Journal of Chromatography, 511 (1990) 257–270
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 412

Packed-column supercritical fluid chromatography–mass spectrometry and supercritical fluid chromatography–tandem mass spectrometry with ionization at atmospheric pressure[a]

ERIC HUANG and JACK HENION*
Drug Testing and Toxicology, Cornell University, 925 Warren Dr., Ithaca, NY 14850 (U.S.A.)
and
THOMAS R. COVEY
Sciex Inc., 55 Glen Cameron Rd., Thornhill, Ontario L3T 1P2 (Canada)
(Received December 12th, 1989)

ABSTRACT

The combination of packed-column supercritical fluid chromatography (SFC) with atmospheric pressure ionization mass spectrometry (SFC–API-MS) is reported. This approach is novel in that the adiabatic expansion of total SFC effluent is subjected to atmospheric pressure chemical ionization (APCI) via a corona discharge with the aid of a heated region around the restrictor. The latter is a laser-drilled stainless-steel pinhole diaphragm which is commercially available, rugged, and easily handled.

Supercritical carbon dioxide was modified with methanol to produce protonated molecular ions via APCI of representative steroids in synthetic mixtures and biological extracts. The separation time for these compounds was within a few minutes so that rapid analyses are possible. The single MS spectra readily indicate the molecular weights of these compounds although little structural information is provided.

In those instances where structural information is desired the technique of tandem mass spectrometry (MS–MS) may be utilized. Thus SFC–MS–MS characterization of trenbolone produced a collision-induced dissociation product ion mass spectrum containing numerous structurally useful fragment ions. SFC–MS–MS analysis of a complex tissue extract fortified with low ppb levels of the same growth-promoting steroid and an internal standard readily revealed the target compound by this technique.

It is suggested that SFC–API-MS is a practical approach to routine SFC–MS. The APCI technique provides reliability and good sensitivity for many classes of compounds while the pinhole diaphragm restrictor is much more rugged and easier to work with than those associated with capillary columns.

[a] Portions of this work were first presented at the *35th ASMS Conference on Mass Spectrometry and Allied Topics, Denver, CO, May 24–29, 1987.*

0021-9673/90/$03.50

INTRODUCTION

Supercritical fluid chromatography (SFC) using bonded stationary phases on either capillary or packed columns has received considerable interest in the last few years. Although there has been some controversy in the literature over whether SFC using capillary or packed columns is preferred, in general most experienced with both techniques admit they each have their own merits and limitations.

The most common detector for capillary SFC has been the flame ionization detector while the UV detector has been favored for packed-column SFC. Each of these modes of detection enjoys acceptance based on ease of use, relatively good sensitivity, wide dynamic range and analytical ruggedness. However, none of the many detectors reported for SFC provide the combination of sensitivity and specificity commonly expected from the mass spectrometer. Although a diversity of reports have appeared which describe the successful combination of SFC with mass spectrometry (MS)[1–3], general acceptance and commercial availability of this desirable combination has lagged expectations.

The majority of the early reports of SFC–MS utilized chemical ionization (CI) instead of the more traditional electron impact (EI). We believe there is good reason for this trend simply because it is much easier to accomplish SFC–MS under CI than EI conditions. In the former the mass spectrometer ion source is operated under pressure conditions which are nearly 1000 times higher than in the latter[4]. This higher gas density in the heated ion source helps transfer heat to vaporize molecules introduced into the mass spectrometer via the adiabatic expansion from the SFC column restrictor. A reagent gas such as methane, isobutane or ammonia is usually admitted which is subjected to primary ionization by high-energy electrons followed by ion–molecule interactions by the ionized excess reagent gas with the analyte molecules[1]. Usually the gas-phase protonation resulting from these processes provides protonated molecular ions with little or no fragmentation. These simple CI mass spectra are of limited value for structural characterization, but readily provide molecular weight determination and relatively good sensitivity.

In contrast to CI experimental conditions, EI ion sources operate under high-vacuum conditions (10^{-5}–10^{-6} Torr) where heat transfer to the analyte, which is necessary for volatilization and subsequent ionization, is very difficult to accomplish. This problem coupled with the additional "gas burden" imposed, for example, by a pressure program during the course of a capillary SFC–MS analysis makes SFC–EI-MS a difficult challenge. Ideally, the EI process requires a constant low pressure in the ion source. Unfortunately, due to the fixed restrictor usually present in such systems the pressure program forces increasingly larger quantities of the expanded mobile phase into the ion source. In fact, since analytes usually elute toward the end of such a pressure program, the conditions in the ion source are at their worse just when they should be at their optimum. Consequently, the limited examples of SFC–EI-MS have not for the most part shown impressive low detection limits especially on "difficult" analytes.

If we remember that SFC with flame ionization detection (FID) appears to work satisfactorily with good reliability and sensitivity perhaps we can consider an alternative approach to SFC–MS. The two obvious facts about the flame ionization detector is that it is maintained at temperatures between 350 and 450°C and it operates

at atmospheric pressure. Thus the tremendous cooling resulting from the supercritical fluid expanding from the restrictor is dealt with by high temperature and gas pressures which facilitate transfer of heat to the analyte. If we could ionize the analyte at atmospheric pressure with sufficient heat for vaporization without thermal degradation then perhaps we could approach FID sensitivities with MS detection. Assuming that ions could be formed, all that remains is a means of sampling them into a vacuum system containing a mass analyzer.

Although these concepts may appear difficult to implement, the task is quite straightforward to accomplish provided a mass spectrometer equipped with an atmospheric pressure ionization (API) ion source is utilized. The focus of this report is to describe how and why SFC–API-MS is perhaps the most logical means of accomplishing routine, high-sensitivity SFC–MS. It is important to note that CI conditions still prevail in this approach, but there are alternative means of providing desired structural information. Also, both capillary- and packed-column SFC are amenable to SFC–API-MS. It is useful to point out that if the API-MS approach can handle SFC flow-rates up to 2 ml/min it can certainly handle the reduced flow-rates of capillary columns. Results of the latter have been accomplished, but will be reported elsewhere.

API-MS is not a new or unproven MS techique. Horning *et al.*[5] reported impressive liquid chromatography (LC)–MS detection limits as early as 1974 while a wide variety of more recent applications and developments have appeared in the last few years[6,7]. The commercial availability of instrumentation used in this work as well as recent developments[8–11] suggests that API techiques will undergo significant developments and be widely applicable in the future. In fact, there is a variety of different ways to form ions at atmospheric pressure.

Historically, ions were formed by emission of β particles from ^{63}Ni foil, but this was later simplified by replacement with a corona discharge which has been utilized in this work. Alternatively, liquids or fluids passing through a capillary held in excess of 3 kV produce charged droplets which emit ions into the gas phase by "ion evaporation". This electrospray approach has been described by ourselves[10] and others[9,11] for the characterization of a wide variety of applications ranging from polar drugs to peptides, proteins and polymers. These approaches to form ions at atmospheric pressure provide unusual flexibility and opportunity for combining separation methods with mass spectrometry. In this report we will focus on packed-column SFC as one example.

EXPERIMENTAL

Chromatography

The packed-column SFC system used in this work was a Hewlett-Packard Model 1084B high-performance liquid chromatography (HPLC) system modified for SFC operation[2]. The variable-wavelength UV detector was equipped with a high-pressure cell and used in-line with the SFC–MS interface in all this work. The exit of the column was disconnected from the conventional backpressure regulator and directed through a 20-μm pinhole centered in a stainless-steel diaphragm restrictor housed in a modified heated pneumatic nebulizer SFC–MS interface (see below). Supercritical fluid conditions were maintained by housing the column in the oven

heated to 80°C while pre-cooled supercritical carbon dioxide (A) was mixed with carbon dioxide doped with 10% methanol (B) and delivered through the column at a flow of 1–2 ml/min. The column used in this work was a standard HPLC column from Shandon (Keystone, State College, PA, U.S.A.) packed with a bonded cyanopropyl stationary phase on 3-μm silica particles (100 × 4.6 mm I.D.). Samples were dissolved in methanol and were injected (5 μl) via a 5-μl loop injector (Model 7125, Rheodyne, Cotati, CA, U.S.A.).

Mass spectrometry

The modified heated pneumatic nebulizer interface was introduced through the standard solids probe inlet of a TAGA 6000E triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (Sciex, Thornhill, Canada). Packed-column SFC flow-rates ranged from 1 to 2 ml/min while supercritical carbon dioxide doped with varying amounts of methanol was continuously introduced into the APCI source under either SFC–MS or SFC–MS–MS conditions. A probe heater temperature of 500°C was used resulting in a measured vapor temperature of about 125°C at the point where the vapor exits from the quartz liner of the heated pneumatic nebulizer SFC–MS interface (see Fig. 3). Liquid nitrogen boil-off gas was introduced into the interface to effect nebulization at a flow-rate of about 7 l/min. Under full-scan SFC–MS operating mode quadrupole 1 was operated beginning at m/z 200 with quadrupole 3 operating in the rf-only mode. When SFC–MS–MS experiments were undertaken, the mass spectrometer was operated in the full-scan daughter ion or selected reaction monitoring (SRM) modes. Unit resolution (full-width at half maximum = 0.6 dalton) was maintained across the mass range scanned by quadrupole 3 in all collision-induced dissociation (CID) work reported here. Under scanning conditions a scan-rate of 3 s per scan was used while under SRM conditions a dwell time of 100 ms was used. For all MS–MS experiments argon was used as the collision gas with an effective target thickness of approximately

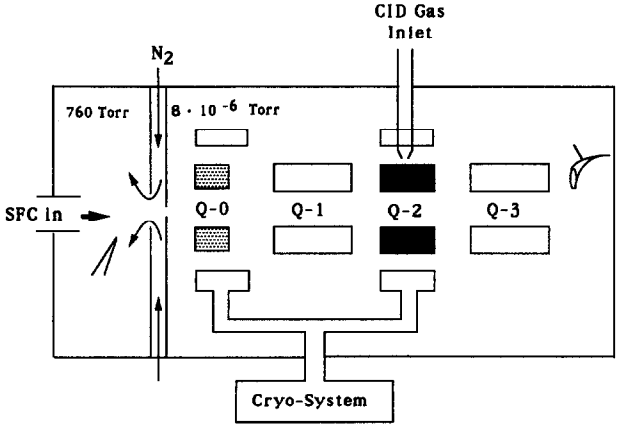

Fig. 1. Simplified diagram of the API tandem triple quadrupole mass spectrometer system used in this work. The API ion source is shown on the left with its associated corona discharge needle. This region is coupled with the the high vacuum system via 100-μm orifice and the nitrogen curtain gas. The vacuum is achieved through a liquid helium cooled cryopump.

$200 \cdot 10^{12}$ atoms/cm^2. The CID experiments were optimized at a laboratory collision energy of 50 eV in order to obtain useful structural information and optimize product ion sensitivity.

The Sciex TAGA 6000E (Fig. 1) is pumped with a two-stage liquid helium cooled cryogenic system. There are two cryoshells, one around the quadrupole lens (Q-0) and the other around the collision cell (Q-2). These cryoshells have a combined pumping speed of more than 60 000 l/s for air. The base vacuum of this system is $5 \cdot 10^{-8}$ Torr, and when the ion sampling orifice is exposed to atmosphere it increases to $7 \cdot 10^{-6}$ Torr. The pitfalls of such a system are that non-condensable gas such as helium cannot be pumped efficiently, and when the cryoshells become saturated, the system needs to be recycled (warmed to room temperature, rough-pumped out, and cooled back down). Recycling takes approximately 10 h and can be done overnight. Usually 50 h of operation are obtained before recycling is required.

Ion extraction with this API-MS system is accomplished through a sampling orifice with an inner diameter of 100 μm (Fig. 2). The atmosphere side of the ion-extraction orifice is bathed with a 0.5–2 l/min flow of dry nitrogen (ultrapure). Without the nitrogen curtain gas ions undergo extensive clustering with water and other polar molecules in the free-jet expansion from atmosphere to high vacuum (see Fig. 2).

Ions are focused into the vacuum via an electrical potential gradient. In positive-ion operation, the corona discharge electrode is operated at 6 kV (constant current control) with the interface plate at 650 V and the orifice at 40 V. When the ions approach the orifice the combination of electrical potential and vacuum pulls them through and into the analyzer. This potential difference can be increased to facilitate CID in the free-jet expansion regin. Low potential differences (20 V or less) impart only enough energy to dissociate hydrogen-bonded clusters and adducts. High potential differences (in excess of 20 V) often fragment molecules and may be used to gain some fragmentation.

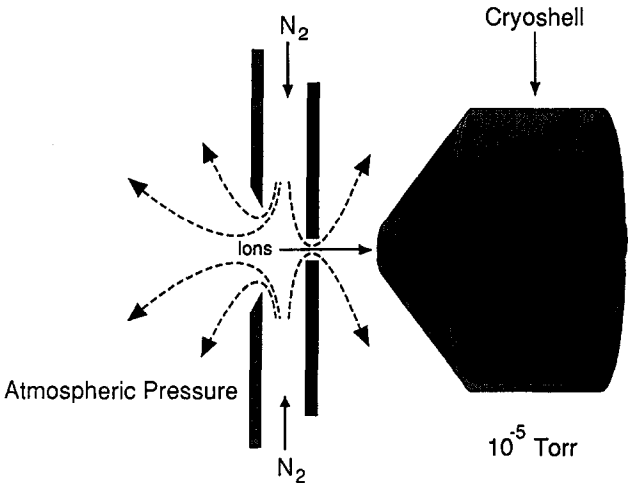

Fig. 2. The API interface which separates the high vacuum mass analyzer system from the API region. Electrical potentials on the lenses focus ions through the ion-sampling orifice and into the mass analyzer.

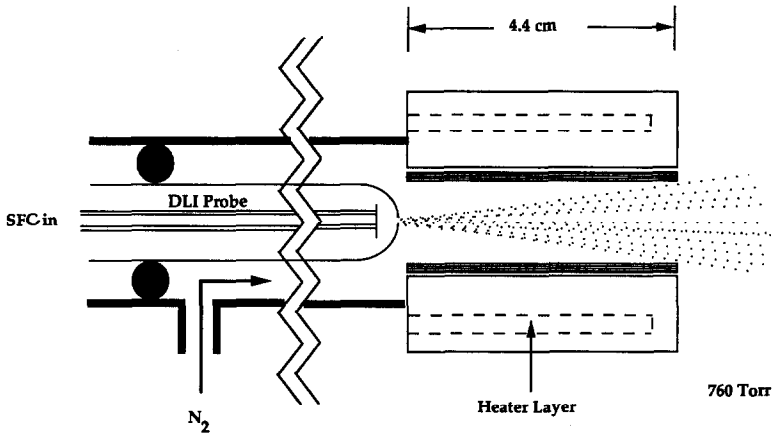

Fig. 3. Combined packed-column SFC restrictor and heated pneumatic nebulizer SFC–MS interface used in this work. The total packed-column SFC effluent passes through the 20-μm pinhole restrictor whereupon adiabatic expansion exposes the analytes to the heated quartz liner region. Following mixing and volatilization by heat the sample is ionized by APCI which is initiated by the corona discharge needle.

The SFC–MS interface

The device or "interface" which can deliver total effluent from the SFC system to the API ion source of the mass spectrometer used in this work is schematically shown in Fig. 3. This system combines the post-column restrictor necessary for maintaining supercritical conditions via a small pinhole diaphragm housed in a direct liquid introduction (DLI) LC–MS probe described previously[13]. This LC–DLI-MS probe was fitted with a 20-μm pinhole diaphragm instead of the 5-μm pinhole used for LC–MS[13]. This probe was held in place via O-rings and housed in an adaptor which placed the probe tip near the opening of a quartz liner maintained at 200°C and allowed co-axial flow of nitrogen nebulizer gas (Fig. 3). The copper heater block which housed the quartz liner was fitted with four cartridge heaters powered by a variable transformer which supplied continuous power to the heaters. Temperature monitoring of this heating block was accomplished via an iron–constantine thermocouple which was also housed in the block. Optimization experiments suggested that a measured temperature of 200°C was adequate to handle the compounds studied in this work. The volatilized analytes eluting from the packed column were ionized via a corona discharge needle maintained at 6 kV (Fig. 1).

The ions formed at atmospheric pressure are then electrically focussed through a 100-μm orifice and through a nitrogen curtain gas into high vacuum conditions (Fig. 2). The nitrogen curtain gas effectively declusters cluster ion adducts with the analyte and minimizes plugging of the small ion-sampling orifice. The small orifice separates the high-vacuum region where the inlet system and ionization region resides. This "decoupling" of the ionization and mass analysis regions provides a flexibility and ease of accomplishing chromatography which is unique from conventional MS systems.

DISCUSSION

APCI

APCI is similar to low-pressure CI in that the analyte of interest must be in the gaseous phase before ionization by ion–molecule reactions can proceed. The ionization process is initiated by an external source of electrons (utilizing β emitters or corona discharge as the primary source). However, because the ions and molecules experience more collisions, APCI is a milder and more efficient process than conventional low-pressure CI. The ionization process of APCI both in positive-ion and negative-ion modes were well documented previously[12]. Under SFC–API-MS experimental conditions, positive reactant ions are formed in ambient air by an electron ionization mechanism and subjected to further collisions to form an equilibrium set of proton hydrates. The analytes in the gas phase then react with these proton hydrates to form protonated molecular ions $(M+H)^+$ and are subsequently mass-analyzed.

Tandem mass spectrometry

The API approach does not directly provide structural information for ionized compounds because little or no fragmentation occurs under the very mild ionization conditions. Although molecular weight information is usually provided, identification of unknown compounds eluting from the SFC system requires plausible fragmentation processes to produce a more useful mass spectrum.

The technique of tandem mass spectrometry (MS–MS) usually involves two mass analyzers in "tandem" and separated by a collision cell[14]. The process involves electrically focussing parent ions such as the protonated molecular ions generated under APCI by the first mass analyzer (Fig. 1, Q-1) into the collision cell. These ions are then subjected to ion–molecule interactions in the collision cell (Q-2) which impart excess energy to the protonated molecular ions. This energy rapidly distributes itself throughout the excited ions and causes breakage of weak bonds resulting in a family of product ions. These ions have different masses than the parent ion and other fragment ions, so another mass analyzer (Q-3) is required to separate these ions. The second mass analyzer produces a full-scan product ion mass spectrum which can be very useful for structural characterization.

When one combines the separation power of SFC with the mixture analysis capability, specificity and sensitivity of tandem mass spectrometry (SFC–MS–MS), we have a very powerful analytical system. In this report we will show how the mild ionization conditions of APCI coupled with the capabilities of MS–MS is a natural combination.

Applications

To illustrate the utility of API for packed-column SFC–MS and SFC–MS–MS we present a few representative examples where these techniques are particularly well-suited. It should be emphasized that compounds of increasing polarity are difficult to chromatograph under SFC conditions even when the mobile phase is modified with methanol or other polar solvents. We suggest that the steroids described below, however, are preferred candidates for SFC and hence SFC–API-MS.

Gas chromatographic (GC) characterization of some steroids either requires

derivatization due to their higher polarity or reduced temperatures due to their thermal instability, while HPLC techniques suffer from occasionally inadequate separation efficiences and longer analysis times. Packed-column SFC, however, provides faster analysis times without derivatization or undue exposure to elevated temperatures. In

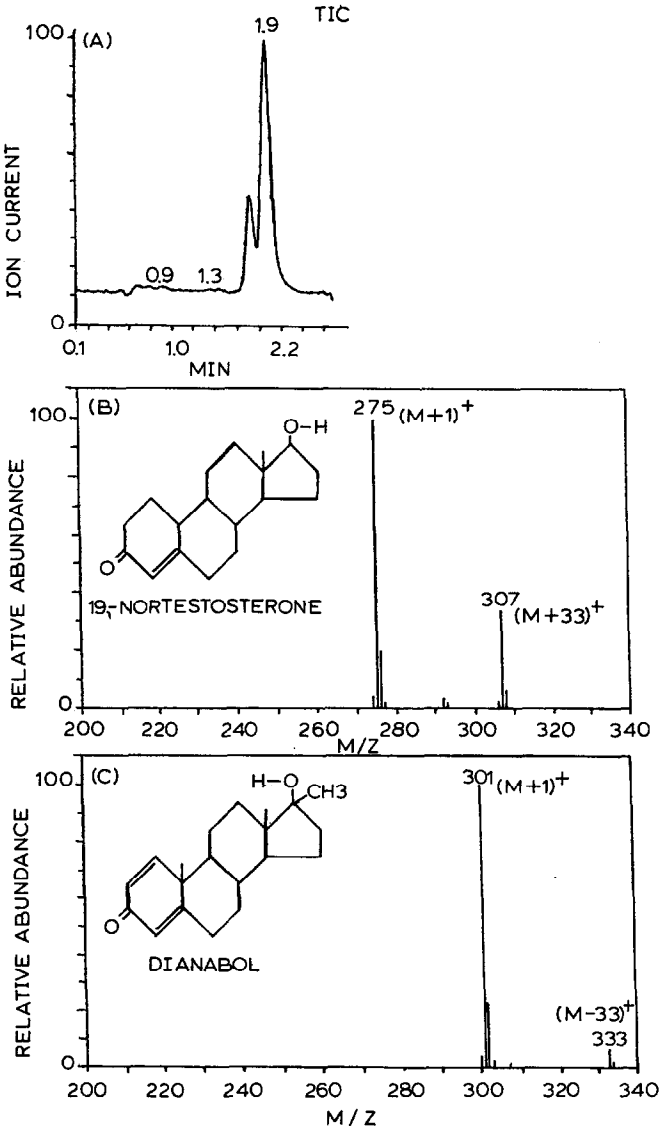

Fig. 4. SFC–API-MS analysis of a synthetic mixture containing 250 and 500 ng of 19-nortestosterone (mol.wt. 274) (B) and Dianabol (mol.wt. 300) (C), respectively. The separation was accomplished on a cyanopropyl column (100 × 4.6 mm I.D.) packed with 3-μm particles using a flow of 1.5 ml/min carbon dioxide–methanol (87:13, v/v) under an operating inlet (outlet) pressure of 3400 (3100) p.s.i. The column was maintained at 80°C and the total effluent was transferred to the API ion source through a 20-μm pinhole restrictor.

the examples of packed-column SFC–MS under APCI conditions described below on both synthetic mixtures and biological extracts the advantages and limitations of this approach will be highlighted.

The on-line packed-column SFI-API-MS analysis of a synthetic mixture

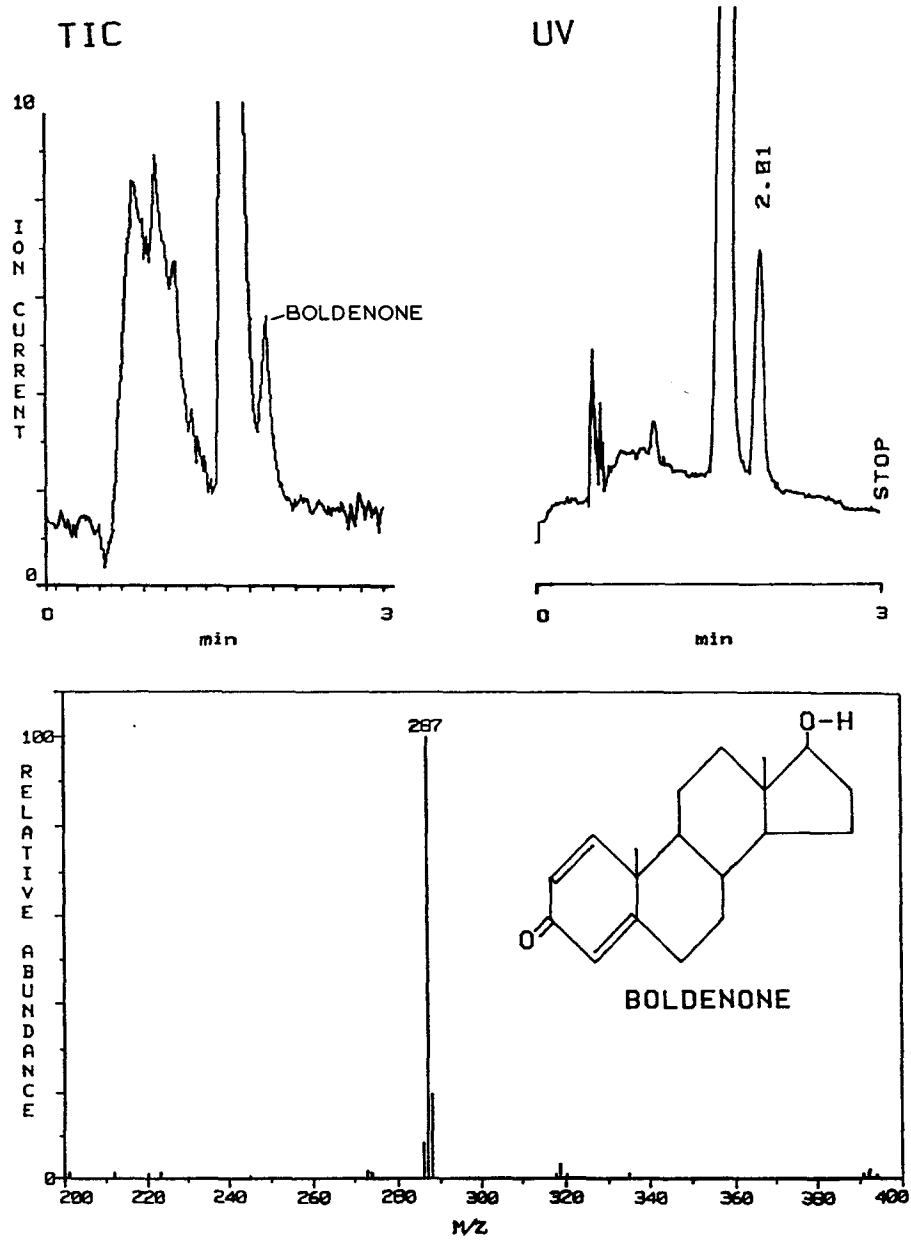

Fig. 5. Packed-column SFC–API-MS analysis of an equine urine extract containing boldenone (mol.wt. 286). Experimental conditions were the same as described for Fig. 4.

containing 250 ng of 19-nortestosterone (mol.wt. 274) and 500 ng methandrostenolone (Dianabol, mol.wt. 300) is shown in Fig. 4. The mass spectrometer was scanned in the single-MS mode from m/z 200 to 500 at a scan-rate of 3 s/scan. The HP 1084B SFC system delivered a 1.5-ml/min flow of carbon dioxide–methanol (87:13, v/v) through a Shandon 3-μm cyanopropyl column (100 × 4.6 mm I.D.) maintained at 80°C. The inlet (outlet) pressure of the column was 3400 (3100) p.s.i. and the total effluent passed through the 20-μm restrictor in the modified DLI probe (Fig. 3). The quartz liner housed in the copper block of the SFC–MS interface (Fig. 3) was maintained at 200°C while the nitrogen make-up gas was set at a flow of 20 standard cubic feet per hour (SCFH).

The total ion current (TIC) from SFC–API-MS analysis of this mixture is shown in Fig. 4A while the corresponding APCI mass spectra for 19-nortestosterone and Dianabol are shown in Fig. 4B and C, respectively. These spectra are dominated by protonated molecular ions which easily provide the molecular weights for these compounds plus adduct ions at $(M+33)^+$ which presumably are proton-bound adducts between the analyte and methanol used as modifier for the carbon dioxide mobile phase. No fragment ions are observed in these mass spectra due to the extremely mild APCI conditions.

Another example of packed-column SFC–MS is shown in Fig. 5 which highlights the rapid and effective means of detecting the anabolic steroid, boldenone, in equine urine. Five microliters of a crude equine urine extract[15] dissolved in methanol were injected under the same SFC–MS conditions as described above for Fig. 4. Fig. 5 shows the corresponding TIC and UV traces from the full-scan SFC–API-MS analysis of the equine urine extract. Note the retention time for boldenone in this experiment is only 2 min, which demonstrates the relatively fast analysis times afforded by packed-column SFC. The unretained endogenous material is adequately resolved from the analyte of interest and the APCI mass spectrum for boldenone shown at the bottom of Fig. 5 readily reveals the $(M+1)^+$ ion at m/z 287 which corroborates its molecular weight of 286 dalton. Forensic confirmation of boldenone in this sample would require structural information in addition to the retenion time and molecular weight information provided by this experiment (see below).

Fig. 6 shows the value of on-line SFC–API-MS–MS analysis of a sample containing the representative anabolic steroid, trenbolone[16]. The TIC and full-scan CID mass spectrum of trenbolone are shown from the analysis of 100 ng trenbolone injected under packed-column SFC–MS conditions identical to those described for Fig. 4. This steroid elutes in 1.2 min and with a collision energy of 70 eV produces the information-rich CID mass spectrum shown in the lower portion of Fig. 6. The m/z 271 ion represents the protonated molecular ion wich corroborates trenbolone's molecular weight of 270 dalton while the abundant fragment ions at m/z 83, 107, 133, 159 and 199 provide structurally important information to facilitate identification of trenbolone in unknown samples.

As a final example of high sensitivity and specificity combined with rapid sample analysis provided by packed-column SFC–MS–MS and APCI we present the data shown in Fig. 7. The upper portion of Fig. 7 shows the UV chromatograms obtained from the analysis of a bovine liver tissue which has been fortified with 20 and 30 ppb[a]

[a] Throughout this paper, the American billion (10^9) is meant.

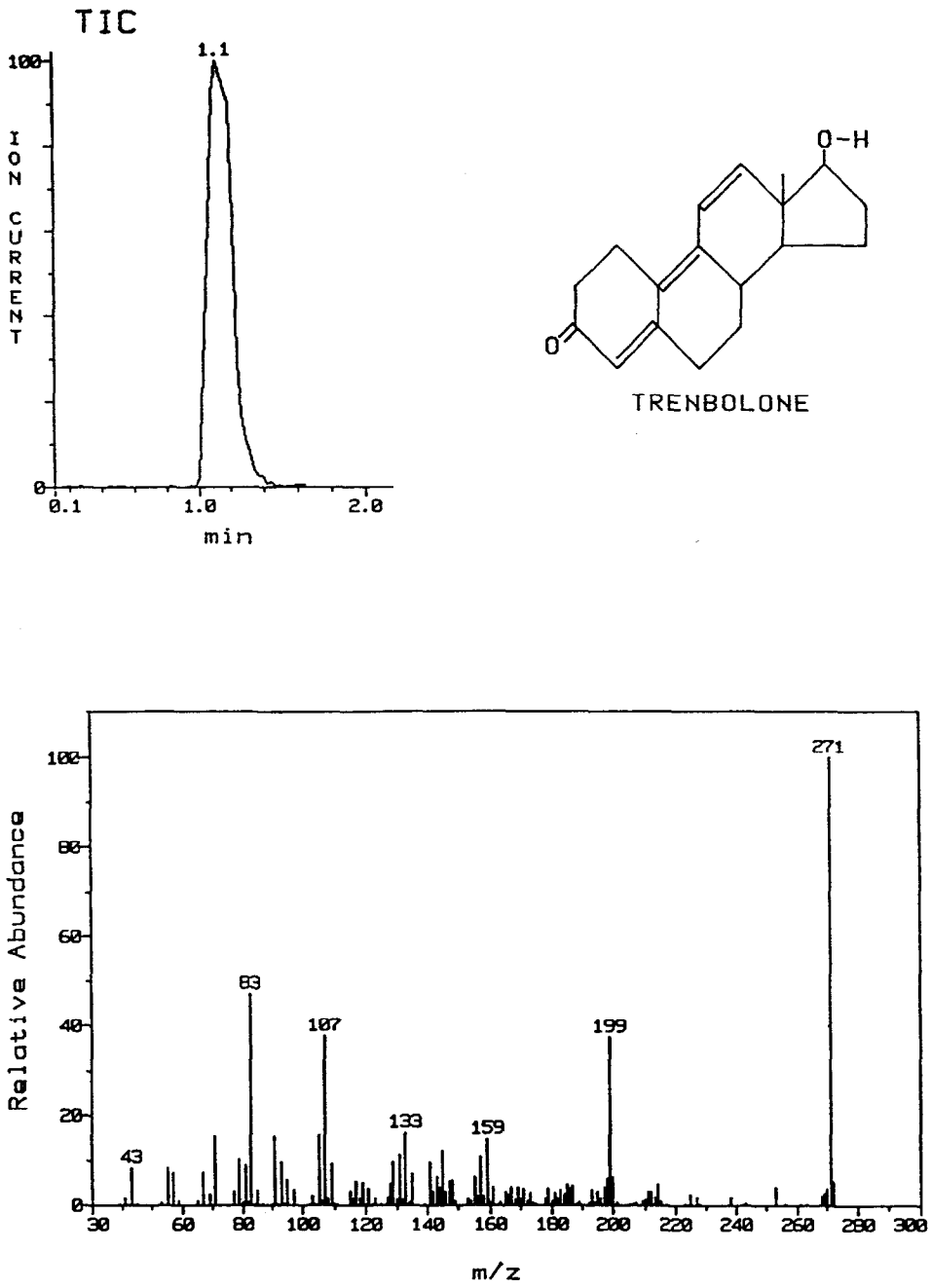

Fig. 6. Packed-column SFC–API-MS–MS analysis of a synthetic standard of the growth promotant, trenbolone (mol.wt. 270). The injected quantity of 100 ng trenbolone produced the TIC (upper portion) and full-scan CID (lower portion) mass spectrum, where the protonated molecular ion at m/z 271 was subjected to CID using argon as the collision gas with a collision energy of 70 eV (laboratory frame). SFC experimental conditions were the same as described for Fig. 4.

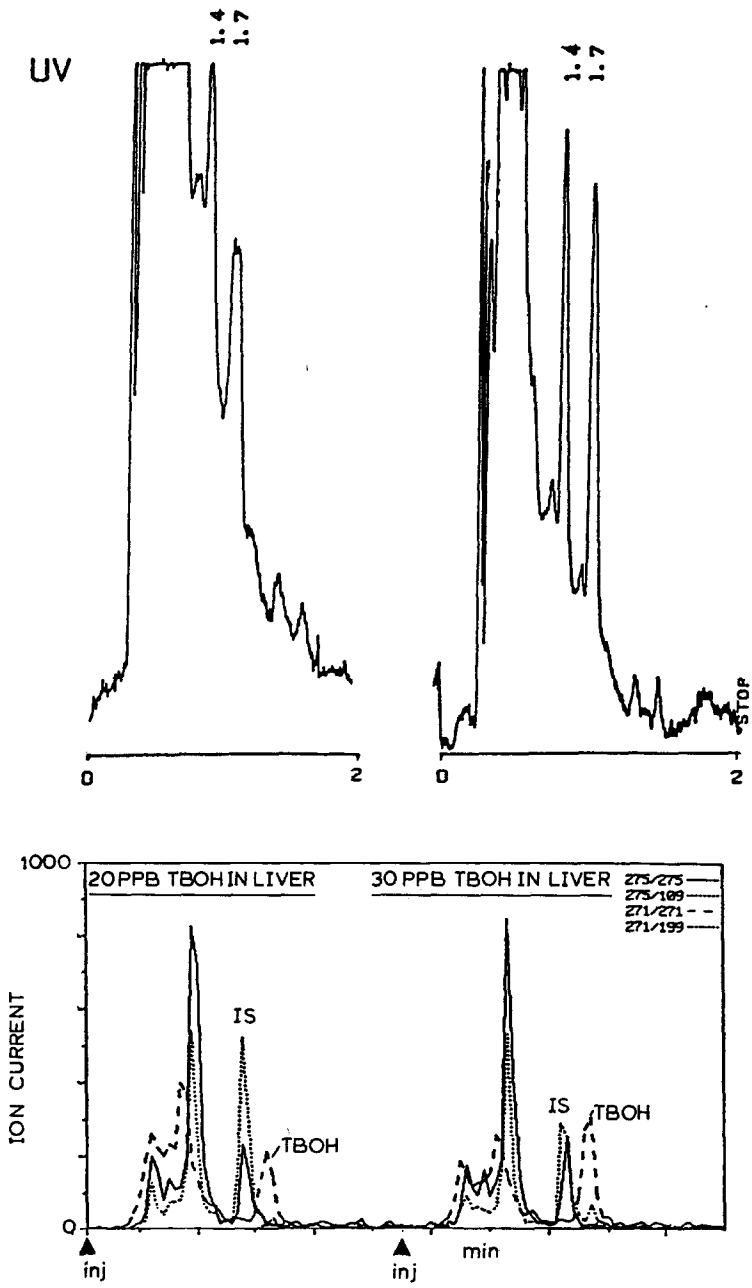

Fig. 7. Packed-column SFC–UV and SFC–API-MS–MS using the SRM mode to detect 20 and 30 ppb, respectively, of trenbolone (TBOH) in a bovine liver tissue homogenate. The internal standard (IS), 19-nortestosterone, was also present at 15 ppb in each sample extract. The experimental conditions were the same as described for Fig. 6.

trenbolone, respectively, and 15 ppb 19-nortestosterone as an internal standard. The liver tissue homogenate was subjected to successive three-phase liquid–liquid extraction followed by liquid–solid extraction as previously reported[16]. A 5-μl volume of the tissue extract was injected under the same conditions as for Fig. 4 and the API-MS–MS system operated in the MS–MS mode under SRM conditions[14]. The SRM experiment is analogous to targeted quantitative GC–selected-ion monitoring (SIM)-MS experiments. The SRM experiment, however, provides an additional element of selectivity due to the mixture analysis capability of tandem mass spectrometry[14].

The internal standard, 19-nortestosterone, and trenbolone are observed at 1.4 and 1.7 min, respectively in the UV chromatograms shown in the upper portion of Fig. 7. The high matrix background signal from the chemically complex tissue extract is evident in both UV chromatograms although both analytes are observed even in the 20-ppb fortified sample. In contrast, however, there is much less interference observed from this matrix in the total selected-ion current profiles shown for these two samples in the lower portion of Fig. 7. This is due to the reduced chemical noise obtained by "selecting" the protonated parent ions and appropriate fragment ions for the internal standard and the target analyte. In this way only these ions are mass-analyzed while all other ions are not observed. This explains the significantly reduced ion current observed in the region where the UV signal is rather high.

The total selected-ion current profiles shown for the 20- and 30-ppb fortified tissue samples shown in the lower portion of Fig. 7 clearly show the separation and detection of the two steroids. In this experiment only the parent ion and the CID fragment ion for each steroid are shown although additional appropriate fragment ions could have been used. These data clearly show differences between the two levels of trenbolone in these samples and could be used for combined qualitative and quantitative analysis of such samples[17]. Particularly evident is the relatively short analysis time under these packed-column SFC–MS–MS conditions where an injection could be made every 2 min. This would allow high sample throughput in suitably equipped laboratories with high sample volume requirements[18].

CONCLUSIONS

Packed-column SFC–MS and SFC–MS–MS analyses under APCI conditions are efficient and rapid means of analyzing chemically complex samples for the determination of unknown and targeted compounds. Sample volumes up to 20 μl may be readily injected onto the column and the elution of relatively polar analytes is not often a problem as can sometimes be the case with capillary SFC columns. The pinhole diaphragm orifice is rugged and commercially available. The corona discharge API system is simple and rugged with near universal response to most analytes. Finally, the API ion source operates at ambient temperature and readily handles packed-column fluid flow-rates up to 2 ml/min so the flexibility of this chromatographic technique may be utilized. It is also a very simple ion source without the cleaning and maintenance problems associated with conventional EI or CI ion sources. The high sample throughput capability of this system could offset the rather high cost of the tandem mass spectrometer system. We suggest that an API ion source with an associated mass analyzer could be the preferred way to accomplish SFC–MS using packed or capillary columns.

ACKNOWLEDGEMENTS

We thank Hewlett-Packard for the generous loan of the modified 1084B SFC system, and the New York State Racing and Wagering Board Equine Drug Testing and Toxicology Program, Sciex Inc., the United States Department of Agriculture Food Safety Inspection Service (FSIS), and the Eastman Kodak Company for financial support. We also thank E. D. Lee for part of the SFC–MS interface design, and Dr. G. A. Maylin, Director of the Laboratory, for his support of this work.

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