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Quantitative electrospray LC–MS and LC–MS/MS in biomedicine[1]

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Abstract

The use of electrospray LC–MS and LC–MS/MS for the quantitative determination of two low molecular weight (< 500 Da) organic compounds in human plasma (Lovastatin) and cell supernatants (Arachidonic acid) and medium molecular weight (> 2000 Da) endogenous peptides (Endothelins) in supernatants of human umbilical vein endothelial cell cultures is reported. These methods make use either of deuterium labelled or structurally similar molecules as internal standards for quantitation and one or more pre-purification steps previous the LC–MS analysis. Linear calibration curves and detection limits around 50 pg ml^{-1} were obtained in all three cases. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

An increasing number of new and challenging biomedical problems call for quantitative methods with high sensitivity and high specificity that are easy to use and rugged enough to exclude any possible interference from complex biological matrices. With the introduction of Electrospray Ionization (ESI) as an effective ionization method, the analysis of polar molecules at high sensitivity has become amenable [1]. Despite the already numerous applications of ESI–MS in the qualitative analysis of small and large molecules, the development of quantitative capabilities has been slow. However, the intrinsic high specificity and sensitivity of ESI in conjunction with MS or tandem MS (MS/MS) analysis suggest it as a powerful quantitative method in biomedicine that will certainly complement other established quantitative tools such as HPLC, RIA, or ELISA.

Quantitative applications of ESI–MS have mainly focused on small molecules, especially on compounds which are relevant in pharmacokinetic studies of drug metabolism. For example, serum and urine levels (equine) of triamcinolone

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acetonide in the low ng ml^{-1} range were determined by LC–ESI–MS/MS [2]. Also, lipid sulphate esters from mouse keratinocytes have been determined specifically in presence of isobaric phospho mono- and diesters at the ng 10^6 cell^{-1} levels by LC–ESI–MS/MS [3]. For a general review on LC–MS related applications see ref. [4].

In the peptide field, ESI–MS has so far mainly been used for qualitative analysis and only in a few cases for the quantification of peptides from biological fluids. For instance, LC–ESI–MS has recently been used for the quantitative determination of a pentapeptide in human and rabbit plasma with column detection limits of 2 ng ml^{-1} (from 8 ml plasma) [5]. A LC–ESI–MS/MS method for the analysis of a 22 amino acid containing peptide showed linearity from 0.1 to 5 ng ml^{-1}. A microcolumn-SPE was used for isolating the peptide from 1.0 ml of plasma with a detection limit of 0.1 ng ml^{-1} [6]. Another group reported an application in which LC–ESI–MS/MS was combined with SPE for the analysis of two opioid peptides from 0.5 ml human plasma. This approach showed linearity from 5 to 1000 ng ml^{-1} with a detection limit of 0.25 ng ml^{-1} [7]. In all these examples, an internal standard was added prior to sample preparation and mass spectrometric analysis.

Quantitative mass spectrometric measurements of high accuracy require the use of an internal standard to account for variations in recovery and in mass spectrometric conditions. Ideally, isotope (deuterium) labelling is used to produce a standard with practically identical properties as the compound of interest. But when an isotope labelled standard is not available, a chemically modified derivative is often used. In the three examples described below we used as internal standards a deuterium-labelled compound, a chemically modified compound and two peptide isoforms, respectively.

In our laboratory we apply LC–ESI–MS and LC–ESI–MS/MS to the quantitative determination of both low and high molecular weight analytes in biological samples in order to be able to solve specific biomedical problems. In this regard, we report herein the use of these techniques for the quantitative determination of i) two low molecular weight (<500 Da) organic compounds in human plasma (Lovastatin) and cell supernatants (Arachidonic acid) and ii) medium molecular weight (>2000 Da) endogenous peptides (Endothelins) in supernatant of human umbilical vein endothelial cell cultures.

Arachidonic acid (AA) is the major polyunsaturated fatty acid in the membrane phospholipids of animal cells. AA is released from the phospholipids and metabolized to give rise to oxidized metabolites such as prostaglandins, thromboxanes, leukotrienes and other hydroxy acids with important biological activities. AA production is usually determined by EI or CI GC–MS methods that require derivatization (e.g. methylation) to render the acid volatile [8,9]. A direct MS analysis can be carried out, however, by direct analysis of the arachidonate anions by LC–ESI–MS. We have developed such a quantitative method using a commercially available deuterated AA standard.

Lovastatin belongs to a family of recently introduced antihypercholesterolemic drugs which are potent inhibitors of HMG–CoA reductase, the rate controlling enzyme in cholesterol synthesis. The drug is hydrolyzed in vivo to mevinolinic acid (MVA), the active dihydroxyacid form which is structurally similar to HMG–CoA. Analytical methods for pharmacokinetic studies of this drug are based on elaborated and time consuming GC–MS procedures involving various derivatization methods with detection limits of 0.2–0.1 ng ml^{-1} in human plasma [10,11]. HPLC using fluorescence detection has also been used for the analysis of the lovastatin analog pravastatin with detection limits of 0.1 ng ml^{-1} from 1 ml plasma, but it required derivatization by *N*-dansylethylendiamine and a column-switching system to eliminate reaction by-products [12]. For routine analysis of lovastatin, we have attempted to simplify the experimental procedures by developing a HPLC–MS/MS method of equal or better sensitivity. As no labelled standard was readily available, we chose the analog methylmevinolinic acid (MMVA) as internal standard.

The endogenous peptides we have selected are the endothelins (ETs), a family of peptides of 21 amino acid residues found in various types of tissues which are the most potent vasoconstricting

substances presently known. They also influence such activities as cell proliferation and hormone production and have been implicated in cardiovascular disorders ranging from stroke to ischaemic heart disease [13]. Conventional methods for the determination of ETs are radioimmunoassay and enzyme-linked immunosorbent assay. Although both methods exhibit high sensitivity, the ability to distinguish between endothelin isoforms relies on the reproducible production of highly specific antibodies and this is not routinely accomplished.

2. Methods

2.1. Basic instrumentation

For HPLC in LC–ESI–MS experiments an ABI 140 solvent delivery system in conjunction with a TRIATHLON sampler (Spark, Emmen, Holland) equipped with a 100 μl sample loop, was used. The TRIATHLON was operated in the μl-pick up mode. The outlet of the column was directly connected to the ESI interface of a Finnigan TSQ 700 mass spectrometer.

2.2. Arachidonic acid

A total of 50 μl (25 pmol) of d_8-AA were added to 200 μl of the cell culture supernatant. 750 μl of water/methanol (6.5:1) were added. The solution was passed through a Bond Elut solid phase extraction cartridge (C18, 100 mg) (Varian). The cartridge was washed with 1 ml of water and eluted with 1 ml of methanol. The eluate was evaporated and redissolved in 100 μl of 70% methanol. 60 μl were analyzed by LC–ESI–MS. HPLC solvent A: $MeOH/H_2O$ with 1% acetic acid (1:1), solvent B: MeOH, gradient: 0 min: 75% B, 3 min: 100% B, 7 min: 100% B, column: Phenomenex Nucleosil 3 μ C18 (50 × 2 mm) 100 Å, precolumn: ODS (10 × 2 mm) (Upchurch). Quantification was carried out by detection of the M–H – signals at m/z 303 (AA) and m/z 311 (d_8-AA).

2.3. Lovastatin metabolite

A total of 100 μl of the internal standard (methylmevinolinic acid, MMVA) were added to 1 ml of human plasma and diluted with 2 ml of water. The sample was then loaded onto a Bond Elut (C8, 200 mg) solid phase extraction cartridge. The cartridge was washed with 3 ml water followed by 3 ml of water/methanol (8:2). Mevinolinic acid and its internal standard were eluted with 2 ml methanol.

After evaporating close to dryness, the sample was redissolved in 100 μl methanol/water (2:1). 25 μl were directly loaded on the LC–ESI–MS system. The two extracted compounds were separated under gradient conditions in less than 8 min on a narrow bore HPLC column (1 × 100 mm ODS). Solvent A was $MeOH/H_2O$ (2:1) with 0.1% of acetic acid and solvent B was MeOH. The gradient started at 30% B and increased to 80% B in 0.1 min where it was held for 5.9 min. The analyte and its internal standard were baseline resolved.

The MS/MS analysis was carried out in the precursor ion mode. The reactions monitored were: MVA m/z 421 → 319, MMVA m/z 435 → 319. Other MS/MS conditions were: CID offset: −20 eV; CID gas pressure: 1.8–2.0 mTorr; Acquisition window: ±1.5 mass units; Scan time: 0.3 s $scan^{-1}$.

2.4. Endothelin isoforms and analogs

Endothelin standards were obtained from Peninsula Laboratories. Cell culture medium of human umbilical vein endothelial cells (HUVEC) were obtained from Dr. Ginés Escolar (Hospital Clínic, Barcelona, Spain). The extraction and purification procedure of cell culture medium was performed as follows: after pH adjustment of culture medium (pH 3) the $Ala^{3,11}$-ET1 standard was added. The sample was loaded on the MeOH-washed and pH 3 conditioned Sep-Pak C18. After washing with 0.1% TFA, endothelins were eluted by acetonitrile/H_2O/TFA (60:40:0.1). The eluate was concentrated in a Speed-Vac.

The first HPLC fractionation step of HUVEC extracts was performed on a KONTRON 325

System equipped with an Uvikon 722LC spectrophotometer for UV detection, and a Hypersil ODS column (3 μm, 4.6 × 60 mm). Solvents were: solvent A: H_2O/acetonitrile/TFA (90:10:0.05), solvent B: H_2O/acetonitrile/TFA (10:90:0.042). Gradient used was: 0 min: 10% B, 5 min: 10% B, 25 min: 100% B. Flow rate: 0.5 ml min^{-1}. Fractions were taken at previously determined retention times for ET standards. Fractions were pooled, evaporated to dryness in a SpeedVac and upon resolvation and addition of VIC standard subjected to a second HPLC step for further separation and on-line mass spectrometric detection. For HPLC–ESI–MS the column used was a Hypersil C4 (300 Å, 5 mm, 2.1 × 150 mm) and the eluents were: solvent A: H_2O/acetonitrile/TFA (90:10:0.02), and solvent B: H_2O/acetonitrile/TFA (10:90:0.016). The linear gradient used was: 0 min: 20% B, 14 min: 95% B, flow: 100 ml min^{-1}. A filter and a precolumn (Hypersil C4, 300 Å, 5 μm) were installed between the injector and the analytical column. Sheath gas and auxiliary gas were optimized using a solution of oxidized b-chain bovine insulin (1 μM) infused at the employed LC flow rate.

MS conditions were: ESI voltage: −4.5 kV; ESI capillary temperature: 250°C; ESI sheath gas (N_2): 65 psi, ESI auxiliary gas (N_2): 25 ml min^{-1}; electrometer gain: 8; multiplier: 1500 V. Ions monitored: $[M+2H]^{2+}$ and $[M+3H]^{3+}$ of ET1, ET2, ET3, $Ala^{3,11}$-ET1 and VIC (SIM, window ±0.5 mass units, 2.5 s $scan^{-1}$).

MS/MS conditions were: ions monitored: ET1 831.3→1145.0, ET2 849.5→1172.0, ET3 881.1→1219.7, $Ala^{3,11}$-ET1 810.6→1113.5 and VIC 858.8→1185.6, (window: ±3.0 mass units, 2.5 s $scan^{-1}$), CID offset: −24 eV; CID gas pressure: 2.1–2.2 mTorr.

3. Results and discussion

3.1. Arachidonic acid

The SIM method developed for AA analysis showed detection limits of 60 pg ml^{-1} (200 fmol ml^{-1}) when 200 μl of the supernatants were analyzed. The low sample volume needed for the MS analysis allowed other parallel analyses (eg. immunoassays) to be carried out with the material from the same culture well (1 ml total volume).

Calibration curves were linear in the range 1–50 pmol ml^{-1} ($r > 0.999$) and the mean intra-assay reproducibility in this interval was calculated to be around 4.6%. The ion chromatograms showing the response obtained from 1 pmol spiked sample and a sample stimulated with calcium ionophore are shown in Fig. 1. Due to the relatively 'clean' nature of this cell supernatants, interference-free analyses are obtained with minimal sample preparation. Additionally, the use of the deuterated internal standard assures highly precise quantitative LC–ESI–MS measurements.

This method has been used in our laboratory for the routine automated analysis of cell culture supernatants with results clearly surpassing those obtained with our classical methods based in techniques such as GC–MS or HPLC–UV.

3.2. Lovastatin metabolite

To establish a method for the analysis of lovastatin metabolite, mevinolinic acid (MVA), we recorded negative ion ESI mass spectra of MVA and of methylmevinolic acid (MMVA) that we used as internal standard. The mass spectra reveal $[M-H]^-$ ions at m/z 421 for MVA and at m/z 435 for MMVA as well as a characteristic common ion at m/z 319. The MS/MS spectrum of product ions from MVA is shown in Fig. 2. The product ion spectrum of MMVA shows an identical pattern except for the $[M-H]^-$ ion at m/z 435 and the 101 ion which is displaced to an m/z 115 due to the extra methyl group in this fragment.

First we tested the sensitivity and specificity of an electrospray LC–MS method (Fig. 3(a)). After solid phase extraction of spiked plasma samples we observed significant interferences from the biological matrix that prevented the use of the LC–MS method without further purification steps. Thus, we decided to apply a LC–MS/MS method which made it possible to achieve a higher specificity of analysis, as illustrated in Fig. 3(b), where specific responses are shown at the expected retention times. This method was used to establish

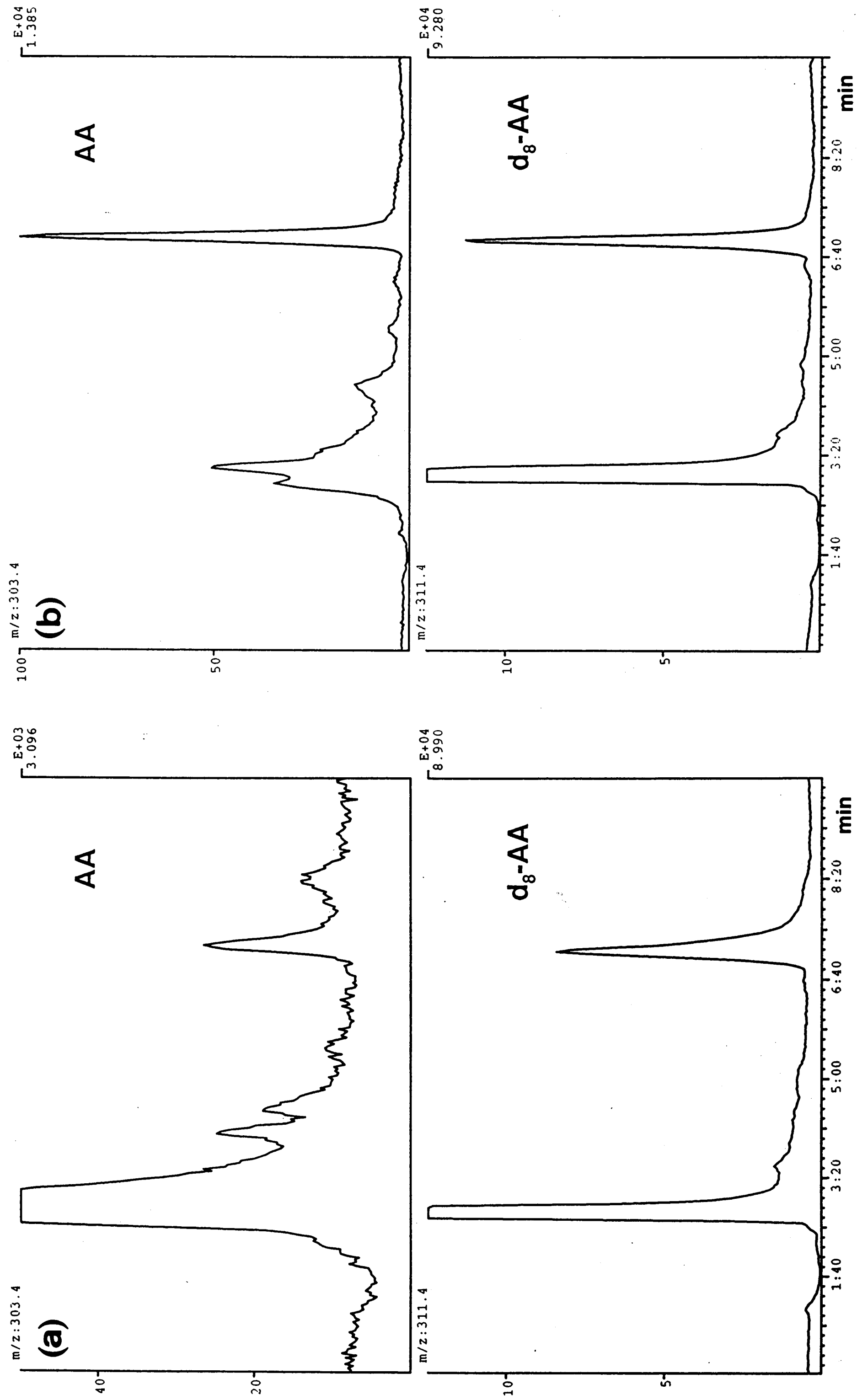

Fig. 1. HPLC–ESI–MS ion chromatograms of AA and the internal standard d_8-AA extracted from cell supernatants: (a) spiked with 1 pmol AA, (b) from cells stimulated with Ca^{2+} ionophore.

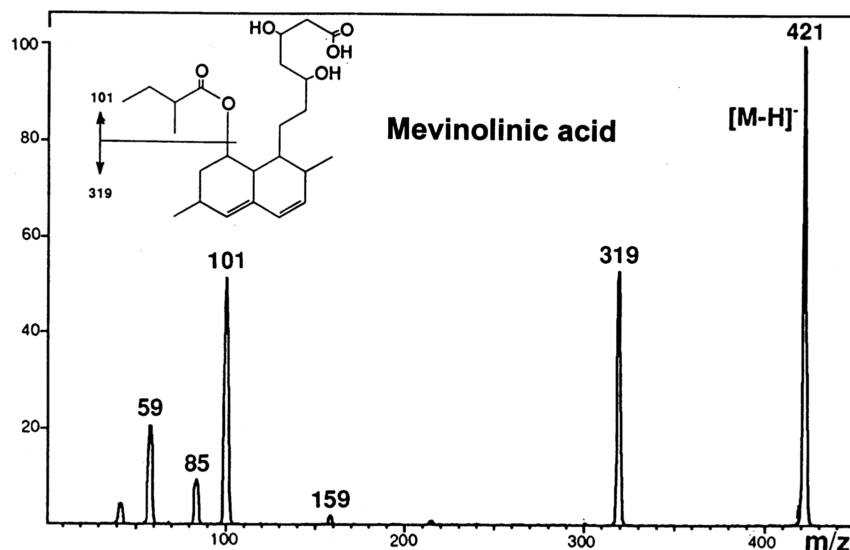

Fig. 2. Negative ion MS/MS spectrum of methylmevinolinic acid (MVA). The parent ion corresponds to the deprotonated molecule at *m/z* 421.

calibration curves, interassay and intraassay variation and stability studies. Calibration curves obtained in three different days showed to be statistically equal. The response was linear within the working range (0.2–10 ng ml^{-1}). The average line obtained was $y = 0.2764x - 0.0069$ (Standard deviations for the slope and intersect were 0.004 and 0.015 respectively). The detection limit was set at 0.05 ng ml^{-1} plasma.

This method has been applied in our laboratory for the routine automated quantitation of MVA in human plasma during pharmacokinetical studies [14]. The use of tandem MS affords the sensitivity and selectivity needed for the analysis. Also, tandem MS allows the quantitation after minor sample preparation providing the speed needed for this kind of analysis.

3.3. Endothelins

Endothelin analogs $Ala^{3,11}$-endothelin-1 ($Ala^{3,11}$-ET1), vasoactive intestinal contractor (VIC), sarafotoxin-S6c, big-endothelin 1 (rat) and big endothelin (porcine) were tested as internal standards. Highest reproducibility of measurements was found for $Ala^{3,11}$-ET1 and VIC. We used $Ala^{3,11}$-ET1 as internal standard for recovery determination and also for internal compensation for variations in recovery in real samples. We used VIC as internal standard for compensation of ESI-MS instabilities in measurements of real samples and of standard solutions.

The solid phase extraction procedure was optimized by addition of $Ala^{3,11}$-ET1 to HUVEC culture medium and monitoring $Ala^{3,11}$-ET1 recovery under different conditions. Recovery was found highest using 0.1% TFA in water for washing and acetonitrile/H_2O/TFA (60:40:0.1) for elution. Although HPLC fractionation prior to HPLC–ESI–MS reduced recovery by 15–20% (absolute), it was found indispensable in real HUVEC samples to avoid column overload and to reduce interferences in HPLC–ESI/MS experiments.

To establish a quantitative method with high sensitivity and high specificity for the analysis of endothelins from HUVEC we compared an electrospray LC–MS and an electrospray LC–MS/MS method. In the LC–MS method selected ions of the $[M+2H]^{2+}$ and $[M+3H]^{3+}$ species of ET1, ET2, ET3, $Ala^{3,11}$-ET1 and VIC were monitored simultaneously (Fig. 4). In the LC–MS/MS method dominant daughter ions that derived from collision-induced dissociation of the $[M+3H]^{3+}$

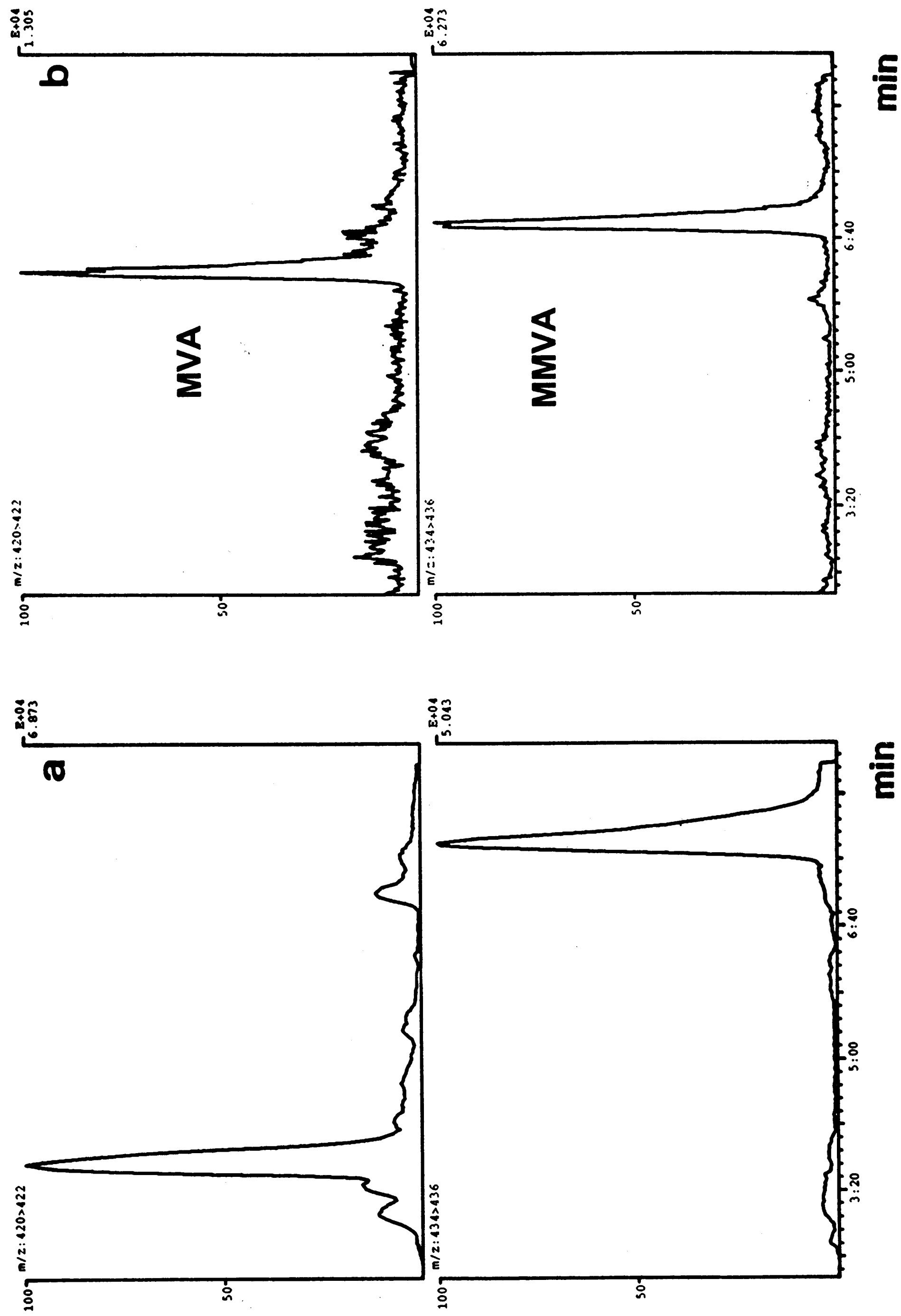

Fig. 3. Sensitivity and specificity of an electrospray (a) LC–MS and (b) LC–MS/MS method for MVA analysis. Ion chromatograms for MVA (upper traces, m/z 421) and the internal standard MMVA (lower traces, m/z 435).

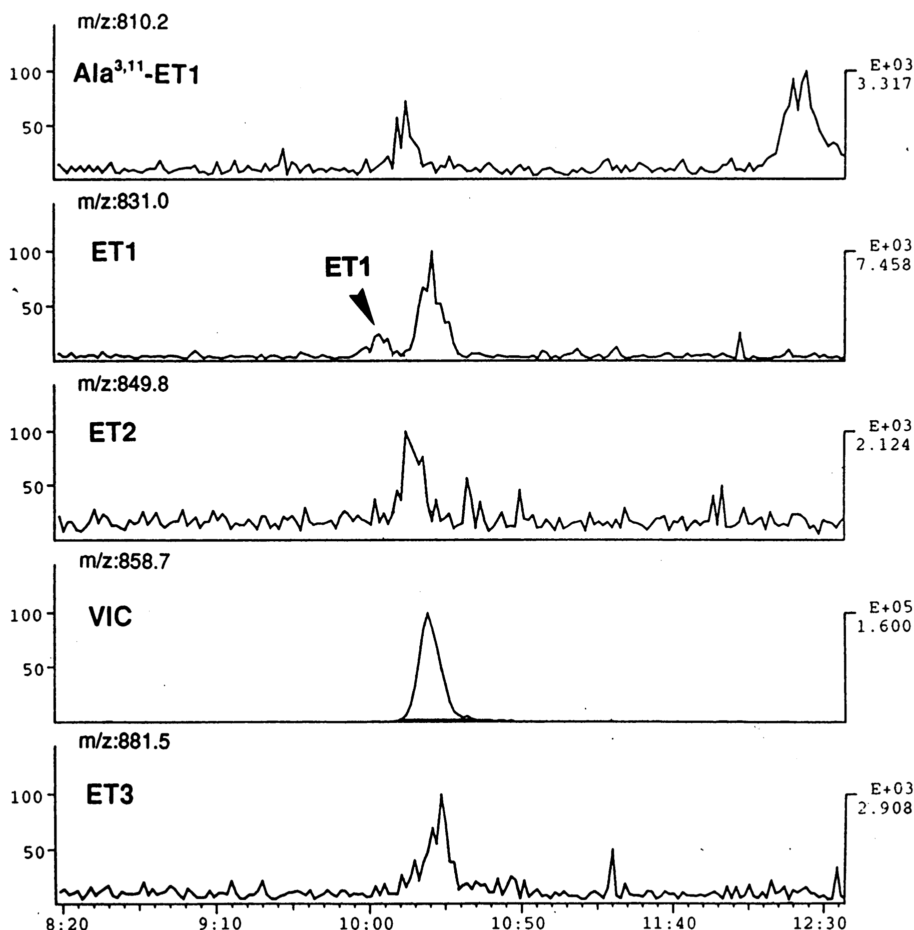

Fig. 4. HPLC–ESI–MS ion chromatograms of 50 fmol ET standards with internal standard VIC (10 pmol).

parent ions were monitored. Concentration–response curves obtained by LC–MS and LC–MS/MS showed a limit of detection for LC–MS at 10 fmol, whereas the LC–MS/MS method yielded only 100–200 fmol. Although the specificity of the LC–MS/MS method was higher, due to the need for high sensitivity we used in subsequent experiments the more sensitive LC–MS method.

Calibration curves for ET standards by LC–ESI–MS were obtained from solutions of ET1, ET2, ET3 and $Ala^{3,11}$-ET1 at various concentration in H_2O/MeOH 1:1 containing 1% AcOH. VIC was added as internal standard. The calibration curves reveal linearity in the range from 50 fmol up to 25 pmol. Accuracy was found to be higher for calibration curves obtained for the $[M+3H]^{3+}$ ion species, which were used for quantitation. Before extraction of HUVEC extracts $Ala^{3,11}$-ET1 was added as internal standard for determination of recovery. Prior to LC–ESI–MS analysis VIC was added as reference standard. For each sample recovery was calculated by comparing the $Ala^{3,11}$-ET1/VIC ratio to the calibration curve. Quantitation was done using the obtained recovery factor and comparing the ET/VIC peak area ratio to the calibration curve. Nevertheless, by monitoring both ion species simultaneously an increase in specificity was accomplished in real samples, which allowed an unambiguous identification of endothelins even in more complex chromatograms.

Quantitation of ETs was carried out by direct analysis of HUVEC extracts, as described above, and also by standard addition methods (Fig. 5). The comparison of amounts of basal values of

ET1 ml^{-1} HUVEC culture medium determined by using the calibration curves (1.78 ± 0.26 pmol ml^{-1}) and by standard addition (1.69 ± 0.22 pmol ml^{-1}) shows good agreement and indicates that the developed method enables quantitation of ET1 by a standard calibration curve. The use of two internal standards allows to omit time-consuming sample preparation steps.

Our failure to observe ions from ET2 and ET3 is in agreement with a recent report by Ashby et al. which used LC–ESI–MS as a method for the identification of ET isoforms in HUVEC cell culture medium [15]. After sample clean-up by passing off the supernatant over a home made anti-ET-1 affinity column, followed by solid phase extraction of the eluate of the affinity column HPLC–ESI–MS SIM analysis revealed only ions of ET1, but not of ET2 and ET3. This is in agreement with our results and earlier reports that ET2 and ET3 are not present in HUVEC cell culture medium.

4. Conclusions

We have demonstrated the usefulness of electrospray LC–MS and LC–MS/MS as a quantitative method in biomedicine. LC–MS can be directly applied to cases such as the analysis of AA in cell supernatants where the relatively simple sample matrix shows no interferences in the analysis and the main concern are low detection limits. In other cases, when a higher specificity is needed, MS/MS is the method of choice as we have shown for the analysis of MVA in plasma samples. These methods are fast, simple and more sensitive than other reported GC/MS methods.

More complex matrices or when, as in the case of endothelin analysis, the relative levels of the target in the sample are very low, require however further off-line sample treatment. For endothelin analysis we have developed a method employing solid-phase extraction, subsequent HPLC fractionation followed by LC–ESI. Relative to our earlier report [16] the method now has been substantially improved by the use of two internal standards, the lowering of the TFA concentration in HPLC–ESI/MS and the simultaneous monitoring of $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions.

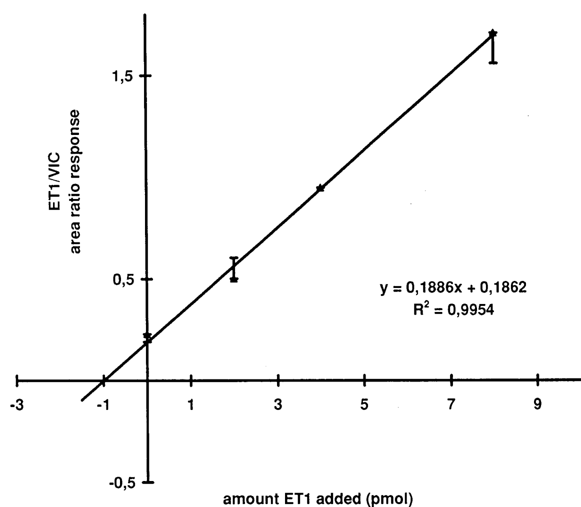

Fig. 5. Standard addition curve of ET1 in HUVEC extracts ($n = 3$).

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References

[1] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64.

[2] K. Metzger, P.A. Rehberger, G. Erben, W.D. Lehmann, Anal. Chem. 67 (1995) 4178–4183.

[3] M.R. Koupai-Abyazani, N. Yu, B. Esaw, B. Laviolette, J. Anal. Toxicol. 19 (1995) 182–186.

[4] E. Gelpí, J. Chromatography A 703 (1995) 59–80.
[5] J. Crowther, V. Adusumalli, T. Mukherjee, K. Jordan, P. Abuaf, N. Corkum, G. Goldstein, J. Tolan Anal. Chem. 66 (1994) 2356–2361.
[6] R.J. White, T.D. Oglesby, P.A. Taylor, J. Florence, J. Wilhelm, in: Proceedings of the 43rd ASMS Conference on Mass Spectrometry and Allied Topics, Atlanta, GA, May 21–26, p. 276.
[7] C. Marquez, P.C. Smith, R.T. Streeper, S.T. Weintraub, in: Proceedings of the 43rd ASMS Conference on Mass Spectrometry and Allied Topics, Atlanta, GA, May 21–26, p. 556.
[8] B.A. Wolf, S.M. Pasquale, J. Turk, Biochemistry 30 (1991) 6372–6379.
[9] S. Masumura, H. Furui, M. Hashimoto, Y. Watanabe, Biochim Biophys. Acta 1125 (1992) 292–296.
[10] M.J. Morris, Biol. Mass Spectrom. 22 (1993) 1–8.
[11] T. Takano, Biomed. Envirom. Mass Spectrom. 19 (1990) 577–581.
[12] C. Dumousseaux, S. Muramatsu, W. Takasaki, H. Takahagi, J. Pharmac. Sci. 83 (1994) 1630–1636.
[13] G.M. Rubanyi, M.A. Polokoff, Pharm. Rev. 46 (1994) 325–415.
[14] R.E. Calaf, M. Carrascal, E. Gelpí, J. Abián, Rapid Comm. Mass Spectrom. 11 (1997) 75–80.
[15] M.J. Ashby, C. Plumpton, P. Teale, R.E. Kuc, E. Houghton, A.P. Davenport, J Cardiovasc. Pharmacol 26 (1995) S247–249.
[16] K. Schneider, D. Canosa, J. Abian, E. Gelpí, 3rd International Symposium on Applied Mass Spectrometry in the Health Sciences, Barcelona, July 9–13, 1995.