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Short communication

Liquid chromatography–tandem mass spectrometry applied to a study of the metabolism of pentamidine Discussion of possibilities and problems

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Abstract

Tandem mass spectrometry is usually employed to achieve rapid screening or structure elucidation. We have used liquid chromatography–tandem mass spectrometry in order to detect metabolites of the antiprotozoal drug pentamidine in urine. Samples of urine from rat and man were analysed both by direct injection and after solid-phase extraction. The present paper discusses advantages and disadvantages of using direct injection of urine samples, optimization of chromatographic conditions with regard to the performance of the mass spectrometer, automation and stability of the entire system. © 1997 Elsevier Science B.V.

Keywords: Pentamidine

1. Introduction

The antiprotozoal drug pentamidine is used for the treatment of African sleeping sickness and *Pneumocystis carinii* pneumonia [1]. The drug has been used since the 1940s, but studies of its disposition has not been performed until recently [2,3]. As a part of these studies we have investigated the metabolism of pentamidine in the rat [2] and in man (results to be published). There are some problems involved in the studies of the metabolism of pentamidine. The drug is a strong base, it tends to adsorb to various materials, some of the metabolites have physicochemical properties that are widely different from the other metabolites and from the parent drug, and the concentration of the metabolites is very low in human material.

We have used liquid chromatography–tandem mass spectrometry in order to achieve an unequivocal identification of the metabolites. The aim of the present paper is to discuss our experiences of the advantages and problems with this technique in connection with studies of pentamidine metabolism. A presentation of the results of in vivo studies of the metabolism of pentamidine in man will be published separately.

2. Experimental

2.1. Solid-phase extraction

The urine samples were injected either directly

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(100–150 μl) after centrifugation or after solid-phase extraction using Sep-Pak (Waters, Milford, MA, USA) with an eluent consisting of 2 ml acetonitrile–water (1:1). Further details will be given in a separate paper dealing with the identification of pentamidine metabolites in man.

2.2. Liquid chromatography

The injector was a CMA 200 autoinjector, (CMA Microdialysis, Solna, Sweden). A personal computer was used to control the system. A Waters 600-MS Silk tertiary gradient system (Waters) and Rheos 4000 gradient system (Flux Instruments, Karlskoga, Sweden) was used. The column used was an Ultrasphere ODS, 5 μm, 15 cm×4.6 mm I.D. (Beckman, CA, USA). The eluent was 3% acetonitrile in a 50 m*M* ammonium acetate buffer at pH 6 with addition of 0.2% TEA (A) and 50% acetonitrile in a 50 m*M* ammonium acetate buffer at pH 6 with addition of 0.2% TEA (B). A gradient was run from A to B.

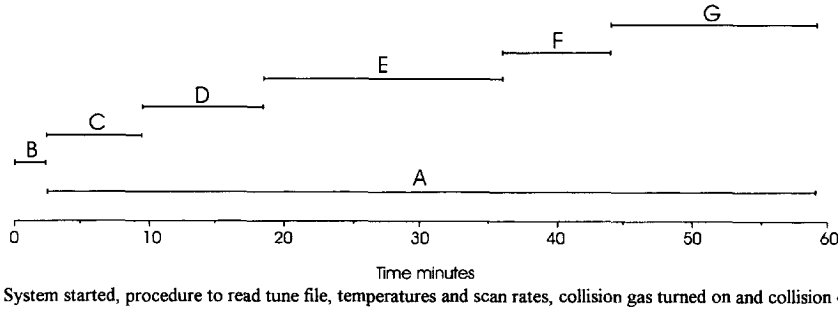
2.3. Mass spectrometry

A Finnigan TSQ 700 tandem mass spectrometer (Finnigan, San Jose, CA, USA) was used. The system was equipped with a Finnigan atmospheric pressure chemical ionization (APCI) interface with the following settings: vaporiser 400°C, heated capillary 200°C, current 5 μA.

The triple quadrupole system was used in either selective ion monitoring (SIM) mode as a single quadrupole system, or in MS–MS mode with alternating daughter and parent scans for selective reaction monitoring (SRM) detection. Ions where detected according to retention time. The eluting peaks where detected both in parent and daughter ion mode. The analytical cycle is shown in the scheme in Fig. 1.

3. Results and discussion

It is always desirable to minimize sample work up when the aim is to detect metabolites. The reason is that it is difficult to identify extraction procedures that give identical recoveries of different analytes. Furthermore, as more sample handling steps are introduced there are inherent risks of sample losses. Ideally, the biological sample should be introduced into the analytical system without any pre-treatment. This is however, not always possible. High selectivity of the analytical system is required, contamination



System started, procedure to read tune file, temperatures and scan rates, collision gas turned on and collision energy set at 20 eV

A. Multiplier turned on. Parent ion of m/z 137 gives a signal for all metabolites except VII m/z 373
B. Eluent from column directed to waste
C. Daugther ions of metabolite IV m/z 137
D. Daugther ions of metabolite VIII m/z 237
E. Daugther ions of metabolite IX m/z 223 and the two isomeric metabolites I and II m/z 357
F. Daugther ions of pentamidine m/z 341
G. Daugther ions of metabolite VII m/z 373 and parent ions m/z 137 of metabolite VII m/z 373

Acquisition stopped, multiplier and collision gas turned off, column equilibration for 1 hour.

Fig. 1. A description of MS activities during an analysis cycle.

may cause technical problems, and the concentration of the metabolites must be sufficiently high.

In our study of the metabolism of pentamidine, the rats were given 6–9 mg pentamidine isethionate per kg body mass and the concentration of the metabolites in rat urine was sufficient to allow direct injection. Humans were given 4 mg pentamidine isethionate per kg body mass, and due to the low concentrations of metabolites in urine, a concentration step was needed. We have thus gained experience of both strategies for metabolite detection.

3.1. Direct injection of urine

The investigation was started by running MS–MS of synthetic references of proposed metabolites [4]. This was done by analysing the different synthetic metabolites and selecting specific MS–MS, daughter and parent, ions for each metabolite. As a second step urine samples spiked with the different metabolites were analyzed using gradient elution. The metabolites were analyzed individually for determination of retention time as well as daughter and parent ion spectra. A 100-μl aliquot of a mixture containing pentamidine and six metabolites was injected. A retention time variability of less than ±5% for the isomeric metabolites with m/z 357 (Fig. 2) was considered acceptable.

The stabilising time after gradient elution was set to 1 h in order to obtain reproducible retention times for all metabolites. For the same reason the TEA concentration had to be constant during the entire cycle.

The effluent from the column was directed to the waste for 2 min by a computer controlled six port valve (Vici Valco, Schenkon, Switzerland) in order to minimise contamination of the mass spectrometer. A blank urine sample was injected between each rat urine sample.

3.2. Solid-phase extraction

Direct injection of human urine was not possible because of the low metabolite concentration. Solid-phase extraction was used for pre-cleaning and enrichment (Fig. 3). Our experience of the advantages and disadvantages of solid-phase extraction compared with direct injection are summarised in Table 1.

3.3. Instrument and parameters

Computer control from the MS system of peripheral devices such as autoinjector, HPLC and valves is of great importance when dealing with complicated analytes like pentamidine metabolites. Every step in the total analysis must be reproducible and this is achieved by invoking control over peripheral devices from the MS system.

The widely differing chromatographic properties of the pentamidine metabolites made it necessary to use gradient elution. Apart from the general and well-known disadvantages of gradient compared with isocratic elution, there is one specific effect that has to be considered when LC is combined with MS. The efficiency of the ionization in the atmospheric pressure ionization (API) source is affected by the amount of organic solvent that enters the source, and consequently, as the concentration of organic modifier increases during gradient elution, the efficiency of the ionization may change.

Most LC–MS systems can only handle volatile buffers, e.g., ammonium acetate. In both API techniques [APCI and electrospray ionization (ESI)] it is recommended to keep the buffer concentration as low as possible.

Our experience has shown that with ESI the buffer concentration should not exceed 5 m*M* when using ammonium acetate. When APCI was first introduced the manufacturers recommendation was to use this technique in the same way as thermospray (TSP) e.g., with 100 m*M* ammonium acetate. Later it was found that better results were obtained if the buffer concentration was kept as low as possible. Some application notes from manufacturers are without any buffer at all, but in practical work it is seldom possible to use such systems.

The use of a competing base such as triethylamine (TEA) is a compromise between optimum sensitivity and optimum chromatographic performance. Without TEA pentamidine and the metabolites showed extremely tailing peaks. TEA decreases the efficiency of the ionization, and may reduce the sensitivity of the mass spectrometric detection drastically. According to the manufacturer, Finnigan, the sensitivity can

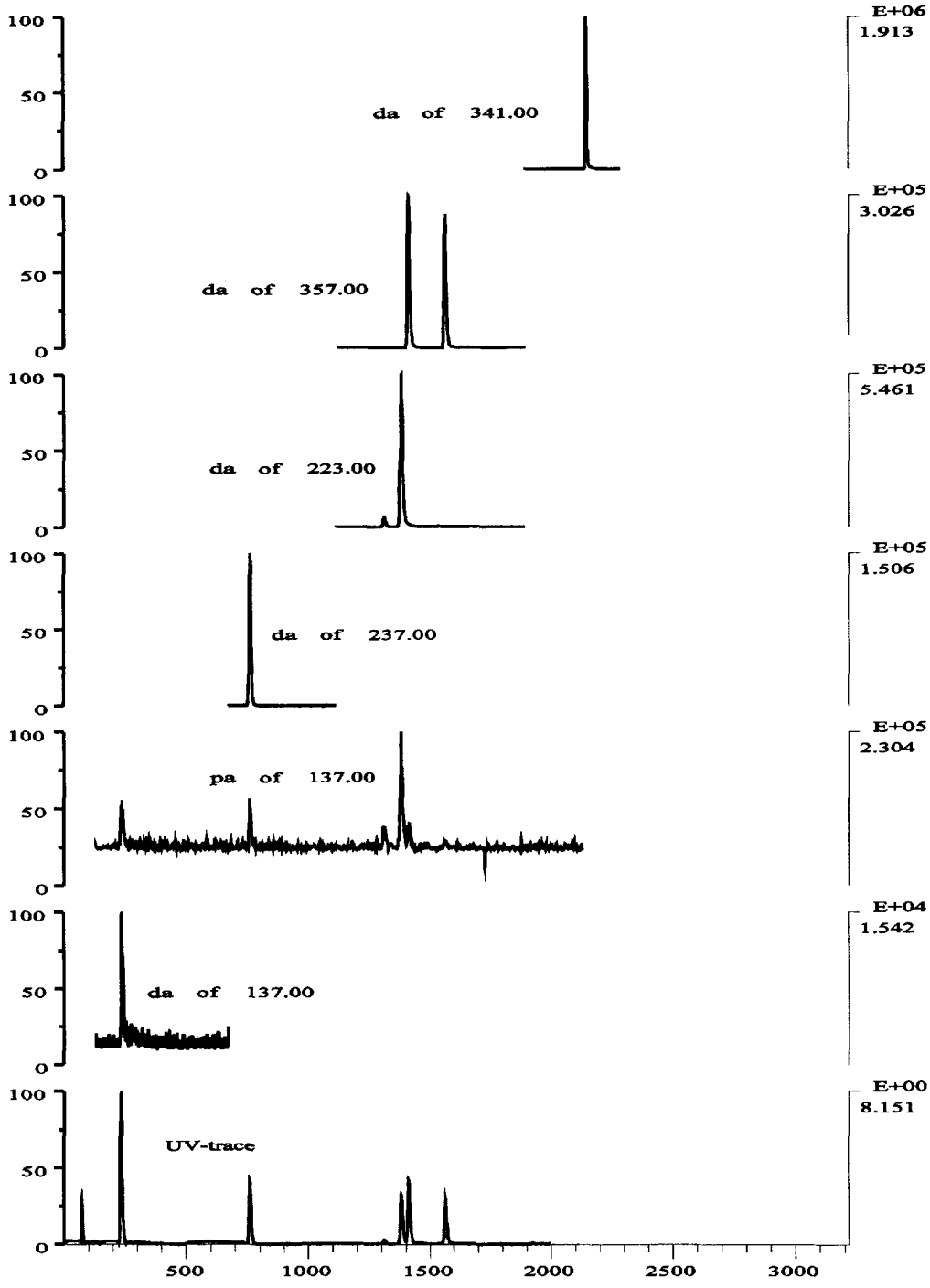


Fig. 2. Pentamidine and metabolites, mixture of synthetic reference compounds. Traces shown are daughter ion (da) and parent ion (pc) chromatogram for the different metabolites.

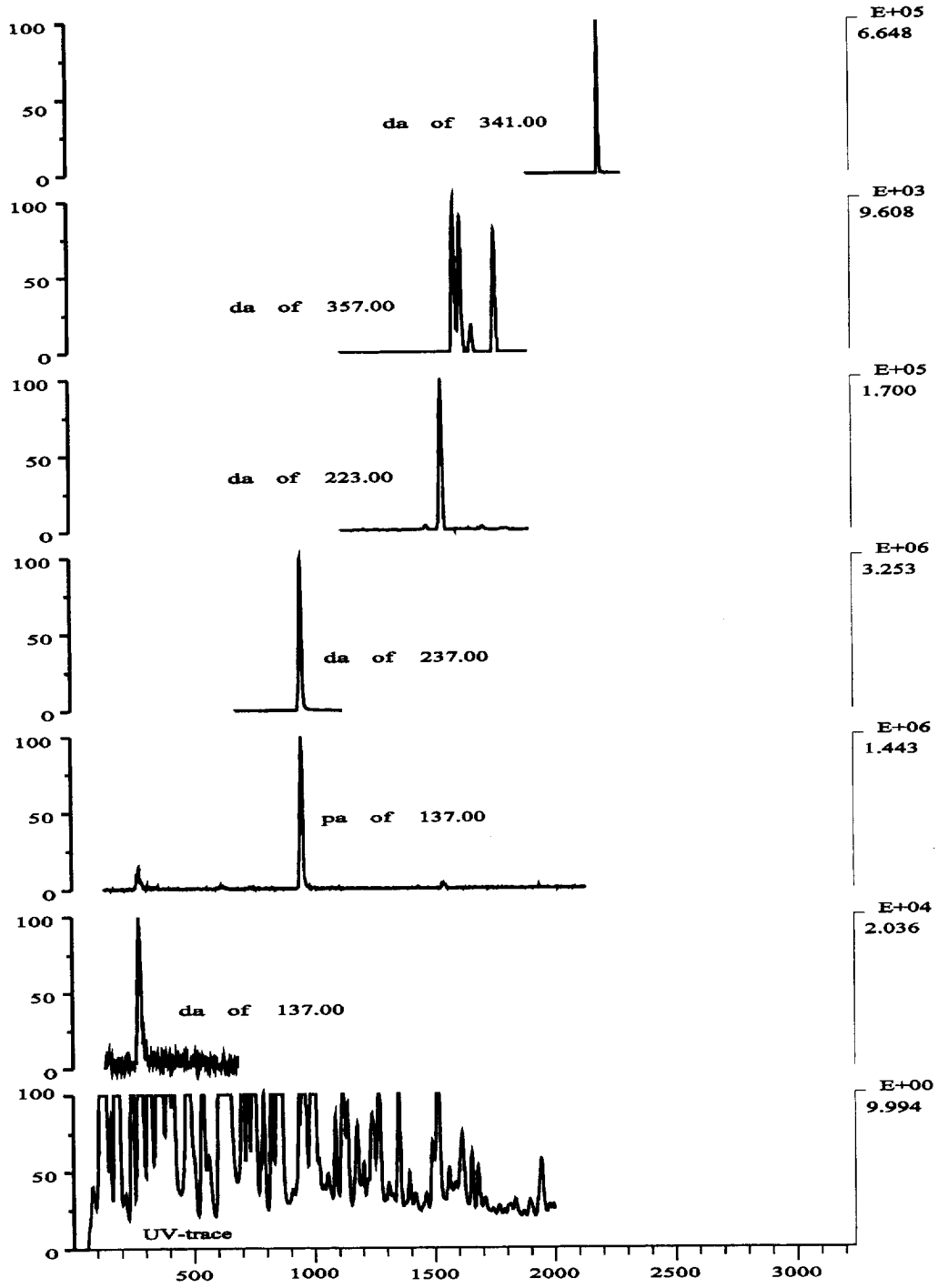


Fig. 3. Pentamidine and metabolites in a urine sample from a patient treated with 4 mg pentamidine isethionate per kg body mass. The sample was subjected to solid-phase extraction.

Table 1
Direct injection of urine samples versus solid-phase extraction: advantages and disadvantages

Direct urine injection into the LC–MS–MS system		Solid-phase extraction prior to LC–MS–MS	
Advantage	Disadvantage	Advantage	Disadvantage
No loss of compounds due to different chemical properties and varying recovery of extraction procedure.	Contamination, especially of the outer surface of the heated capillary and the API housing.	Cleaning up of samples, less risk for technical problems.	Recovery may not be reproducible.
	Matrix effects: To many molecules competing for the ionization.	Enrichment of samples.	Difficult to obtain good recovery for all metabolites because of different chemical properties.
	Only possible with sufficiently concentrated samples.		

decrease as much as 70% when TEA is used. We have noticed a decrease in sensitivity but thanks to better peak shape the detectability increased so that the net effect in our case was an improvement.

3.4. Reproducibility

Several different factors affect the reproducibility of the MS system: (1) stability of the APCI source: (a) contamination and (b) life time of parts in APCI source; and (2) MS–MS stability and reproducibility: (a) calibration, (b) tuning, (c) stability of collision gas pressure, (d) possibility of automation and (e) quality of quantitation package, software and hardware compatibility.

The stability of the MS system is very important and we have found scan rates, collision gas pressure and calibration and tuning to be extremely stable. The major source of variations and problems in our study has been the API interface. While running crude samples, there is a high risk for contamination, especially of the outer surface of the heated capillary and API source, which could cause a decrease in the sensitivity. Problems like clogged heated capillary, which seems to be a problem in peptide analysis, have not occurred. The heated capillary has, especially after running under acidic conditions (acetic acid pH~3), shown corrosion of the surface with loss of sensitivity. We have found that re-tuning of the system only has a minor effect on the sensitivity, while deterioration of the heated capillary could cause up to hundred-fold decrease in sensitivity. A way of overcoming the problem may be to frequently implement automated washing procedures during and after a batch run. We have also noticed that the vaporizer in APCI could cause decrease in sensitivity. Replacement of the vaporizer restored the sensitivity without any other notable parameter change. The skimmer has also become contaminated, in most cases by a thin water-soluble film. This effect increased with old heated capillaries or bad vaporizers.

A good way of frequently checking the performance of the system is to run control samples with a low concentration of the compound of interest. It should be noted that the fact that the instrument meets the specifications when running a test compound does not necessarily mean that the sensitivity is optimal for all other compounds. This could be a problem in discussions with the manufacturer.

The LC–MS–MS system is well suited for automatic long term analysis, for quantitative work an internal standard should be preferable. Buffer concentration should be kept as low as possible to minimize competition of ionization with sample. TEA can be used but with care.

In our study tuning, calibration and collision gas pressure have been very stable.

The weakest point in our system has been the API interface, especially the heated capillary.

Acknowledgments

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References

[1] Y. Aden Abdi, L.L. Gustafsson, Ö. Ericsson and U. Hellgren, Handbook of Drugs for Tropical Parasitic Infections, Taylor and Francis, London, 2nd ed., 1995, p. 117.

[2] U. Bronner, L.L. Gustafsson, F. Doua, Ö. Ericsson, T. Miézan, M. Rais and L. Rombo, Br. J. Clin. Pharmacol., 39 (1995) 289.

[3] U. Bronner, Ö. Ericsson, J. Nordin, I. Wikström, Y. Aden Abdi, J.E. Hall, R.R. Tidwell and L.L. Gustafsson, Pharmacol. Toxicol., 77 (1995) 114.

[4] B.J. Berger, N.A. Naiman, J.E. Hall, J. Peggins, T.G. Brewer and R.R. Tidwell, Antimicrob. Agents Chemother., 36 (1992) 1825.