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HPLC/atmospheric pressure chemical ionization-mass spectroscopy of eight regulated sulfonamides

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Abstract

Reversed phase high performance liquid chromatography coupled with on-line atmospheric pressure chemical ionization mass spectrometry, HPLC/APCI-MS, has been applied to a mixture of eight sulfonamides. In full scan mode, extracted ion chromatograms produced minimum detectable quantities (MDQ) of 0.8 ng on column, for six of the eight regulated sulfonamides investigated. Selected ion monitoring yielded a 50 pg MDQ for sulfamerazine, sulfadiazine and sulfamethazine, while, the other compounds presented higher values. Analysis of supercritical fluid extracts of chicken liver containing sulfadimethoxine were found to be easily detected by HPLC/APCI-MS. In extracts of chicken liver spiked with 25 μg kg^{-1} (25 ppb) of sulfadimethoxine this compound could be detected in selected ion mode, while 100 pg μl^{-1} was detectable in either full scan or single ion modes. The analysis method for extracted sulfadimethoxine also demonstrated good linearity and reproducibility in both single ion and scan mode.
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1. Introduction

The use of selective detectors for chromatography is a key approach in analytical chemistry. Valuable information about complex samples is more easily obtained with selective detectors than with conventional detectors which often require extra time and skill to achieve efficient separation of interfering analytes. The mass spectrometer is such a detector.

The mass spectrometer offers both selective and molecular information from the same chromatographic separation during scanning conditions. Atmospheric pressure ionization, either electrospray or atmospheric pressure chemical ionization (APCI), is rapidly becoming one of the primary ionization methods for HPLC/MS. APCI uses a heated nebulizer which vaporizes both the mobile phase and sample. Ionization is accomplished with a corona discharge pin that emits electrons which collide with the abundant solvent ions. Solvent ions can then collide with the analyte causing ionization. Since ionizaton is due to molecular collisions, APCI is considered to be a relatively soft albeit exothermic ionization technique.

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Henion et al. [1] have used HPLC/APCI-MS-MS for the analysis of sulfonamides. They did not report on the sensitivity of their system but showed the fragment ions of several sulfonamides. Analysis of racehorse urine contaminated with three sulfonamides was shown to be feasible. Horie et al. [2] have described the detection of sulfonamides in meat by means of HPLC and thermospray mass spectrometry. Chromatographic analyses of incurred swine tissue at 1 μg g^{-1} were shown. Pleasance et al. [3] have reported the separation and identification of sulfonamides by reversed phase liquid chromatography and ion-spray mass spectrometry. Later Volmer [4] reported the simultaneous multiresidue analysis of 21 sulfonamides in milk using a fast short column HPLC separation in conjunction with electrospray tandem mass spectrometry. Doerge et al. [5] three years later demonstrated the potential analytical sensitivity for detection of sulfonamides in spiked milk extracts by HPLC coupled to atmospheric pressure chemical ionization mass spectrometry. The ease of use of HPLC/APCI-MS suggested that this technique may be practical for routine screening and confirmation analysis of trace milk contaminants. In all these five studies the positive ion spectrum yielded mainly protonated molecular ions.

In this work we have evaluated HPLC/APCI MS detection for sulfonamides in biological tissue. System sensitivity in full scan and selected ion modes was determined using standard solutions. In addition, supercritical fluid extracts (SFE) of chicken liver containing sulfadimethoxine as low as 25 pg μl^{-1} were also evaluated.

2. Experimental

A Fisons Instruments (Altincham, UK) VG Platform single quadrupole mass spectrometer was interfaced to a 1050 series HPLC pump and a model 1050 variable wavelength UV detector (Hewlett Packard, Little Falls, DE) via the APCI interface probe from VG shown in Fig. 1. The APCI probe was heated to 400°C to ensure complete vaporization of the column effluent. Corona pin voltage was set at 3.0 kV for all analyses. Extraction cone voltage was set at 30 V and the source temperature was set to 120°C. A scan time of 1 s and interscan delay of 0.05 s were used for scan mode (*m*/*z* 110–320) and a 1 s dwell time was used for selected ion monitoring. The selected ions were the base peak for each sulfonamide previously determined in full scan mode. For sulfadizine, sulfachlorpyridazine, and sulfathiazole *m*/*z* 156 was monitored. Ion monitoring for sulfamerazine (*m*/*z* 265), sulfapyridine (*m*/*z* 250), sulfamethazine (*m*/*z* 279), sulfaquinoxaline (*m*/*z* 300) and sulfadimethoxine (*m*/*z* 310) was based upon the molecular ion. Only 310 *m*/*z* was monitored for quantitative analysis of sulfadimethoxine in chicken liver extracts. Both drying gas and sheath gas were nitrogen from the gas outlet of a liquid nitrogen dewar. Optimal gas flows were adjusted daily using a simple optimization procedure described later. Samples were injected onto the column via a Rheodyne model 7125 injection valve with a 20 μl sample loop.

A Prodigy C18 (250 × 4.6 mm, 5 μm dp) column purchased from Phenomenex (Torrence, CA) was used for all HPLC separations. Chro-

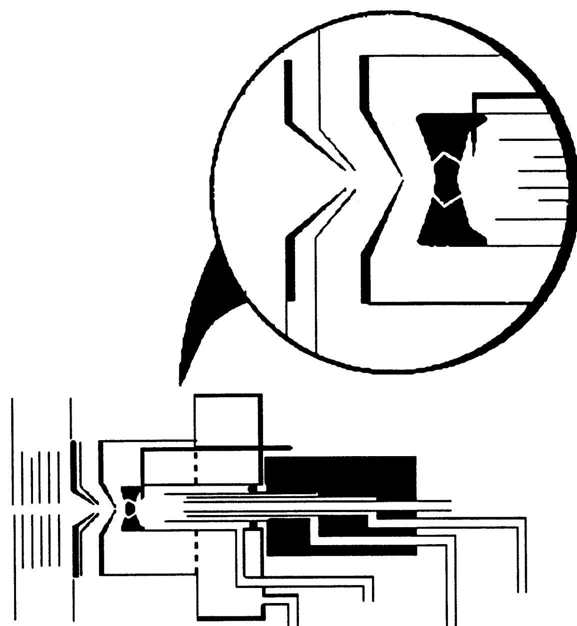

Fig. 1. Atmospheric pressure chemical ionization probe. Taken from VG platform technical users manual.

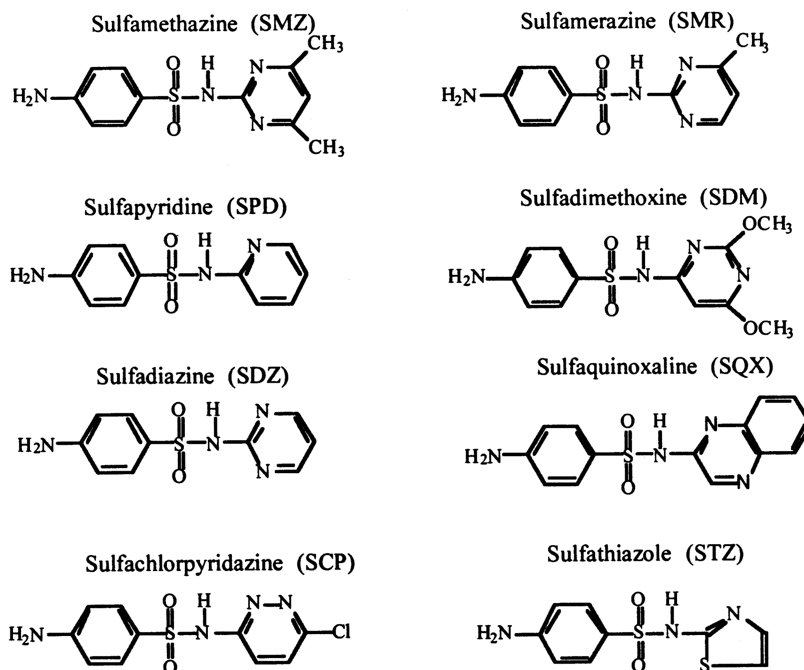

Fig. 2. Structure of eight currently regulated sulfonamides.

matographic conditions for the first 8 min consisted of 85/15 8 mM ammonium acetate/acetonitrile adjusted to pH 6.5 with acetic acid. At 15.5 min the mobile phase was 60/40 8 mM ammonium acetate/acetonitrile (pH 6.5) after a linear gradient of 100% A (85/15) to 100% B (60/40). At 23 min, the mobile phase was returned to initial conditions. Additional chromatography of solely sulfadimethoxine for both minimum detection studies and detection in chicken liver extracts consisted of an isocratic elution using 65% 8mM ammonium acetate/35% acetonitrile (pH 6.5). A flow of 1 ml min^{-1} was used throughout. The entire column flow could be added directly to the APCI probe without flow splitting.

All sulfonamides and SFE chicken liver extracts were obtained from Robert Maxwell at the USDA/ARS in Philadelphia, PA. The SFE procedure has been published previously [6]. The solid phase, alumina in situ trap, which was used during SFE was rinsed with various mobile phases including 70/30 water/methanol, 50/50 water/methanol, and 100% methanol. HPLC grade methanol and acetonitrile were purchased from EM (Gibbstown, NJ). Reagent grade ammonium acetate was purchased from Aldrich (Milwaukee, WI). All chemicals were used without further purification.

3. Results and discussion

Initially, the minimum detectable quantity for the eight currently regulated sulfonamides [7] (Fig. 2) was determined by HPLC/APCI-MS. Ion transmission of the LC interface was optimized by adjusting the sheath gas and drying gas flows (Fig. 1), as well as, the position of the APCI probe via the positioning screws. This optimization procedure was performed each day prior to analyzing any samples. Maximum ion current signified the best sensitivity of the system. The MDQ was first determined for all eight sulfonamides in scan mode. MDQs ranged from 100 to 10 ng viewed directly from the TIC. However, very little mass spectral data manipulation is performed directly from the TIC. The extracted ion chromatogram plots a single ion from the total scan

Table 1
Minimum detectable quantity (S/N = 2) of eight regulated sulfonamides by HPLC/APCI-MS in full scan mode from 110 to 300 m/z and extracted ion mode

Compound	TIC (ng)	EIC (ng)
SDZ	10	0.8 (156)[a]
SCP	100	10 (156)
STZ	20	1 (156)
SMR	10	0.8 (265)
SPD	10	0.8 (250)
SMZ	10	0.8 (279)
SQX	10	0.8 (300)[b]
SDM	10	0.8 (310)[b]

Sulfadiazine (SDZ), sulfachlorpyridazine (SCP), sulfathiazole (STZ), sulfamerazine (SMR), sulfapyridine (SPD), sulfamethazine (SMZ), sulfadimethoxine (SMZ) and sulfaquinoxaline (SQX).
[a] Number in parentheses is m/z used for extracted ion chromatograms.
[b] SQX and SDM MDQ determined using isocratic elution of 65/35 ammonium acetate/ acetonitrile adjusted to pH 6.5. All other MDQ determinations performed using gradient elution as described in the Experimental.

Table 2
Minimum detectable quantity (S/N = 2) of eight regulated sulfonamides by HPLC/APCI-MS in selected ion recording mode

Compound	Selected ion (SIR) (ng)
SDZ	0.8 (156)[a]
SCP	6 (156)
STZ	0.8 (156)
SMR	50 pg (265, 250)[b]
SPD	50 pg (265, 250)
SMZ	50 pg (279)
SQX	200 pg (300)[c]
SDM	100 pg (310)[c]

Sulfadiazine (SDZ), sulfachlorpyridazine (SCP), sulfathiazole (STZ), sulfamerazine (SMR), sulfapyridine (SPD), sulfamethazine (SMZ), sulfadimethoxine (SMZ) and sulfaquinoxaline (SQX).
[a] Number in parentheses is m/z used for single ion monitoring.
[b] Both 265 and 250 m/z monitored simultaneously.
[c] SQX and SDM MDQ determined using isocratic elution of 65/35 ammonium acetate/acetonitrile adjusted to pH 6.5. All other MDQ determinations performed using gradient elution as described in the Experimental.

range versus time. In so doing, much less noise and better signal-to-noise (S/N) ratios can be obtained. Mass spectra of each compound were, subsequently, obtained from which the base peak for each compound was used for generation of the reconstructed ion chromatograms. Table 1 lists the MDQ for each of the eight compounds as well as the extracted ion used. As can be observed, the reconstructed ion mode produced much lower MDQs than total ion mode.

Atmospheric pressure chemical ionization is a relatively soft ionization technique producing only a protonated molecule and a few fragment ions. The background subtracted mass spectrum of sul-

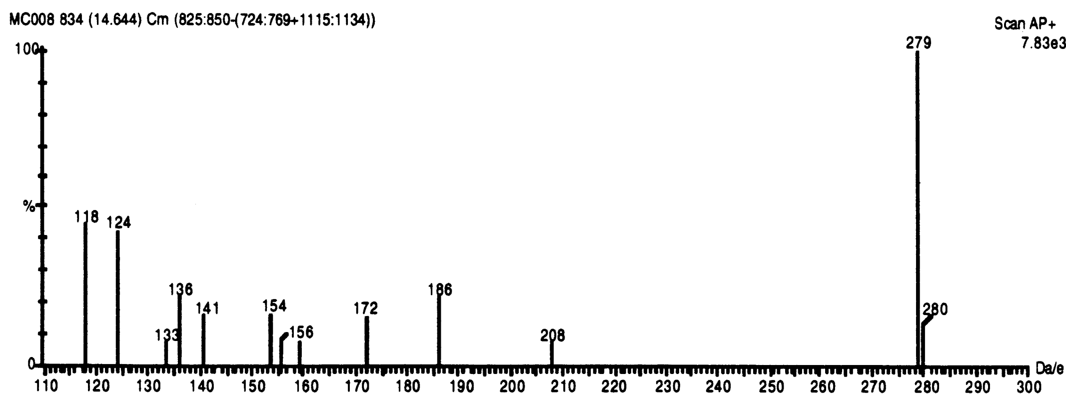

Fig. 3. HPLC/APCI mass spectrum of sulfamethazine (10 ng of each). Full scan m/z 110–320. Column Conditions: 0–8 min. 100% 85/15 8 mM ammonium acetate/acetonitrile then a gradient to 100% 60/40 8 mM ammonium acetate/acetonitrile during the next 7.5 min followed by a hold to 23 min; flow, 1.0 ml min^{-1}; 250 × 4.6 mm i.d., 5 µm Prodigy C18. Injection: 20 µl in methanol. MS Conditions: APCI probe 400°C; corona pin, 3.0 kV; extraction cone, 30 V; source, 120°C.

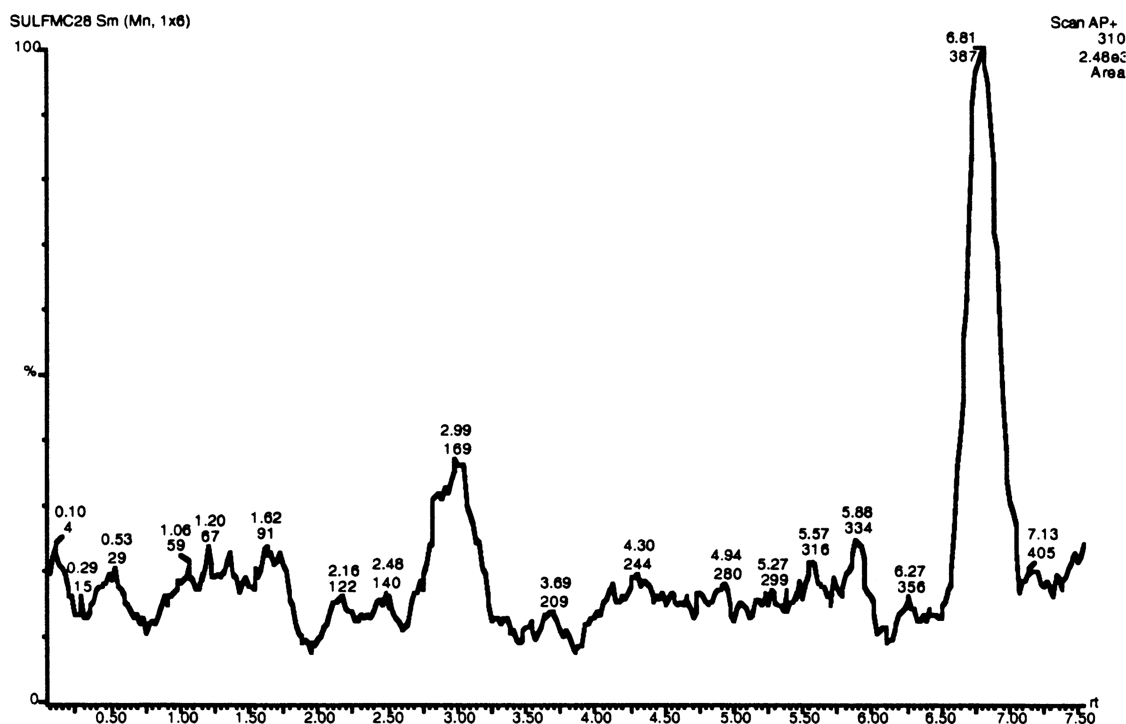

Fig. 4. HPLC/APCI-MS of a supercritical fluid extract from chicken liver. Trap was rinsed with 50/50 methanol/water thus yielding a solution containing of 100 pg μl^{-1} of sulfadimethoxine (SDM) (full scan mode, m/z 110–320). Column Conditions: 65/35 8 mM ammonium acetate/acetonitrile pH adjusted to 6.5 with acetic acid; flow, 1.0 ml min^{-1}; 250 × 4.6 mm i.d., 5 μm Prodigy C18. Injection: 20 μl in methanol. MS Conditions: APCI probe 400°C; corona pin, 3.0 kV; extraction cone, 30 V; source, 120°C. * Numbers above the peak indicate retention time and scan number.

famethazine at 10 ng on column is shown in Fig. 3. The protonated molecule at 279 is clearly observed and is the most abundant protonated peak. Other sulfonamides however, did not yield such abundant molecular ions. Extraction cone voltage has been shown to affect analyte fragmentation such that higher voltages cause more fragmentation [8]. A cone voltage of 30 V was used in this study since it is relatively mild, however, it appeared that some fragmentation still occurred for several of the sulfonamides investigated. The peak at m/z 156, the base peak for several of the sulfonamides, is common for all sulfonamides. It results from the cleavage of the sulfur–nitrogen bond on the sulfonamide. This fragmentation has been shown previously in both electron impact (EI) ionization [9] spectra and APCI-MS-MS [1] spectra of sulfonamides.

Next, a MDQ was determined using selected ion recording (SIR), where only one ion is mass spectrometrically monitored over a given period of time. SIR yields a lower MDQ since more time can be spent dwelling on a single ion of the compound instead of scanning masses where the compound is not present. Table 2 shows the results for each compound using the same chromatographic conditions. SIR was able to produce a lower MDQ than the extracted ion mode for almost all compounds. In most cases SIR yielded at least five times better sensitivity than scan mode. A previous report of HPLC/APCI-MS of sulfonamides [1] did not report the sensitivity of the system, but μg quantities of material were routinely analyzed in this study. However, in this study a 10% split in the column flow was necessary which no doubt reduced the observed sensitivity. On the other hand, some researchers have

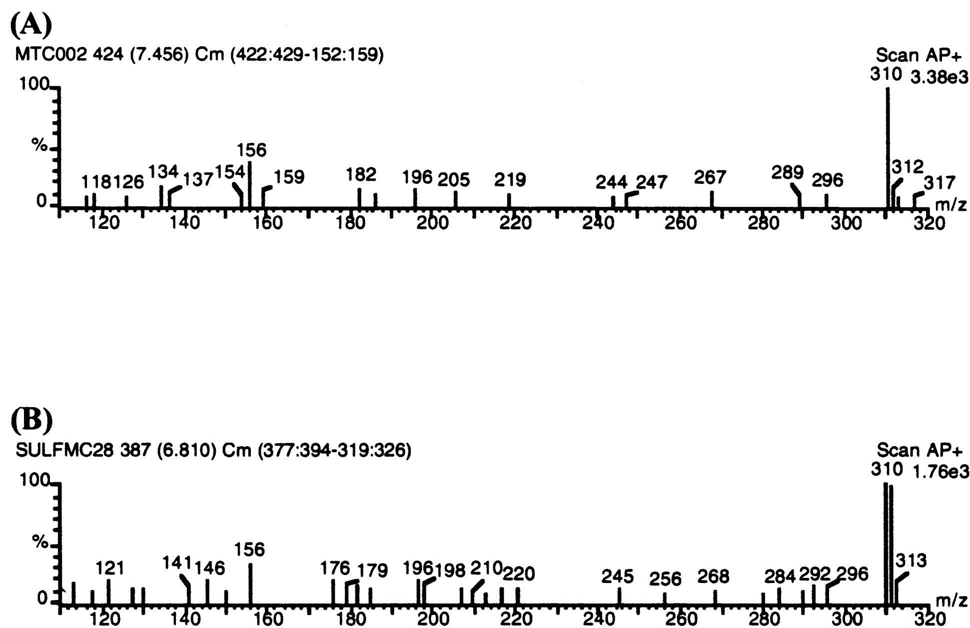

Fig. 5. Background subtracted mass spectra of 100 pg μl^{-1} sulfadimethoxine in full scan mode (*m*/*z* 110–320). (A) Standard solution (B) chicken liver extract.

observed that splitting does not reduce sensitivity in many cases (i.e. the ms detector behaves as a concentration based detector). The SIR method used in our study, therefore, appears to offer considerable flexibility and very sensitive mass spectral detection of sulfonamides.

The analysis of sulfonamides present in the supercritical fluid (SF) extractions of chicken liver was performed via HPLC/APCI-MS. Extracts of the chicken liver which had been fortified with SDM were provided by Robert Maxwell of the USDA/ARS in Philadelphia, PA. Their SFE extraction procedure which calls for the collection of the sulfonamides inside the extraction vessel on an alumina trap has been published in detail previously [6]. The alumina in-line trap was rinsed with 50/50 water/methanol. Fig. 4 shows the HPLC/APCI-MS chromatogram (in full scan mode (*m*/*z* 110–320)), of a SF extract prepared to yield a final solution concentration of 100 pg μl^{-1} (100 ppb) of SDM (2 ng injected). A peak for SDM can be clearly seen in the SIR mode, while the mass spectrum obtained (Fig. 5) is similar to that of the SDM standard alone. This extract showed no effect due to the presence of co-extractive material and the mass spectrometer exhibited sufficient sensitivity for routine sample analysis at this concentration level. The same sample assayed in SIR mode (*m*/*z* 310) also showed a sizeable peak at the regulatory level of SDM in chicken liver extracts (Fig. 6). In fact, the SIR mode exhibited sufficient sensitivity such that a sample solution consisting of SDM in a chicken liver extract at 25 pg μl^{-1} was still detectable with a S/N greater than 4:1. This was the lowest concentration extract provided to us, however, it is believed that lower concentrations would be detectable. It can be envisioned that a 1 g tissue extract containing sulfadimethoxine at the regulatory limit of 100 ng g^{-1} [3] rinsed into 1 ml of solution would have a concentration of 100 pg μl^{-1}. Therefore, this procedure offers a potential assay method for certain sulfonamides of regulatory interest.

The effect of co-extractive material on the detection of sulfonamides in chicken liver tissue was also of interest. To investigate this, the in-line alumina trap was rinsed with 100% methanol

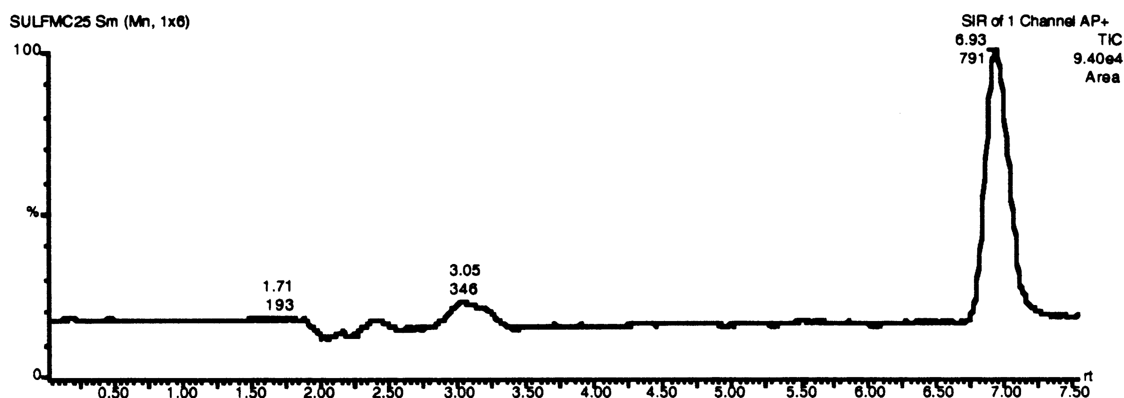

Fig. 6. HPLC/APCI-MS of a supercritical fluid extract from chicken liver rinsed with 50/50 methanol/water containing 100 pg μl^{-1} of sulfadimethoxine (SDM) (single ion mode, 310 *m*/*z*). Column Conditions: 65/35 8 mM ammonium acetate/acetonitrile pH adjusted to 6.5 with acetic acid; flow, 1.0 ml min^{-1}; 250 × 4.6 mm i.d., 5 μm Prodigy C18. Injection: 20 μl in methanol. MS Conditions: APCI probe 400°C; corona pin, 3.0 kV; extraction cone, 30 V; source, 120°C. * Numbers above the peak indicate retention time and scan number.

rather than 50/50 methanol/water to more effectively rinse any non-polar material from the trap into solution. No co-extractive interference was observed either in SIR mode or in full scan mode. A sample containing only 25 pg μl^{-1} of SDM was readily observed which demonstrates the applicability of HPLC/APCI-MS for the routine analysis of sulfonamides in the presence of non-polar co-extractive material.

Although the HPLC/APCI-MS method suggested adequate sensitivity for routine assay of sulfonamides, quantitative capabilities of the system were vitally important. In an effort to evaluate the system, a series of standards of SDM in 70/30 methanol/water were injected on-column with the mass spectrometer both in full scan (130–524 ppb) and SIR modes (32.5–260 ppb). Fig. 7 shows calibration curves from both modes for a series of standards. The correlation coefficient in either method was greater than 0.99, which indicated good linearity. In addition, replicate analyses of a 130 ppb solution of SDM performed in SIR mode possessed a RSD less than 7% for three injections, which indicated good sample to sample repeatability even at low levels.

4. Conclusions

HPLC/APCI-MS, has been shown to produce very low MDQs using both full scan and selected ion modes for the eight regulated sulfonamides investigated. Selected ion mode yielded an MDQ of 50 pg for three of the eight sulfonamides investigated. The analysis of supercritical fluid extracts of chicken liver containing sulfadimethoxine at 100 pg μl^{-1} or less was found to be feasible by HPLC/APCI-MS. In addition, the method also demonstrated good linearity and reproducibility for the detection of a representative sulfonamide in both full scan and single ion modes. HPLC/APCI-MS offers promising results for the routine identification and analysis of sulfonamides from biological matrices [10].

Acknowledgements

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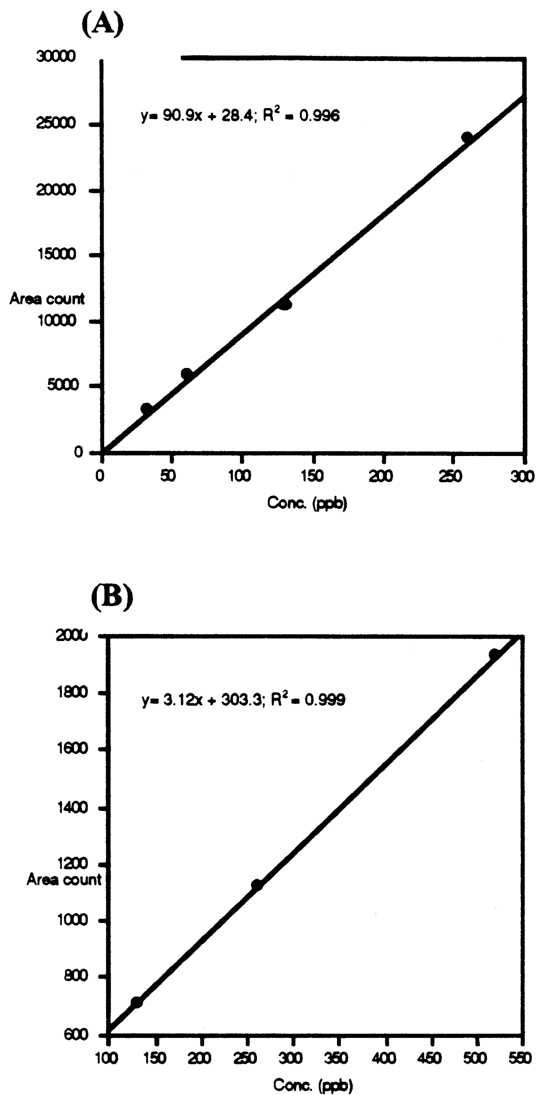

Fig. 7. Calibration plots for (A) single ion mode using m/z 310 (32.5–260.8 ppb) and (B) scan mode using the extracted ion m/z 310 (130.4–521.5 ppb).

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