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CORONA DISCHARGE IONIZATION LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY INTERFACE FOR TARGET COMPOUND ANALYSES

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SUMMARY

The design and construction of a dedicated liquid chromatography–mass spectrometry instrument for detecting and assaying trace-level target compounds are described. It employs a microbore high-performance liquid chromatograph interfaced directly to a point-to-point high voltage corona discharge ionization source with a quadrupole mass analyser. Applications are described using reversed-phase chromatography for the detection of route-specific impurities in formulated medicinal preparations at the 5 ppb level for patent protection, and for the detection of biogenic amines in blood plasma without derivatization.

INTRODUCTION

Mass spectrometry (MS) is an analytical technique of the highest specificity and sensitivity, but it is generally unsatisfactory for the analysis of mixtures or for the detection of target compounds in mixtures, owing to the complexity of the spectral data for each component. This problem was tackled effectively in the 1960s for volatile compounds by the coupling of gas chromatography (GC) with MS, allowing MS analysis of each component in a mixture. The newly developed soft ionization techniques have simplified the spectra and concentrated the signal for each component into a pseudomolecular ion, thereby increasing sensitivity. Liquid chromatography has long been an important separative method, but it was through the development of high-performance liquid chromatography (HPLC) that it came to rival GC in terms of speed and resolution. HPLC also has a substantial advantage over GC in being able to handle polar, involatile and thermally unstable materials without derivatization.

Unfortunately there is a basic incompatibility between HPLC and MS in that

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one operates in the condensed liquid phase and the other at high vacuum. For HPLC the ideal interface should not restrict the solvent systems, the use of buffers, or the solvent flow-rate, and it should not impair chromatographic integrity. It should be capable of the efficient transfer of the entire chromatographic eluent for compounds that vary widely in polarity, volatility and stability. From the MS point of view the interface should not restrict the method of ionization, and electron impact (EI), chemical ionization (CI), fast atom bombardment (FAB), etc. should be operable in both positive and negative ion modes. The solvent must not impair the high vacuum requirement in critical regions or cause maintenance problems or loss of sensitivity. The mode of scanning the mass spectrometer should not be restricted, *i.e.* full scans, single ion monitoring, linked scans and other tandem modes should all be available[1–6].

Following pioneering work by Tal'roze[7], many approaches have been adopted to the solution of these problems but none meets all of the requirements specified above. The early use of direct liquid introduction (DLI) into the ion source resulted in high pressures, even with the low flow-rates achieved by 1:1000 splitting of the eluent, and only CI was possible, with the solvent acting as the reagent gas[8]. This compromised both the HPLC and the MS operations as only a limited range of solvents was acceptable. The transport interface (moving belt) also relied on a volatile solvent system but it allowed a range of ionization techniques to be employed[9]. Early versions were ineffective for reversed-phase chromatography and involatile compounds, but more recently FAB has been added to the range of ionization techniques applicable[10].

Thermospray is currently the most widely used technique[11]. This development from DLI, in which most of the solvent is pumped away leaving ionized solute, enjoys the advantages of not requiring solvent splitting for conventional flow-rates of 1 cm^3/min and being able to handle reversed-phase chromatography with high aqueous phase solvents, buffers and polar solutes. However, for the analysis of non-polar compounds a secondary ionization method, such as corona discharge, is required. Continuous-flow FAB offers exceptional promise for the analysis of involatiles[12,13], but the very low flow-rates of < 10 μl/min make direct interfacing to standard analytical columns difficult, and although packed fused-silica microbore columns can be directly coupled to mass spectrometers[14], the technical problems associated with handling these miniature LC systems makes them undesirable for routine use. Ionization has also been achieved at atmospheric pressure using radioactive decay[15], corona discharge[16], thermospray[17], and ion spray[18,19]. This eliminates the problem of pumping away the solvent, but still requires the efficient transfer of the ions into the mass spectrometer. The monodispersed aerosol generation interface combining HPLC and MS (MAGIC)[20], which allows both EI and CI data to be obtained, gives spectra that can be subjected to library search and is therefore suited to the identification of unknown compounds.

In the present study it was desired to construct an instrument that would provide high sensitivity detection and assaying of known target compounds at trace levels in pharmaceutical formulations and in biological fluids, often for screening with a minimum of sample work-up and without derivatization. The target compounds could vary widely in terms of polarity and volatility and would not necessarily be amenable to thermospray. It was decided that microbore HPLC at 50–100 μl/min

flow-rates using a DLI interface with corona discharge would best meet these requirements, giving soft ionization with little or no fragmentation and therefore being ideal for single or multiple ion monitoring.

DESIGN AND CONSTRUCTION

The corona discharge LC–MS system was designed to be flexible. Variable dimensions in the ion source were the electrode gap, the position of the nebulizer with respect to this, the ion exit aperture and the distance between it and the entrance to the quadrupole analyser. The dimensions given in this section were found to give optimum performance with the initial design, and these were used for the two studies below. Subsequent changes to the pumping improved the performance as detailed later.

The instrument has two vacuum housings connected only by a 0.3-mm diameter aperture. The ion source housing is pumped by a 2000 l/s diffusion pump giving a pressure of $1–5 \cdot 10^{-4}$ Torr at a solvent flow-rate of 100 μl/min. There is no direct measurement of the pressure within the ion chamber but it is calculated to be *ca.* 10 Torr. The analyser region is pumped by an Edwards EO4 diffusion pump (70 l/s) and cold trap, which maintains a pressure of $1 \cdot 10^{-5}$ Torr. The gas-tight ion chamber in Fig. 1 is a high-grade non-magnetic stainless-steel block with a cylindrical cavity, 38 mm diameter × 24 mm deep with a 2 mm diameter ion exit aperture. The corona electrodes are pointed stainless-steel rods with a diameter of 1 mm, mounted on either side of the chamber, one being electrically insulated. The electrode gap was optimized at 14 mm. The LC eluent is introduced into a nebulizer consisting of a fused-silica tube carrying the liquid, surrounded by a larger bore silica tube through which heated nitrogen gas is blown. The outer tube extends slightly beyond the inner tube, preventing drops formation and ensuring spray formulation. The nebulizer is mounted through the wall of the ion chamber and extends in by *ca.* 10 mm, from where the spray is directed into the electrode gap. As reported by others[21], experiments without the nebulizer suffered from pulsing due to drop formation. The ion chamber can be

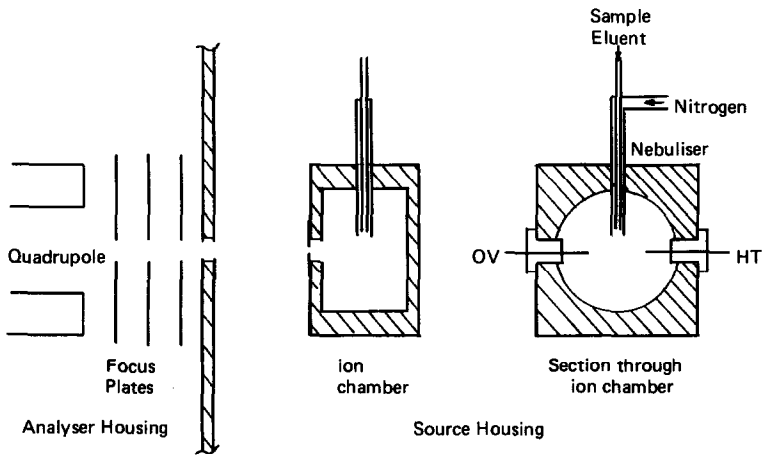


Fig. 1. Schematic diagram of the ion source.

moved to vary the distance between the ion exit aperture and the analyser entrance aperture, which was optimized at 14 mm. The chamber support assembly provides electrical isolation and ensures precise alignment of these two apertures with the focus plates and the quadrupole analyser.

In the experiments described here the chamber was held at ground potential, although later experiments with a small positive potential showed enhanced sensitivity due to the improvement in ion abstraction efficiency, 70 V giving a two-fold increase in signal strength. The corona is struck by applying 5 kV to the insulated electrode, giving a corona current of 300 μA under normal operating conditions. As reported before[22], the relationship between current and voltage is non-linear, and higher voltages give significantly higher currents, but 5kV was adopted as it gives reliable operation without electrical breakdown. The position and influence of the negative glow region of the corona is crucial in maximizing the positive ion current detected. Ions passing through the small aperture into the analyser region are focused into the quadrupole by a three-element lens, the grounded aperture being the first element. The centre element is held at $-$ 70 V and the third element is at $-$10 V, the same as the bias on the quadrupole. The quadrupole in the experiments described here was a VG QXK400, mass range 0–400 a.m.u.

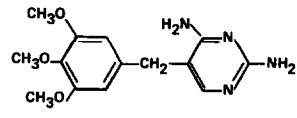
CHROMATOGRAPHY

Most HPLC analyses in the pharmaceutical industry are carried out under reversed-phase conditions with solvent systems such as methanol–water or acetonitrile–water, often with buffers. Here two different methanol–water systems were used, in one case with buffers. The equipment comprised a DuPont 8800 pump with a Chrompak 25 cm $\times$ 1.35 mm I.D. CP-Spher-C_{18} 8-μm column. Samples were introduced through a Rheodyne 7520 microinjection valve using either a 1-μl or a 5-μl loop. The flow-rate was 100 μl/min, and the column eluent flowed through a micro UV cell (8 μl) before introduction into the mass spectrometer. A series of compounds was examined to evaluate the chromatographic integrity of the LC–MS system, and no measurable broadening of mass spectral peaks was observed when compared with the UV signal. Separations were carried out at 22°C, and UV absorption was measured at 280 nm using a DuPont 852 UV detector.

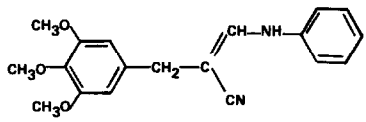
PATENT PROTECTION—IMPURITIES IN TRIMETHOPRIM

The financial investment required to successfully produce a new medicinal agent is very high, and companies protect this investment through the medium of patents. It may be possible to prove that a competitor's product infringes patents that protect manufacturing methods by detecting route-specific trace impurities, *i.e.* starting materials, intermediates or side-products[23]. Preparation of trimethoprim (TMP) (**1**)[24], an important antibacterial agent, by the so-called anilino route gives rise to the impurity TAA, β-anilino-α-(3,4,5-trimethoxybenzyl)acrylonitrile (**2**), whereas an alternative route can give the benzal impurity, β-methoxy-α-(3,4,5-trimethoxybenzylidene)propionitrile (**3**).

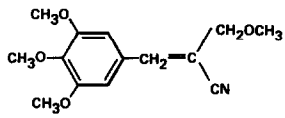
The calibration curves illustrated in Fig. 2 were constructed for TAA and benzal. For both compounds these show linear responses for protonated molecular ion



Trimethoprim (1)



TAA (2)



Benzal (3)

current *versus* amount on column over the range 50–750 pg (absolute measurements, with no internal standards), and 50 pg (150–200 fmol) represents the lower limit of detection as the signal-to-noise ratio was reduced to 2:1. This represents a substantial increase in sensitivity compared with UV detection, for which the lower limit was 1 ng injected on column. TMP is usually formulated with sulphamethoxazole as Co-trimoxazole. For the analysis of Co-trimoxazole tablets an initial solvent extraction was carried out to remove the bulk of the TMP and the sulphamethoxazole, and 6.5 g of crushed Co-trimoxazole tablets containing 1 g of TMP were shaken with 20 cm^3 of

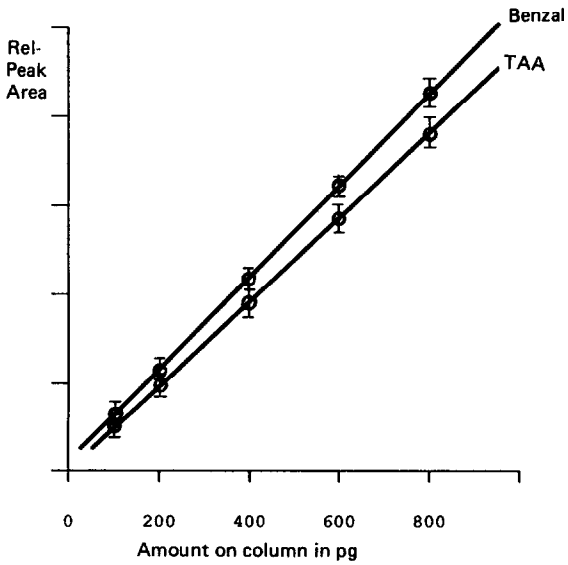


Fig. 2. Calibration plots for TAA and benzal.

dichloroethane for 10 min. The supernatant liquid was filtered off and reduced almost to dryness in a stream of dry nitrogen. Methanol (100 μl) was added and 1 μl was injected into the chromatograph. The signals were monitored at *m/z* 264 (benzal), 291 (TMP) and 235 (TAA). The traces are shown in Fig. 3.

Despite the extraction the strongest signal was observed for TMP at a retention time of 9 min, and this was also seen by the UV detector at 280 nm. The retention times for TAA and benzal are 12 and 14 min. A peak was observed for TAA but not for benzal, confirming that the preparation employed the anilino route. It is noteworthy that the UV trace does not show a peak for TAA, and indeed a weak peak would be obscured by the tailing of the TMP peak. Multiple ion monitoring by MS eliminates such interferences. This experiment was designed to confirm the presence or absence of target compounds and was not an accurate assay as no internal standards were employed. However, reference to the calibration curve indicated that *ca.* 220 pg of TAA were injected, being 1% of the total sample, therefore *ca.* 22 ng of TAA had been extracted from the 6.5 g of tablets. Separate experiments have shown the extraction to be *ca.* 70% efficient for TAA, so this shows the detection of TAA in formulated TMP preparations at a level down to *ca.*5 ppb. This gives a rapid and sensitive method of screening pharmaceutical products for known route-specific impurities.

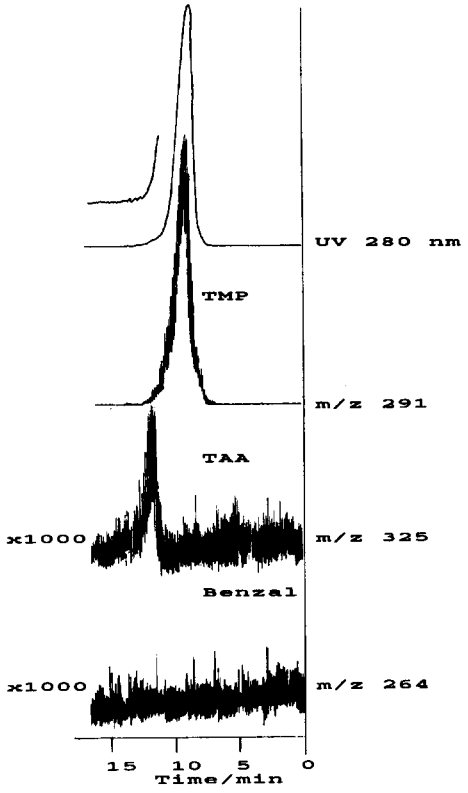


Fig. 3. Multiple ion monitoring traces dor Co-trimoxazole extracts.

BIOGENIC AMINES IN BLOOD PLASMA

The function of adrenaline and noradrenaline as chemical mediators of most of the postganglionic fibres of the sympathetic division of the autonomic nervous system is well known[25]. O-Methylation is an important metabolic pathway for catecholamine hormones[26], O-methyl transferase being the primary enzyme that inactivates circulating and possible locally released catecholamines. Detection and quantitation of adrenaline and metanephrine in tissue, blood plasma and urine are important for recognizing pheochromocytoma and also as a reflection of adrenergic and adrenomedullary stimulation[27]. GC–MS is effective for such assays but derivatization is necessary to aid volatility and thermal stability[27–29]. HPLC with electrochemical detection gives high sensitivity but it lacks the specificity of MS[30–32]. Consequently an LC–MS method is being developed.

The amines were extracted from 5 cm^3 samples of rat plasma by a published method[33], using an alumina column (Bondulate). They were absorbed onto the column from alkaline buffered plasma (pH 8.6) and after the column had been washed they were desorbed with 0.5 *M* acetic acid. The volume was reduced under vacuum to 10 μl and 5 μl were injected on column. The solvent system was methanol–0.1 *M* sodium orthophosphate–0.05 *M* citric acid (40:20:40). The sample HPLC column was used as in the previous experiment. Multiple ion monitoring was carried out at *m/z* 184 and 198 for the protonated molecular ions of adrenaline and metanephrine respectively. The traces are shown in Fig. 4 for the analysis of the plasma of two rates.

Calibration curves showed that adrenaline and metanephrine gave linear responses for ion current *versus* amount injected, and they could be detected down to a level of 100 pg (S/N = 2:1), whereas UV detection gave a lower limit of 10 ng from blood plasma. The extract from a healthy rat (A) gave a signal for adrenaline corresponding to 250 pg on column, which, assuming 100% efficiency of extraction, is 100

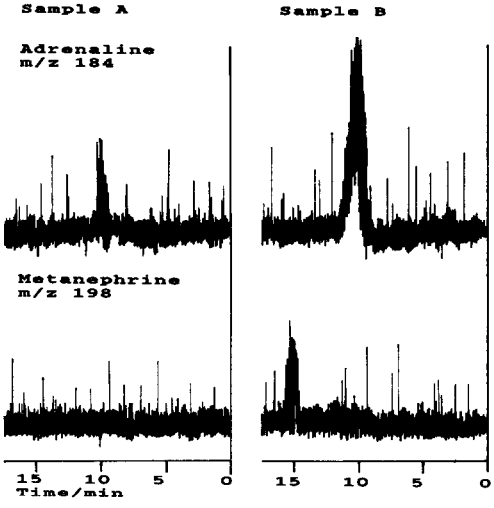


Fig. 4. Multiple ion monitoring traces for extracts from rat serum.

pg/cm^3 in plasma; there was no detectable signal for metanephrine. The extract from a hypertensive rat (B) showed an increase in adrenaline to 250 pg/cm^3 and metanephrine was detected at 80 pg/cm^3. These results may be subject to the well-known errors associated with assays carried out without internal standards, and a suitable standard is now being developed. However, these preliminary data agree well with observations by GC–MS and HPLC with electrochemical detection and they demonstrate the sensitivity attainable with a relatively simple extraction procedure and without derivatization.

IMPROVEMENTS AND MODIFICATIONS

A programme of modifications and improvements was carried out to further enhance the performance, the most important being to optimize ion transfer from the ion chamber to the analyser. The pumping of the analyser region was up-rated with the replacement of the original 70 l/s diffusion pump by a 700 l/s Edwards Diffstak. This reduced the analyser pressure from $1{\cdot}10^{-5}$ to $1{\cdot}10^{-6}$ Torr and increased the detected ion current for 100 pg of TAA by a factor of 1.7 as losses due to ion–molecule collisions were reduced.

The improved pumping also allowed better ion transmission to be achieved by increasing the size of the analyser entrance aperture from 0.3 to 0.9 mm, giving a nine-fold increase area, the signal for 100 pg of TAA increasing by a factor of 7. It is believed that further improvements to the pumping in the region of these apertures will provide a further sensitivity gain. The original quadrupole analyser had a very limited mass range, restricting the applications of the instrument. It is now being replaced by a VG 12/12 with a mass range of 2000 a.m.u. Finally, a computer-based control and data acquisition system is being installed.

CONCLUSIONS

The LC–MS system described has been demonstrated to provide high sensitivity specific detection and assay of target compounds in complex mixtures with the minimum of sample work-up. Unlike earlier corona sources with point-to-plane discharges, this source uses a precisely controlled point-to-point discharge that allows the region of maximum ion generation to be focussed coaxially with the ion slit and the quadrupole analyser. The use of corona discharge provides a soft ionization method that gives strong protonated molecular ions for both polar and non-polar compounds with little fragmentation, ideal for single or multiple ion monitoring. It can cope with normal or reversed-phase chromatography, with or without added buffers, and although not reported here it is amenable to gradient operation. As well as the studies reported above, the instrument has been used equally effectively for aromatic hydrocarbons and peptides, showing the wide range of compound polarities amenable to analysis.

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