcolumn addition of alkali metal chlorides and cobalt chloride in LC–ESI-MS–MS of carbohydrates has been reported [6]. The technique was used to enhance sensitivity and also, in the case of cobalt coordination, to assist in carbohydrate structural elucidation. We also studied the 'wrong-way-round' ionization of sulfate conjugates derived from dehydroepiandrosterone (DHEA; I) and its metabolites. LC–ESI-MS of steroid sulfate conjugates has traditionally been carried out using ammonium acetate buffer [7–12] in combination with methanol [7–10] or acetonitrile [9–12]. Murray et al. reported [13] microbore LC–ESI-MS of twenty-five steroid sulfates using acetonitrile–water as the mobile phase. We observed that a mixture of steroid sulfates can be satisfactorily resolved in HPLC and estimated by ESI-MS by addition of acetic acid (~3%) to the mobile phase.

The present study is an offshoot of our ongoing efforts to find active metabolites of DHEA (I), which might qualify for the appellation 'steroid hormone'. Because large doses of DHEA (I) are needed to produce observed pharmacological effects [14–21], it may serve as a precursor of steroids with other activities just as it serves as a precursor of testosterone and estrogens. We were particularly distressed by the lack of sensitivity of LC–MS in ESI mode for nonpolar and weakly polar compounds such as DHEA, Δ^5-Diol, and fatty acid esters of DHEA and its derivatives. This necessitated frequent change of ionization chambers from ESI to APCI resulting in longer shut down times, besides it was difficult to analyze a sample which contained a mixture of nonpolar and polar derivatives of DHEA. The hydrolysis of fatty ester derivatives permitted their analysis in ESI mode but it resulted in loss of vital structural information. This publication is a successful culmination of our efforts to increase the sensitivity of weakly polar and nonpolar compounds in LC–ESI-MS by using volatile organic acids and silver metal salts.

2. Experimental

2.1. Chemicals

DHEA (I) and 5α-H-Diol (XI, 5α-H-androstane-3α,17β-diol) were purchased from Steraloids, USA. Δ^5-diol (II, androst-5-ene-3β,17β-diol), 7α-OH-DHEA (III), 7-oxo-DHEA (IV), 7β-OH-DHEA (V), 7-oxo-diol (VI, 3β,17β-dihydroxyandrost-5-en-7-one), 7α-Triol (VII, androst-5-ene-3β,7α,17β-triol), and 7β-triol (VIII, androst-5-ene-3β,7β,17β-triol)

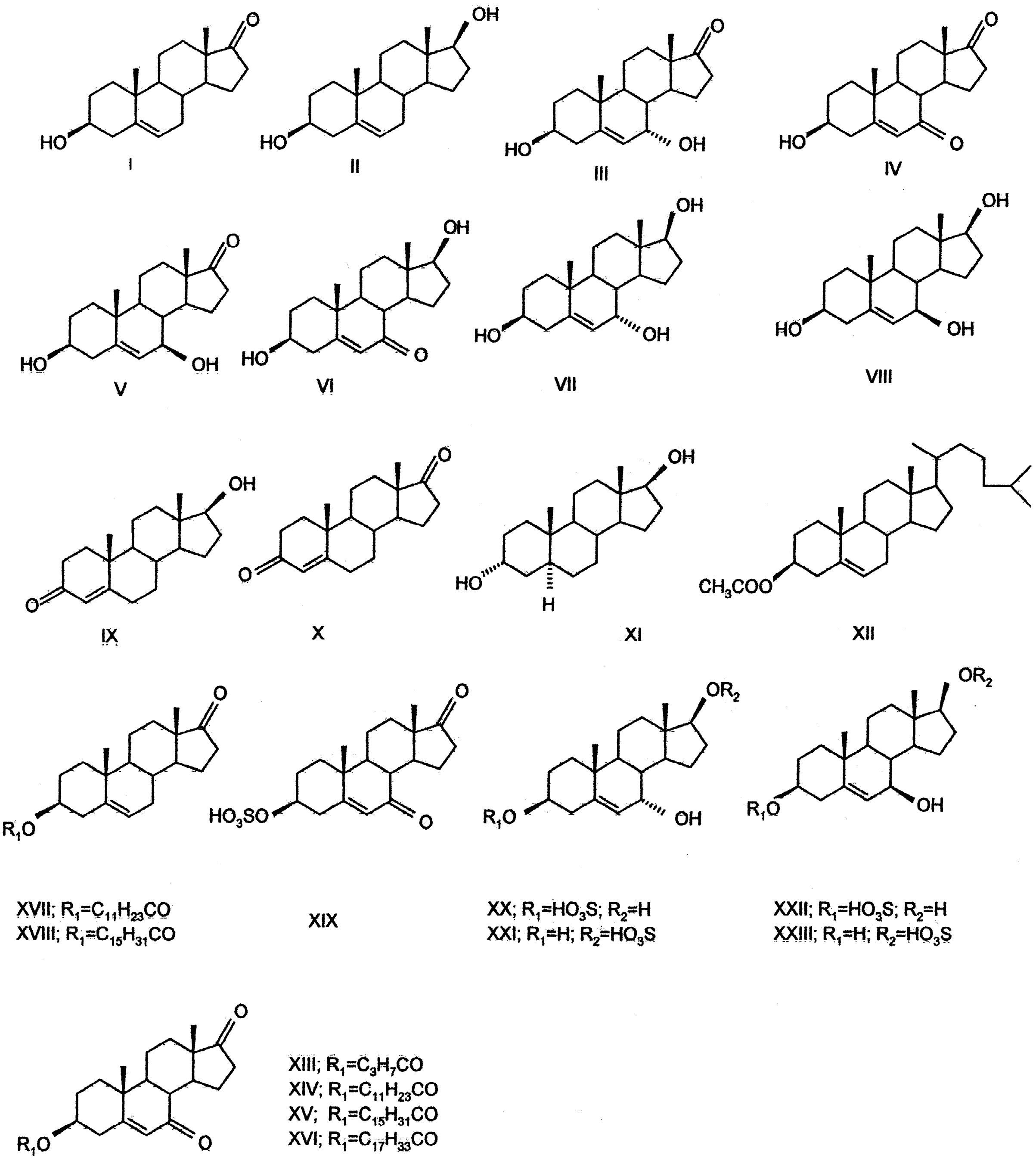

Fig. 1. Chemical structures of the steroids used in the study. I, DHEA; II, Δ^5-diol; III, 7α-OH-DHEA; IV, 7-oxo-DHEA; V, 7β-OH-DHEA; VI, 7-oxo-Diol; VII, 7α-Triol; VIII, 7β-Triol; IX, testosterone; X, Δ^4-dione; XI, 5α-H-diol; XII, cholesterol acetate; XIII, 7-oxo-DHEA-3β-butyrate; XIV, 7-oxo-DHEA-3β-laurate; XV, 7-oxo-DHEA-3β-palmitate; XVI, 7-oxo-DHEA-3β-stearate; XVII, DHEA-3β-laurate; XVIII, DHEA-3β-palmitate; XIX, 7-oxo-DHEA-3β-sulfate; XX, 7α-triol-3β-sulfate; XXI, 7α-triol-17β-sulfate; XXII, 7β-triol-3β-sulfate; XXIII, 7β-triol-17β-sulfate.

were synthesized by known procedures as reported earlier [22]. Testosterone (IX) and Δ^4-dione (X, androst-4-ene-3,17-dione) were purchased from Sigma. Cholesterol acetate (XII), 7-oxo-DHEA-3β-butyrate (XIII), 7-oxo-DHEA-3β-laurate (XIV), 7-oxo-DHEA-3β-palmitate (XV), 7-oxo-DHEA-3β-stearate (XVI), DHEA-3β-laurate (XVII) and DHEA-3β-stearate (XVIII) were synthesized in our laboratory [23,24]. The structures of the synthesized compounds were confirmed by nuclear magnetic resonance (^{1}H and ^{13}C) and mass spectrometric data; their purity was checked by TLC and by LC–MS, and was found to be more than 99%. 7-oxo-DHEA-3-S (XIX, 7-oxo-DHEA-3β-sulfate), 7α-triol-3β-S (XX, 3β-sulfooxyandrost-5-en-7α,17β-diol), 7α-triol-17β-S (XXI, 17β-sulfooxyandrost-5-en-3β,7α,-diol), 7β-triol-3β-S (XXII, 3β-sulfooxyandrost-5-en-7β,17β-diol), 7β-Triol-17β-S (XXIII, 17β-sulfooxyandrost-5-en-3β,7β-diol) were synthesized in our laboratory and their identity and structure confirmed by their mode of synthesis [24] and by an on-line UV diode array detection system (DAD) spectra and LC–ESI-MS. Fig. 1 gives the structures of various steroids used in the present study. Sodium acetate, methanol and acetonitrile were of HPLC grade (Aldrich). Acetic acid (Fisher) was of HPLC grade and trifluoroacetic acid (Fluka) was of spectrophotometric grade. All other reagents were of analytical-reagent grade and were used as such.

2.2. Instrumentation

The chromatographic system consisted of an Agilent 1100 series LC–MS system, comprised of a capillary pump (G1376A) with an on-line microdegasser (G1379A) operated in normal mode, a quaternary pump (G1311A) with an on-line degasser (G1322A) used for postcolumn additions, a column oven (G1316A), an autosampler (G1313A), a diode array detection system (G1315A), a mass detector (G1946A) and a Gilson fraction collector (FC-203B). Data were acquired and processed using LC–MSD CHEMSTATION version A.08.03 software.

2.3. Chromatographic conditions

The chromatographic conditions used in the study were as varied as the nature of the compounds. Flow-injection analysis (FIA) was extensively used to optimize the LC–MS parameters of chemically pure steroid derivatives i.e. DHEA (I) and its polar and nonpolar derivatives, and to study the influence of various modifiers and additives on the sensitivity. FIA was carried out using Zorbax SB-C_{18} and SB-CN cartridge columns and methanol–water or acetonitrile–water as mobile phases. A mixture consisting of DHEA (I) and nine of its metabolites was resolved on Zorbax SB C_{18} column. Fatty acid esters of DHEA (I) and 7-oxo-DHEA (IV) were resolved on a cyano column (Zorbax-CN, 75×4.6 mm, 3.5 μm) using a methanol–water gradient in the ESI and APCI modes and acetonitrile–water gradient in the ESI mode. Sulfate conjugates (XX–XXIII) of 7(α+β)-triol (VII+VIII) were resolved on a Zorbax-SB C_{18} column (75×4.6 mm, 3.5 μm) using acetonitrile and 3% aqueous acetic acid gradient. The steroids were monitored at 205 and 240 nm with a reference wavelength of 360 nm. The neutral compounds were analyzed using ESI in the positive mode. Operating conditions were optimized by FIA. The nitrogen gas flow was maintained at 10–13 l/min with a drying temperature of 350 °C and nebulizer was maintained at 40–50 p.s.i. (276–345 kPa) in the ESI mode. The samples were run in the scan and the SIM (selected ion monitoring) modes. The sulfate conjugates were analyzed in the negative ion mode (ESI). DHEA (I) was also analyzed by LC–APCI-MS. The operating conditions were (FIA): N_2 gas flow 3.5 l/min at 350 °C; nebulizer 30 p.s.i. (207 kPa); capillary voltage 300 V; fragmentor 80 V; corona current 6 μA; vaporizer temperature 450 °C. The HPLC and MS conditions used for various compounds are summarized in Table 1.

The postcolumn additions were made at ~10–25% of the HPLC flow-rate (0.075–0.2 ml/min). The volatile organic acids were either mixed in mobile phase or were added postcolumn. Sodium acetate, lithium acetate, cesium chloride, lanthanum chloride, silver nitrate, etc. were all freshly prepared solutions (filtered through 0.45-μm membrane) of 1 m*M* concentration. Lower concentrations were prepared by serial dilution. Silver nitrate solution was protected from light by covering the containers with aluminum foil and by switching off overhead lights. The metal salts solutions were added postcolumn only. The ESI ion source was cleaned every day

Table 1
LC–MS conditions for the compounds

Compound[a]	Column[b]	Column temp. (°C)	Mobile phase[c]	Flow (ml/min)	Fragmentor (V)	V_{cap} (V)
I	C-1	25	M-1	0.6	80	5000
I	C-1	25	M-2	0.6	80	5000
II	C-1	25	M-1	0.6	130[d]	5000
II	C-1	25	M-2	0.6	60	5000
VIII	C-2	25	M-3	0.6	80	4500
VIII	C-1	25	M-4	0.6	80	4500
IX	C-3	40	M-5	0.8	80	4500
XI	C-1	25	M-6	0.6	60	5000
XII	C-2	25	M-7	0.8	100	5000
XIII–XVIII	C-4	30	M-8	0.8	100	4500
XIII–XVIII	C-4	35	M-9	0.6	100	4000
XIII–XVIII	C-4	35	M-9	0.6	100	3000[e]
XIX	C-1	25	M-10	0.5	80	3000[f]
XX–XXIII	C-3	40	M-11	0.8	80	3000[f]

[a] Compounds: I = DHEA; II = Δ^5-Diol; III = 7α-OH-DHEA; IV = 7-oxo-DHEA; V = 7β-OH-DHEA; VI = 7-oxo-diol; VII = 7α-Triol; VIII = 7β-triol; IX = testosterone; X = Δ^4-dione; XI = 5α-H-diol; XII = cholesterol acetate; XIII = 7-oxo-DHEA-3β-butyrate; XIV = 7-oxo-DHEA-3β-laurate; XV = 7-oxo-DHEA-3β-palmitate; XVI = 7-oxo-DHEA-3β-stearate; XVII = DHEA-3β-laurate; XVIII = DHEA-3β-palmitate; XX = 7α-triol-3β-S; XXI = 7α-triol-17β-S; XXII = 7β-triol-3β-S; XXIII = 7β-triol-17β-S.

[b] Columns: C-1 = Zorbax SB C_{18}, 4.6×12.5 mm, 5 μm; C-2 = Zorbax SB CN, 4.6×12.5 mm, 5 μm; C-3 = Zorbax SB C_{18}, 4.6×75 mm, 3.5 μm; C-4 = Zorbax SB CN, 4.6×75 mm, 3.5 μm.

[c] Mobile-phases: M-1 = methanol–water (70:30) isocratic; M-2 = acetonitrile–water (50:50) isocratic; M-3 = methanol–water (50:50) isocratic; M-4 = acetonitrile–water (30:70) isocratic; M-5 = acetonitrile–water gradient, 20–50% acetonitrile in 30 min; M-6 = methanol–water with 0.002% acetic acid (70:30) isocratic; M-7 = acetonitrile–water (80:20) isocratic; M-8 = acetonitrile–water gradient, 50–100% in 10 min; M-9 = methanol–water gradient with 0.002% acetic acid, 80–90% in 10 min; M-10 = acetonitrile–water (25:75) isocratic; M-11 = acetonitrile–water gradient with 3% acetic acid, 10–35% acetonitrile in 25 min.

[d] Fragmentor voltage with 0.1 m*M* silver nitrate at 66 μl/min postcolumn addition; and 60 V without postcolumn addition of silver nitrate.

[e] APCI mode: nebulizier 20 p.s.i. (138 kPa), N_2 gas flow 4 l/min at 350 °C, vaporizer 350 °C, corona 6 μA.

[f] Negative ion mode.

when Ag^+ (1–10 n*M*) was used. The use of higher concentrations of Ag^+ (~100 n*M*) required more frequent cleaning.

2.4. Stock solutions

The standard stock solutions (1 mg/ml) were prepared by dissolving accurately-weighed 10–25 mg of steroid samples in a volume of methanol to form 1 mg/ml solutions. Working solutions were prepared by serial dilution in the concentration range of 1–1000 ng in 10 μl methanol.

2.5. Data analysis

The peak heights of compounds were compared to determine the increase in the sensitivity. All comparisons were made intra-run analysis only. The analyses were carried out in duplicate or triplicate.

3. Results and discussion

3.1. Addition of trifluoroacetic acid, acetic acid and formic acid

The signal suppression by volatile organic acids normally employed in LC–MS in general, and by trifluoroacetic acid even at low concentrations of ~1000 ppm in particular, is well known, and has been discussed in the Introduction. However, we observed that sensitivity of neutral steroid molecules can be increased several fold by adding the same acids at low concentrations (~1–500 ppm depending on the acid). We studied the effect of organic modifiers (methanol and acetonitrile) on the sensitivity in presence of different acids. Metal salts were added postcolumn. The organic acids were added either postcolumn or mixed with mobile phase. Therefore, we also studied the effect of postcolumn addition of water to rule out change in sensitivity caused by postcolumn addition of water. Postcolumn addition of water in general resulted in some increase in sensitivity (~10–50%). The mobile phase usually tolerated ~10–25% addition of water, the actual value depended on the nature and concentration of organic modifier, and it was practically evaluated before carrying out analysis of individual compounds. In the FIA involving short columns and isocratic system the solvent composition was decided by first carrying out the analysis on regular columns by gradient analysis normally employed by us for our metabolic studies. The solvent composition for the peak of interest was determined and was used for the FIA analysis.

Acetonitrile–water systems usually caused signal suppression for various ergosteroids, sometimes by more than an order of magnitude when compared to methanol–water systems. However, chromatographic requirements do not always permit the use of methanol–water system as the mobile phase of choice. We have reported earlier that we were not able to sufficiently resolve 7α-triol (VII) from 7β-triol (VIII) using methanol–water gradient but the resolution could be easily achieved by replacing methanol with acetonitrile [22]. Postcolumn addition of TFA to the mobile phase even at 1 ppm resulted in a dramatic increase in the signal response for several of the ergosteroids; the best response (>10 fold increase in sensitivity) was achieved at ~10 ppm concentration of TFA. Fig. 2 shows the effect of TFA concentrations on the signal response [total ion current (TIC) mode] of DHEA (I), Δ^5-diol (II) and 7β-triol (VIII). In case of 7β-triol (VIII), a polar metabolite of DHEA (I), maximum response peaked at ~5 ppm concentration of TFA. Higher concentrations of TFA (>5 ppm in this case and >20 ppm

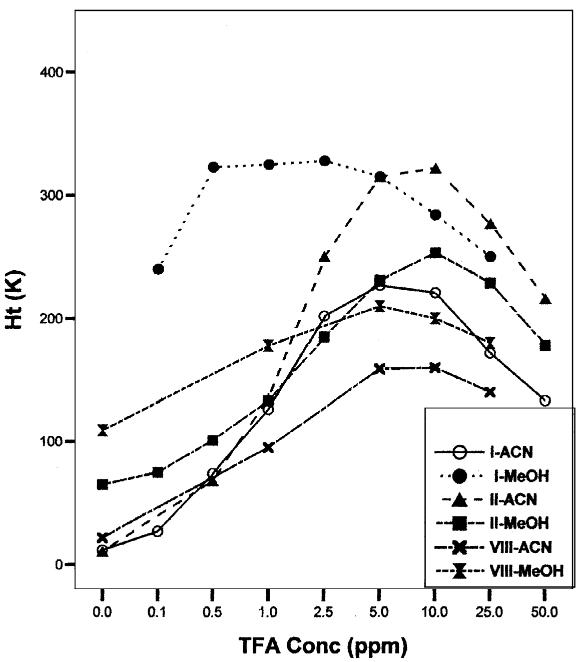

Fig. 2. Effect of trifluoroacetic acid on the signal response of DHEA (I), Δ^5-diol (II) and 7β-triol (VIII) in LC–MS using methanol–water and acetonitrile–water systems. The *x*-axis is categorical.

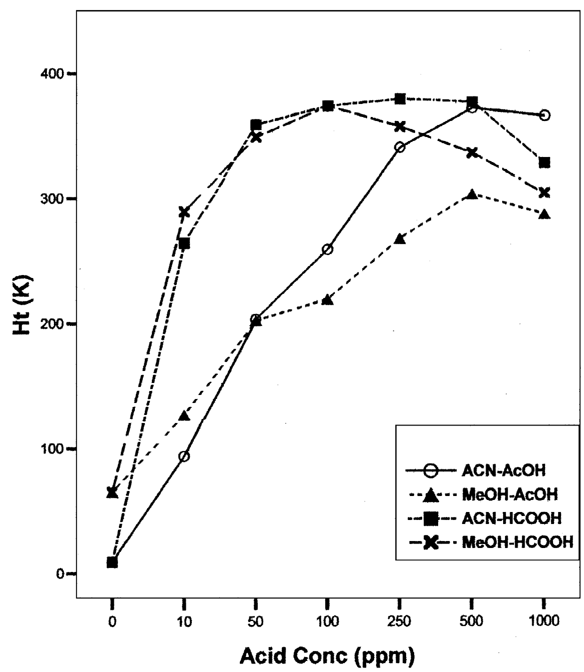

Fig. 3. Effect of formic acid and acetic acid on the signal response of DHEA (I) in LC–MS using methanol–water and acetonitrile–water systems. ACN, acetonitrile; MeOH, methanol; AcOH, acetic acid. The *x* axis is categorical.

in general) were found to cause signal suppression in LC–MS. At high concentrations (>0.1% or >1000 ppm) baseline noise increased several fold making the whole exercise futile. This behavior of TFA at normally employed concentrations of TFA (0.1–0.5%) has been documented [1,25,26] and has been attributed to high conductivity and surface tension of eluents.

Such a dramatic increase in mass signal of neutral steroid molecules caused by minuscule amounts of TFA (1–10 ppm) prompted us to study the effect of other commonly used organic acids such as acetic acid and formic acid on the sensitivity of ergosteroids. Both acids performed better than TFA with formic acid being the best (Figs. 3–5), though in the case of 7β-triol (VIII), acetic acid was marginally better (~5%) than formic acid when methanol was used as organic modifier (Fig. 5). The concentration required for optimum performance was 50–200 ppm for formic acid and 200–500 ppm for acetic acid. Higher concentrations caused peak suppression; and generally methanol was the better mobile phase modifier.

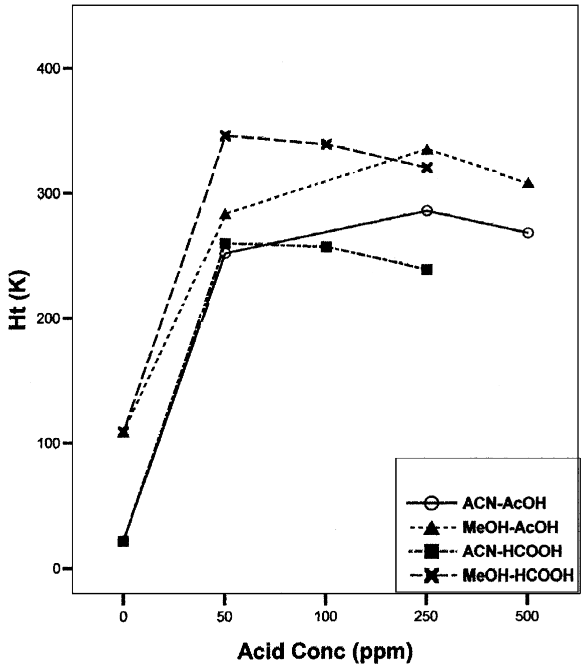

Fig. 5. Effect of formic acid and acetic acid on the signal response of 7β-triol (VIII) in LC–MS using methanol–water and acetonitrile–water systems. ACN, acetonitrile; MeOH, methanol; AcOH, acetic acid. The *x*-axis is categorical.

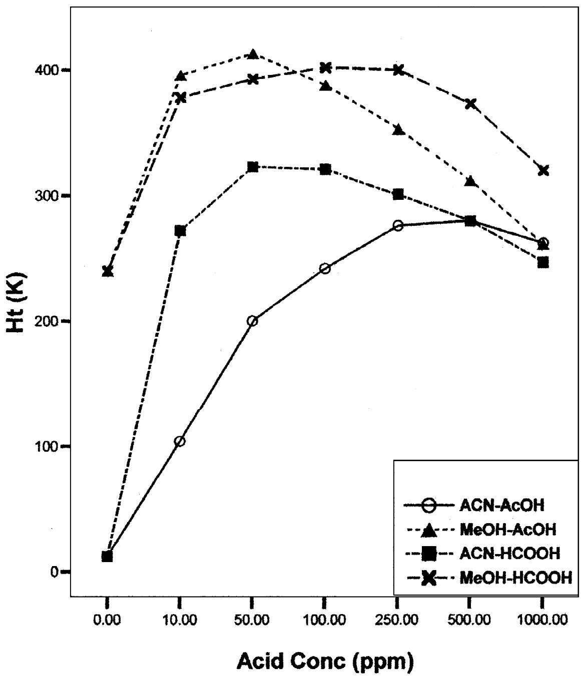

Fig. 4. Effect of formic acid and acetic acid on the sensitivity of Δ^5-Diol (II) in LC–MS using methanol–water and acetonitrile–water systems. ACN, acetonitrile; MeOH, methanol; AcOH, acetic acid. The *x*-axis is categorical.

Fig. 6 shows an extracted ion chromatogram of DHEA (I) and nine of its metabolites namely DHEA [I, *m/z* 271 $(M+H-H_2O)^+$], Δ^5-diol [II, *m/z* 273 $(M+H-H_2O)^+$], 7α-OH-DHEA [III, *m/z* 269 $(M+H-2H_2O)^+$], 7-oxo-DHEA [IV, *m/z* 303 $(M+H)^+$], 7β-OH-DHEA [V, *m/z* 269 $(M+H-2H_2O)^+$], 7-oxo-diol [VI, *m/z* 305 $(M+H)^+$], 7α-triol [VII, *m/z* 271, $(M+H-2H_2O)^+$], 7β-triol [VIII, *m/z* 271 $(M+H-2H_2O)^+$], testosterone [IX, *m/z* 289 $(M+H)^+$] and Δ^4-dione [X, *m/z* 287 $(M+H)^+$]. The liquid chromatography was conducted on a C_{18} reversed-phase column using an acetonitrile–water gradient. Fig. 6a shows the extracted ion chromatogram with postcolumn addition of water at 10% of the flow-rate while Fig. 6b shows the chromatography of the same mixture with postcolumn addition of formic acid (0.1% formic acid at 11% of the flow-rate thereby giving an effective concentration of 100 ppm, v/v). As seen from Fig. 6a and b, the

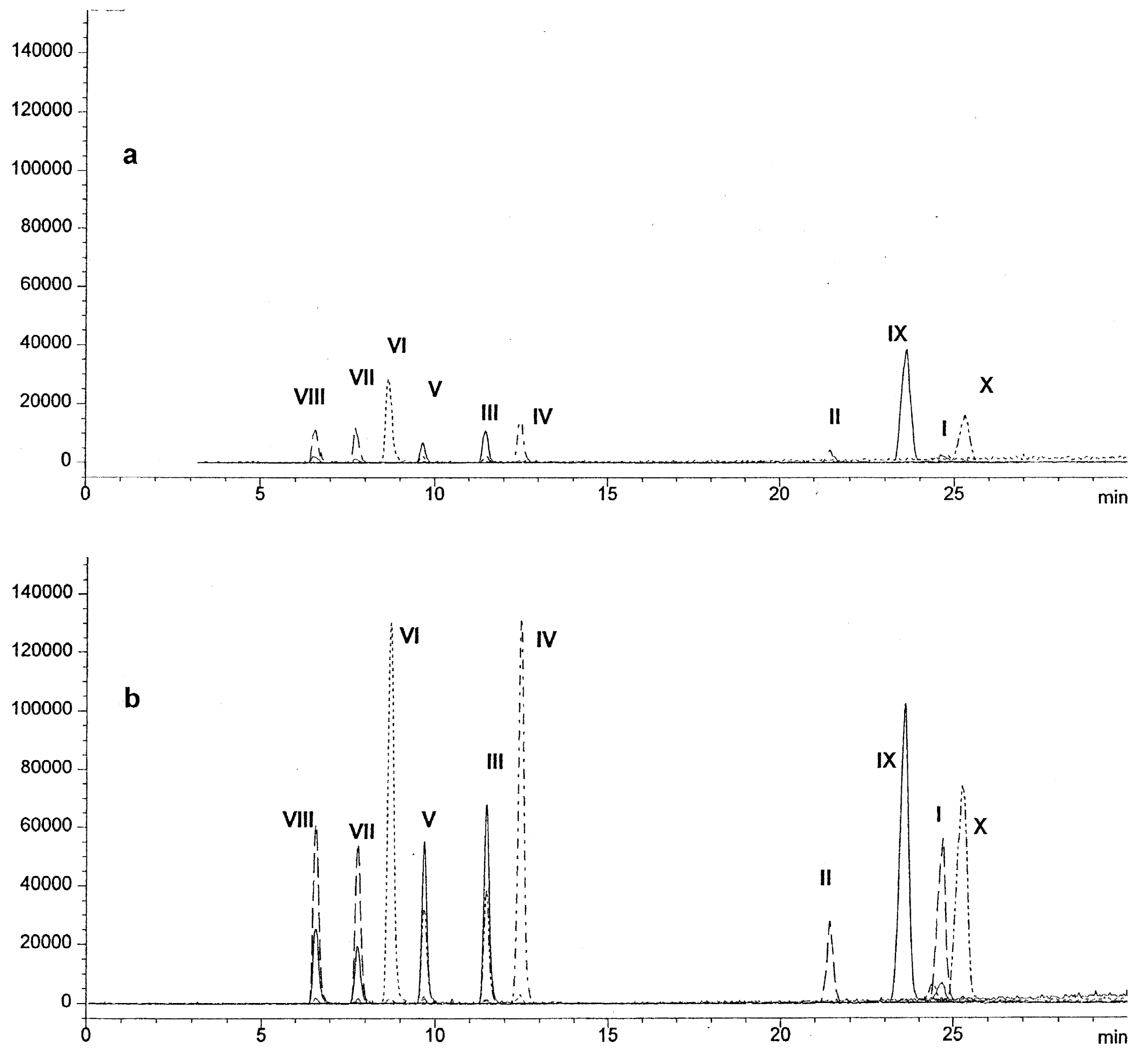

Fig. 6. (a and b) Increase in the sensitivity of DHEA (I) and nine of its metabolites in the presence of formic acid (100 ppm). Extracted ion chromatogram. Compounds, m/z (extracted ion): I, DHEA, 271 $(M+H-H_2O)^+$; II, Δ^5-diol, 273 $(M+H-H_2O)^+$; III, 7α-OH-DHEA; 269 $(M+H-2\ H_2O)^+$; IV, 7-oxo-DHEA, 303 $(M+H)^+$; V, 7β-OH-DHEA, 269 $(M+H-2H_2O)^+$; VI, 7-oxo-diol, 305 $(M+Na)^+$; VII, 7α-Triol, 271 $(M+H-2H_2O)^+$; VIII, 7β-triol 271 $(M+H-2H_2O)^+$; IX, testosterone, 289 $(M+H)^+$; X, Δ^4-dione, 287 $(M+H)^+$, 50 ng each. LC–MS conditions: Column Zorbax-SB C_{18}, 75×4.6 mm, 3.5 μm at 40 °C; Mobile phase 20–50% acetonitrile in 30 min at 0.8 ml/min flow-rate; Nebulizer 50 p.s.i. (345 kPa); Drying gas (N_2) 13 l/min at 350 °C; Fragmentor 80 V; V_{cap} 4500 V; scan mode. Postcolumn addition of water (a) and 0.1% formic acid (b) at 0.088 ml/min.

addition of formic acid resulted in several fold increases in the sensitivity of all ten compounds. This also allowed us to conclude that trace level concentrations of acids are effective in generating the positive ions of neutral molecules in ESI techniques.

We next devoted our attention to the LC–ESI-MS study of nonpolar derivatives of DHEA (I) and related compounds and in presence of small amounts of volatile organic acids. It is well known that neutral weakly polar compounds are better substrates for APCI [27]; for nonpolar fatty acid derivatives of DHEA (I) and related compounds, APCI is the only technique of choice. During a study of the hydrolysis of fatty acid derivatives of ergosteroids by isolated rat brain and liver [28], we encountered mixtures of nonpolar and polar steroids. The fatty esters present in the mixture could not be analyzed by ESI, and this necessitated frequent change of ionization chambers. So we decided to study the nonpolar compounds by ESI after addition of small amounts of acids to the eluent expecting that the oxygen atom present in ester function will act as a substrate for the proton. To check this hypothesis we first studied the LC–ESI-MS of cholesterol acetate (XII), in presence of organic acids. Fig. 7a shows FIA analysis (in triplicate) of cholesterol acetate (1 ng on column) in SIM mode (m/z at 451). Addition of formic acid (200 ppm) resulted in more than twenty-fold increase (Fig. 7b) in the signal thereby making it possible to analyze this compound by ESI in subnanogram quantities. With the top of the line mass detectors now available in the market, it should be possible to achieve another 5–10-fold increase in the sensitivity. We next studied the analysis of a mixture of ester derivatives of DHEA (I) and 7-oxo-DHEA (IV). The mixture consisted of 7-oxo-DHEA-3β-butyrate (XIII), 7-oxo-DHEA-3β-laurate (XIV), 7-oxo-DHEA-3β-palmitate (XV), 7-oxo-DHEA-3β-stearate (XVI), DHEA-3β-laurate (XVII) and DHEA-3β-stearate (XVIII). Attempts to resolve them on a C_{18} column failed, but we were able to separate these esters on a cyano column using methanol–water and acetonitrile water gradients. The acetonitrile–water gradient did not resolve 7-oxo-DHEA-3β-palmitate from DHEA-3β-laurate, but it was still possible to differentiate between them on the basis of extracted ion analysis. Fig. 8 illustrates that the postcolumn addition of formic acid (200 ppm) greatly improved the signal response (TIC) of the above esters, so that it became possible to analyze them by ESI mode. The LC–MS data (retention time, λ_{max}, characteristic ions in mass and relative abundances) of these ester derivatives have been summarized in Table 2. An important feature in the analysis of these esters was

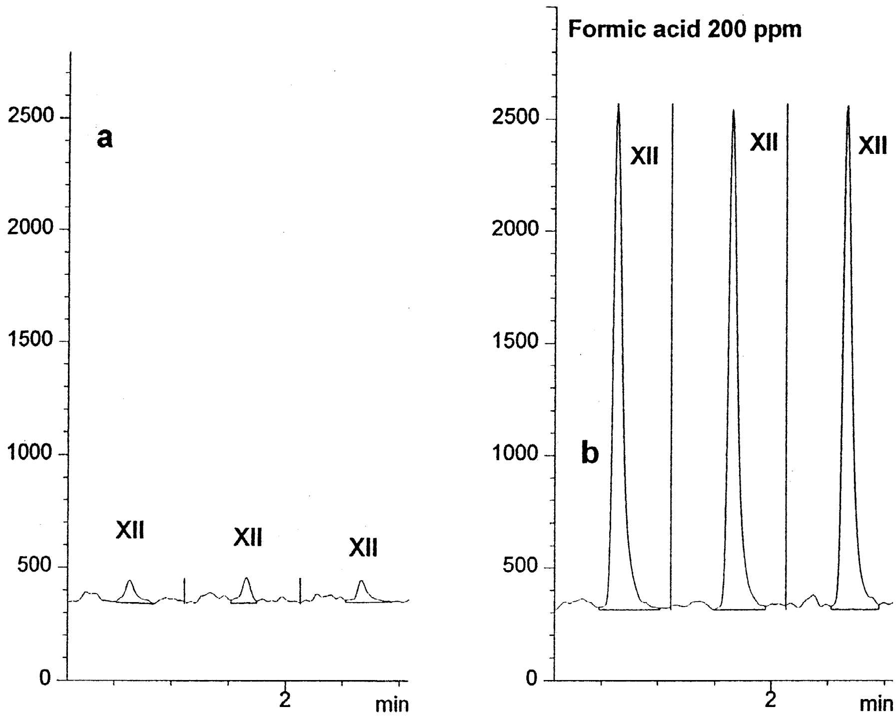

Fig. 7. (a and b) Flow-injection analysis of cholesterol acetate (XII, 1 ng) in selected ion monitoring mode at m/z 451 $(M+Na)^+$ with (b) and without (a) addition of formic acid (100 ppm). LC–MS conditions: Column Zorbax-SB CN, 12.5×4.6 mm, 5 μm at 25 °C; Mobile phase acetonitrile–water (80:20) at 0.8 ml/min flow-rate; Nebulizer 45 p.s.i. (310 kPa); drying gas (N_2) 12 l/min at 350 °C; fragmentor 100 V; V_{cap} 5000 V. Postcolumn addition of water–0.1% formic acid at 0.2 ml/min.

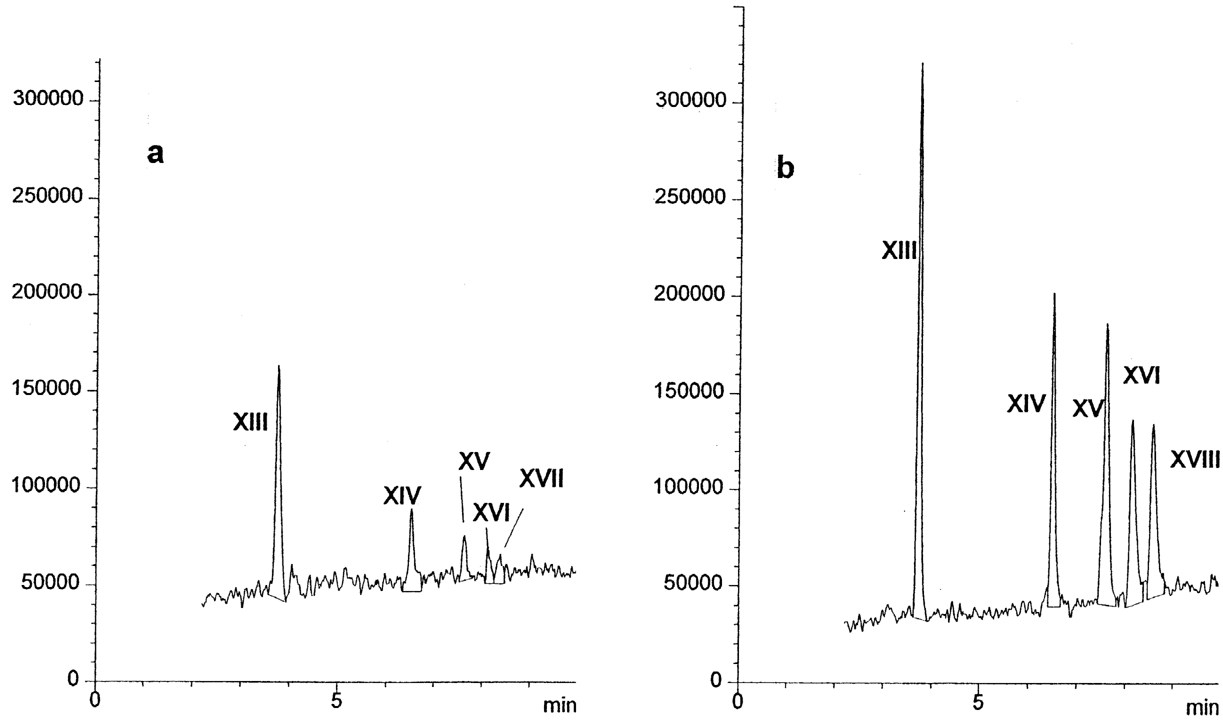

Fig. 8. (a and b) Increase in the signal response of carboxylic acid esters of DHEA (I) and 7-oxo-DHEA (IV) in presence of formic acid (200 ppm); 40 ng each. Compounds: XIII 7-oxo-DHEA-3β-butyrate; XIV 7-oxo-DHEA-3β-laurate; XV 7-oxo-DHEA-3β-palmitate; XVI 7-oxo-DHEA-3β-stearate; XVIII DHEA-3β-palmitate. LC–MS conditions: column Zorbax-SB CN, 75×4.6 mm, 3.5 μm at 30 °C; Mobile phase 50–100% acetonitrile in 10 min at 0.8 ml/min flow-rate; Nebulizer 40 p.s.i. (276 kPa); Drying gas (N_2) 12 l/min at 350 °C; Fragmentor 100 V; V_{cap} 4000 V. Postcolumn addition of water (a) and 0.1% formic acid (b) at 0.2 ml/min.

the presence of molecular ions (as sodium adduct) in their ESI mass spectra. In the APCI mode we found it difficult to obtain molecular ion of these compounds under a single set of parameters. The parent compound DHEA (I) was analyzed using ESI-MS as well as APCI-MS using methanol–water as mobile phase. DHEA was found to be somewhat more sensitive (~25%) in the ESI-SIM mode with post-column addition of formic acid. The compounds need to be studied individually to arrive at appro-

Table 2
LC–MS analysis of esters of DHEA (I) and 7-oxo-DHEA (IV)

Compound[a]	t_R (min)	Characteristic ions in mass m/z, relative abundance	DAD λ_{max} (nm)
XII	3.72[b], 2.57[c]	285 $(M+H-\text{butyric acid})^+$, 100; 395 $(M+Na)^+$, 27	238
XIV	6.50, 4.82	285 $(M+H-\text{lauric acid})^+$, 100; 507 $(M+Na)^+$, 35	236
XV	7.60, 6.82	285 $(M+H-\text{palmitic acid})^+$, 100; 563 (M+Na), 33	236
XVI	8.14, 8.00	285 $(M+H-\text{stearic acid})^+$, 100; 591 $(M+Na)^+$, 43	236
XVII	7.60, 6.30	271 $(M+H-\text{lauric acid})^+$, 28; 493 $(M+Na)^+$, 100	–
XVIII	8.57, 8.60	271 $(M+H-\text{palmitic acid})^+$, 41; 549 $(M+Na)^+$, 100	–

[a] Compounds: XIII, 7-oxo-DHEA-3β-butyrate; XIV, 7-oxo-DHEA-3β-laurate; XV, 7-oxo-DHEA-3β-palmitate; XVI, 7-oxo-DHEA-3β-stearate; XVII, DHEA-3β-laurate; XVIII, DHEA-3β-palmitate.
[b] Acetonitrile–water gradient, 50–100% in 10 min.
[c] Methanol–water gradient, 80–90% in 10 min.

priate conclusions, as the sensitivity of the mass detector is highly compound specific and dependent on the experimental conditions. The detection limits (defined as signal-to-noise ratio of 3 to 1) do not necessarily parallel the TIC sensitivities. The LC–MS analysis of a group of sixty steroids in the APCI and ESI modes has been reported [5].

3.2. Metal ion adducts

The formation of adduct ions in ESI-MS has been well documented [29,30]. The formation of sodium adduct ions of corticosterone was exploited by us for its quantitative determination in plasma at picogram levels [31]. We observed that sodium adduct formation is a function of mobile phase [5] as well as of structure of the ergosteroids. In general, the methanol–water system favored the formation of sodium adduct when compared to the acetonitrile–water system. As we discussed in an earlier publication, presence of a hydroxyl group at position sixteen favored the formation of a sodium adduct, but not when the hydroxyl group was present at the seven position [22]. In the present study, we investigated adduct formation by postcolumn addition of alkali metal salts (Li^+, Na^+, K^+, Cs^+) and transitional metal salts (Ag^+, La^{3+}) with a view to improve the signal response of steroid molecules. The metal salts were added as 1 m*M* solutions at ~10% of the HPLC flow-rates. DHEA (I) and Δ^5-Diol (II) were selected for the study.

Addition of Li^+ resulted in exclusive formation of $(M+Li)^+$ ion, but there was no improvement in the sensitivity. Addition of sodium acetate did not help either. We found that K^+ was not strong enough to displace Na^+, and gave a mixture of $(M+K)^+$ and $(M+Na)^+$ in the mass spectrum resulting in lower sensitivity in the SIM mode. We did not observe any adduct formation with Cs^+ or La^{3+}. However addition of Ag^+ suppressed the formation of other

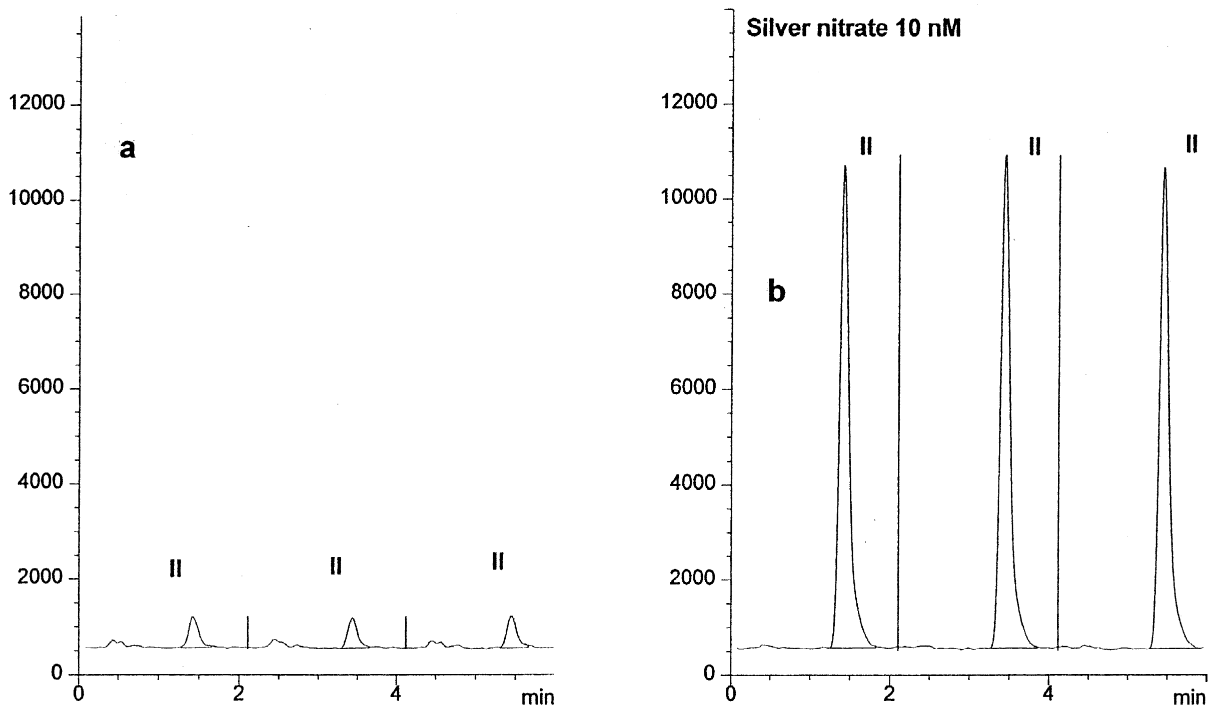

Fig. 9. (a and b) Signal enhancement in LC–MS-ESI by postcolumn addition of silver nitrate (1 m*M* at 50 μl/min). Compound: Δ^5-diol (II); LC–MS conditions: column Zorbax-SB C_{18}, 12.5×4.6 mm, 5 μm at 25 °C; Mobile phase methanol–water (65:35) at 0.5 ml/min flow-rate; Nebulizer 40 p.s.i. (276 kPa); Drying gas (N_2) 11 l/min at 350 °C; Fragmentor 60 V without postcolumn addition (a) of Ag^+, and 130 V with postcolumn addition (b); V_{cap} 5000 V.

adducts and a strong intense doublet corresponding to $(M+Ag)^+$, characteristic of silver isotopes, was exclusive and total. Presence of Ag^+ even at 1 n*M* concentration in the mobile phase significantly improved the signal response. The increase in signal response was highly compound-dependent. We observed more than ten-fold increase (Fig. 9a and b) in the signal response (SIM) of Δ^5-diol (II) by postcolumn addition of silver nitrate at 1–100 n*M* concentration of Ag^+ in mobile phase. Less than 1 n*M* or more than 100 n*M* concentration of Ag^+ resulted in a lower signal response of Δ^5-diol (Fig. 10). There was only ~85% increase in the signal response of DHEA (I) in the presence of silver nitrate (100 n*M* in mobile phase). The presence of a double bond in the steroid molecule was not necessary. 5α-H-diol (XI), a compound with no double bond, strongly complexed with silver ion. Regardless of the improvement in the signal response, the silver adducts require higher fragmentor voltage than normally needed for the molecule. The use of a higher voltage in mass suppresses baseline noise, which may further help in improving the signal-to-noise ratio. These characteristic features of silver adduct formation are clearly visible in Fig. 11, which shows FIA analysis of Δ^5-diol (II) as a function of fragmentor voltage with and without postcolumn addition of Ag^+. The optimum fragmentor voltage required in the case of postcolumn addition of silver ion was 135±5 V, which is ~75 V more than what was needed in the absence of silver. The postcolumn addition of silver ion significantly reduced the base line noise (by ~80%). To the best of our knowledge, the use of silver ion for enhancing the sensitivity of compounds in LC–ESI-MS has not been reported previously.

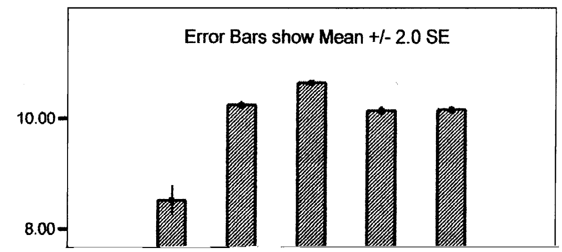

Fig. 10. Effect of the concentration of the Ag^+ on the signal enhancement of Δ^5-diol (II); 2 ng on column. Selected ion monitoring at *m/z* 273 $(M+H-H_2O)^+$ and *m/z* 397 $(M+Ag)^+$. LC–MS-ESI conditions as in Fig. 9.

3.3. 'Wrong-way-round' ionization of sulfate conjugates

'Wrong-way-round' electrospray ionization, in the present context refers to observation of intense $[M-H]^-$ ions from strongly acidic solutions. The phenomenon was first noticed by Loo et al. [32] who published a positive ion ESI spectrum of horse-heart cytochrome C at pH 11, but did not elaborate. Recently Boyd et al. discussed in detail the insensitivity of ESI abundances of analyte ions of amino acids to pH of the pre-spray bulk solution [33]. 'Wrong-way-round' ionization of oxanilic and sulfonic acid herbicides has been reported [26]. We recently reported the LC–MS analysis of polar sulfate conjugates of DHEA and its metabolites in negative ion mode and that too in highly acidic medium [22]. We used acetic acid (3.0%, v/v) to suppress peak tailing and to achieve better separation and resolution of these polar compounds using acetonitrile as organic modifier. We studied signal response of sulfate conjugates using 7-oxo-DHEA-3β-sulfate (XIX.). Use of trifluoroacetic acid (0.2%, v/v) caused more than 95% decrease in signal sensitivity than was observed in absence of an acid. Formic acid (0.8%) caused ~90% decrease in signal response. The signal response obtained by addition of acetic acid (3%, v/v) was five times better than the response produced by addition of formic acid (0.8%, v/v). Acetic acid was able to suppress peak tailing caused by the presence of highly polar sulfate

[2] S.K. Chowdhury, B.T. Chait, Anal. Chem. 63 (1991) 1660.
[3] A.U. Mirza, B.T. Chait, Anal. Chem. 66 (1994) 2898.
[4] D. Temesi, B. Law, LC·GC 17 (1999) 626.
[5] Y.-C. Ma, H.-Y. Kim, J. Am. Soc. Mass. Spectrom. 8 (1997) 1010.
[6] M. Kohler, J.A. Leary, Anal. Chem. 67 (1995) 3501.
[7] H. Zhang, J. Henion, Anal. Chem. 71 (1999) 3955.
[8] C.H.L. Shackleton, C. Kletke, S. Wudy, J.H. Pratt, Steroids 55 (1990) 472.
[9] L.D. Bowers, Sanaullah, J. Chromatogr. B, 687 (1996) 61.
[10] K. Shimada, Y. Mukai, K. Yago, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 765.
[11] Q. Jia, M. Hong, Z. Pan, S. Orndorff, J. Chromatogr. B 750 (2001) 81.
[12] M. Nakajima, S. Yamato, K. Shimada, Biomed. Chromatogr. 12 (1998) 211.
[13] S. Murray, N.B. Rendell, G.W. Tayler, J. Chromatogr. A 738 (1996) 191.
[14] DHEA and aging, in: F.L. Bellino, R.A. Daynes, P.J. Hornsby, D.H. Lavrin, J.F. Nestler (Eds.), Ann. NY Acad. Sci, Vol. 774, 1995.
[15] M.P. Cleary, in: H. Lardy, F. Stratman (Eds.), Hormones, Thermogenesis and Obesity, Elsevier, New York, 1989, p. 365.
[16] C.-Y. Su, H. Lardy, J. Biochem. (Tokyo) 110 (1991) 207.
[17] D.L. Coleman, R.W. Schwizer, E.H. Leiter, Diabetes 33 (1984) 26.
[18] M. Ben-David, S. Dikstein, G. Bismuth, F.G. Sulman, Proc. Soc. Exp. Biol. Med. 125 (1967) 1136.
[19] R.M. Loria, T.H. Inge, S.S. Cook, A.K. Szakal, W. Regelson, J. Med. Virol. 26 (1988) 301.
[20] A.G. Schwartz, Cancer Res. 39 (1979) 1129.
[21] J. Shi, S. Schulze, H. Lardy, Steroids 65 (2000) 124.
[22] A. Marwah, P. Marwah, H. Lardy, J. Chromatogr. B 767 (2002) 285.
[23] H. Lardy, N. Kneer, Y. Wei, B. Partridge, P. Marwah, Steroids 63 (1998) 158.
[24] P. Marwah, A. Marwah, H. Lardy, unpublished results.
[25] A.P. New, J.-C. Wolff, S. Crabtree, L.F. do Santos, G. Okafo, J. Lee, K. Divan, J. Chromatogr. A 913 (2001) 205.
[26] J. Eshraghi, S.K. Chowdhury, Anal. Chem. 65 (1993) 3528.
[27] E.M. Thruman, I. Ferrer, D. Barcelo, Anal. Chem. 73 (2001) 5441.
[28] A. Marwah, P. Marwah, H. Lardy, Bioorg. Chem. (2002) in press.
[29] B. Law, D. Temesi, J. Chromatogr. B 748 (2000) 21.
[30] M.W. Crowther, T.R. O'Connell, S.P. Carter, J. Am. Oil Chem. Soc. 75 (1998) 1867.
[31] A. Marwah, P. Marwah, H. Lardy, J. Chromatogr. B 757 (2001) 333.
[32] J.A. Loo, H.R. Udseth, R.D. Smith, Rapid Commun. Mass Spectrom. 2 (1988) 207.
[33] B.A. Mansoori, D.A. Volmer, R.K. Boyd, Rapid Commun. Mass Spectrom. 11 (1997) 1120.