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Bioanalysis of erythromycin 2'-ethylsuccinate in plasma using phase-system switching continuous-flow fast atom bombardment liquid chromatography–mass spectrometry

P. S. KOKKONEN

TNO CIVO-Institutes, P.O. Box 360, 3700 AJ Zeist (The Netherlands) and Centre for Bio-Pharmaceutical Sciences, Leiden University, P.O. Box 9502, 2300 RA Leiden (The Netherlands)

W. M. A. NIESSEN* and U. R. TJADEN

Centre for Bio-Pharmaceutical Sciences, Leiden University, P.O. Box 9502, 2300 RA Leiden (The Netherlands)

and

J. VAN DER GREEF

TNO CIVO-Institutes, P.O. Box 360, 3700 AJ Zeist (The Netherlands) and Centre for Bio-Pharmaceutical Sciences, Leiden University, P.O. Box 9502, 2300 RA Leiden (The Netherlands)

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ABSTRACT

A specific method for the determination of erythromycin 2'-ethylsuccinate (EM-ES) in plasma is described. The method involves a liquid–liquid extraction procedure followed by the analysis of extracts using phase-system switching (PSS) continuous-flow fast atom bombardment (CF-FAB) liquid chromatography–mass spectrometry (LC–MS). In PSS EM-ES is enriched after analytical separation on a short trapping column, from which it is desorbed to the LC–MS interface. In this way, favourable mobile phases can be used for the LC separation and for the MS detection. Using the PSS approach a flow-rate reduction from 1.0 ml/min in the LC system to 15 μl/min going into the mass spectrometer was achieved without splitting. The determination limit for EM-ES was 0.1 μg/ml.

INTRODUCTION

Erythromycin (EM) is a macrolidic antibiotic that has been used for the treatment of various infectious diseases since 1952. EM can be administered as acid-resistant esters (*e.g.*, ethylsuccinate, acistrate), which are partly hydrolysed to EM in plasma and body fluids. Because these esters are antibacterially inactive, it is important that an analytical method is capable of separating the intact pro-drug and its hydrolysed active product, the EM base.

EM and/or its esters have been determined in biological samples using high-performance liquid chromatographic (HPLC) separation and UV absorbance [1],

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fluorimetric [2] or electrochemical detection [3–6]. Improved specificity for the determination of erythromycin 2′-ethylsuccinate (EM-ES) and EM in plasma has been achieved using fast atom bombardment mass spectrometry (FAB MS), based on simultaneous monitoring of the characteristic protonated molecules of the analytes [7].

EM-ES has also been determined in plasma using continuous-flow FAB (CF-FAB) LC–MS [8]. CF-FAB LC–MS is a powerful technique for the on-line separation and detection of polar and thermally labile compounds [8–11]. However, the main disadvantage of the technique is that the maximum allowable flow-rate is *ca.* 15 μl/min [12], leading to the necessity to split the eluent after conventional LC columns (3–4.6 mm I.D.) [11]. As the mass spectrometer is a mass flow-sensitive detector, the concentration detection limits are consequently increased by a factor of the reciprocal splitting ratio. For the determination of EM-ES in plasma using CF-FAB LC–MS a 1:160 splitter was applied [8].

The phase-system switching (PSS) approach has been designed to solve mobile phase [13-17] and flow-rate incompatibilities in LC–MS [11,15,17–19]. PSS has been applied for flow-rate reduction in CF-FAB from 1 ml/min in the HPLC system to 15 μl/min going into the mass spectrometer without a splitter using EM-ES as a model compound [19]. In PSS the compound of interest, in this instance EM-ES, is enriched after the analytical separation on a short trapping column, from which it is subsequently desorbed with a solvent composition and flow-rate that are favourable for the LC–MS interface.

The applicability of the PSS CF-FAB LC–MS method for quantitative bioanalysis was studied by determining EM-ES in plasma.

EXPERIMENTAL

PSS equipment

The PSS system, a schematic diagram of which is given in Fig. 1, consisted of an HPLC unit and a trapping and desorption (TD) unit, and was connected to a Finnigan MAT (Bremen, Germany) CF-FAB probe by means of a 1.2 m × 75 μm I.D. fused-silica capillary (SGE, Melbourne, Australia).

The HPLC unit consisted of a Model 302 pump (Gilson, Middleton, WI, U.S.A.) (pump 1), a Gilson Model 802C manometric module, a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 injector (20 μl), a ChromSep reversed-phase C_8 column (indicated as AC) (100 mm × 3 mm I.D., packed with 10-μm material) (Chrompack, Middelburg, The Netherlands), an LKB Model 2151 UV detector operated at 225 nm and a Kipp & Zonen (Delft, The Netherlands) Model BD8 single-channel flat-bed pen recorder.

The TD unit consisted of a Gilson Model 302 pump (pump 2), a Brownlee Labs. MicroGradient system (ABI Analytical, Kratos Division, Ramsey, NJ, U.S.A.) (pump 3), two Rheodyne Model 7010 manual switching valves (V1 and V2), a stainless-steel union-T (TEE.0.20″, Upchurch Scientific, Oak Harbor, WA,

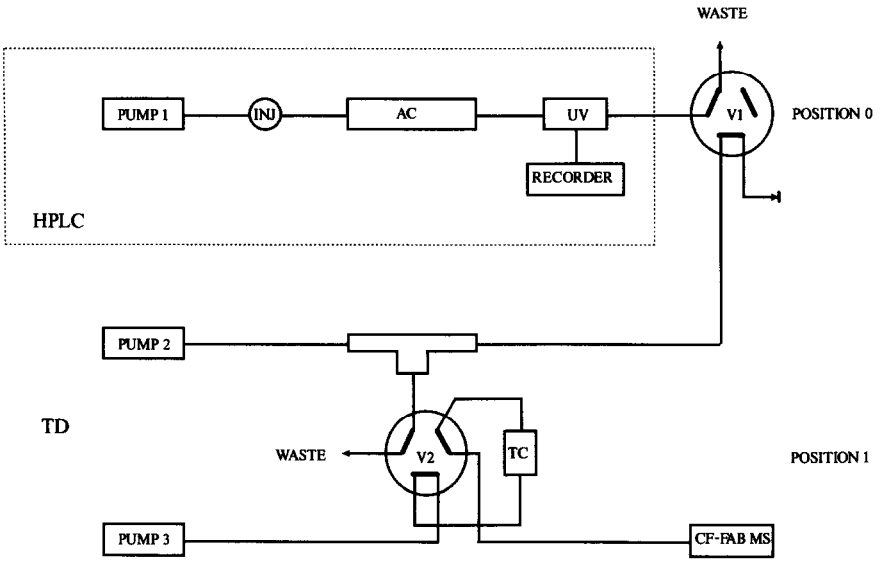

Fig. 1. Schematic diagram of the PSS system containing an HPLC unit and a trapping and desorption unit (TD). The diagram shows the procedure for the desorption of the target compound from the trapping column (TC). AC = analytical column.

U.S.A.) and a trapping column (indicated as TC). The trapping column consisted of a preconcentration column holder (Chrompack) and a 5 mm × 1.0 mm I.D. laboratory-made metal cartridge. The connnection between the union-T and valve 2 was made with a 1 m × 220 μm I.D. fused-silica capillary.

PSS procedure

EM-ES and erythromycin [$^2H_5$]-2′-ethylsuccinate (EM-[$^2H_5$]-ES, the internal standard) were separated from EM and the biological matrix by means of a mobile phase (pump 1) consisting of methanol–0.05 *M* sodium phosphate buffer (pH 7.1) (7:3, v/v) at a flow-rate of 1.0 ml/min.

The enrichment of EM-ES and the internal standard on the trapping column was realized by the post-column addition of water (pump 2) at a flow-rate of 2.0 ml/min. The analytes were desorbed from the trapping column with a mobile phase (pump 3) consisting of methanol–glycerol (92:8, w/w) at a flow-rate of 15 μl/min.

The TC column was packed under reduced pressure with Amberlite XAD-2 resin (30–40 μm) (Rohm & Haas, Philadelphia, PA, U.S.A.).

Mobile phases were filtered over a nylon filter (0.45 μm) (Millipore, Bedford, MA, U.S.A.) and degassed ultrasonically before use. The mobile phase containing the phosphate buffer was flushed with helium during the experiments. The mobile phases were stored in a refrigerator at 8°C to avoid bacterial growth and mould formation.

Mass spectrometry

The CF-FAB probe was fitted on to a Finnigan MAT 90 double-focusing mass spectrometer, operating at 5 kV. The Finnigan MAT ICIS data system was used. The instrument was equipped with a FAB gun (Ion Tech, Teddington, U.K.) using xenon and producing a beam of neutral atoms with 7 kV energy. The FAB spectra were recorded in the positive-ion mode by scanning from m/z 50 to 1100 with a scan speed of 0.7 s per decade. The protonated molecules of EM-ES at m/z 862 and EM-[$^2H_5$]-ES at m/z 867 were monitored in the selected-ion monitoring mode (SIM). The resolution was 1000 (10% valley definition) during the measurements.

A stainless-steel target with a gold-plated drain channel was used [12]. An exchangeable ion volume with a wick was applied to reduce contamination problems. The wick, made from compressed paper, was positioned at the bottom of the ion volume. Additional vacuum pumping at the ion-source housing was obtained by a liquid nitrogen trap. The temperature of the ion source was kept at 75°C to improve the evaporation of the mobile phase [8].

Under these conditions it was possible to work continuously for 2 h, after which the wicks were replaced and the target was quickly flushed with methanol, chloroform and acetone. After a 5-min stabilization time in the MS vacuum, the system was ready for use.

Reagents

EM-ES, EM and EM-[$^2H_5$]-ES were kindly supplied by the Research Center of Orion Pharmaceutica (Espoo, Finland). Sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dihydrate, methanol and diethyl ether were obtained from Merck (Darmstadt, Germany). Glycerol (98% chemically pure) was purchased from Lamers & Pleuger ('s-Hertogenbosch, The Netherlands), sodium carbonate (AnalaR) from BDH (Poole, U.K.), and acetonitrile (ChromAR) from Promochem (Wesel, Germany). The pH of the sodium phosphate buffer was adjusted to 7.1 by mixing 0.05 mol/l aqueous solutions of sodium dihydrogenphosphate and disodium hydrogenphosphate. Water was distilled before use.

The isotopic purity of labelled internal standard was determined from the peak intensities in the mass spectrum of the standard solution of EM-[$^2H_5$]-ES. The contribution of EM-[$^2H_5$]-ES to the m/z 862 peak was 1.1%.

Sample preparation

Stock solutions of EM-ES (1.05 mg/ml) and EM-[$^2H_5$]-ES (0.75 mg/ml) were prepared in acetonitrile. Plasma samples containing 0.105, 0.210, 0.525, 1.05, 2.10, 5.25 and 10.5 μg/ml EM-ES were prepared by adding the appropriate amount of EM-ES in 50 μl of acetonitrile to 1.0 ml of blank human plasma.

A liquid–liquid extraction was performed as sample pretreatment. A solution of 3.75 μg of EM-[$^2H_5$]-ES in 50 μl of acetonitrile was added to 1.0 ml of sample

as an internal standard. Saturated sodium carbonate in water (100 μl) and diethyl ether (4 ml) were added. After rapid mixing, the tubes were gently shaken on a horizontal shaker for 10 min. After centrifugation for 5 min at 1000 *g*, the organic layer was separated and evaporated to dryness in a gentle stream of helium at 40°C. The residue was dissolved in 50 μl of acetonitrile. The extracts were stored in a refrigerator at 4°C until analysed. After melting, an aliquot of 20 μl was injected onto the HPLC column. This procedure is a modification of the method described by Ottoila and Taskinen [7].

RESULTS AND DISCUSSION

Analysis of plasma samples

The PSS CF-FAB LC–MS method was developed for the determination of EM-ES in plasma. The PSS system is based on the valve-switching technique and is described in detail elsewhere [19]. A schematic diagram of the PSS system is shown in Fig. 1. After extraction, EM-ES is separated from EM and the complex plasma matrix in the analytical column and monitored on-line using the UV detector in the HPLC unit. A buffer in the HPLC eluent is needed to separate EM from its esters [1–6,8]. The retention times of EM and EM-ES are 2.2 and 3.5 min, respectively. The retention times of EM and EM-ES were checked daily to secure the correct timing of sequencing.

After the separation, the EM-ES peak is isolated by heart cutting and enriched on the trapping column in the TD unit. The resolution for the separation of EM and EM-ES is 1.0, which is sufficient for heart cutting. The enrichment procedure is started *ca.* 50 s before and is finished 90 s after the centroid of the peak of EM-ES is monitored on the UV detector. Addition of water to the HPLC eluent is used to increase the retention of EM-ES and the internal standard on the trapping column [19]. After enrichment, the trapping column is washed for 1 min to remove the phosphate buffer and other water-soluble interferences. In this work, EM-ES was heart cut and EM and the disturbing background were directed to waste. However, it is also possible to heart cut EM in addition to its ester using enrichment on the same or different trapping columns [16,17].

EM-ES and the internal standard are desorbed from the trapping column in the back-flush mode with a flow-rate of 15 μl/min and a mobile phase optimized for CF-FAB MS containing methanol and glycerol [8,12,19]. Using XAD as packing material the best desorption properties and the most intense and narrow peak for EM-ES are achieved [19]. It takes *ca.* 5.5 min before the analyte and the internal standard are desorbed from the trapping column and eluted to the CF-FAB target. This time delay is due to the low flow-rate used, the desorption characteristics of EM-ES and the residence time in the system [19].

Total ion current chromatograms of a blank plasma and a spiked plasma sample are shown in Fig. 2. No interfering background peaks are observed in the chromatogram of the blank plasma sample. Only a slight increase in the chemical

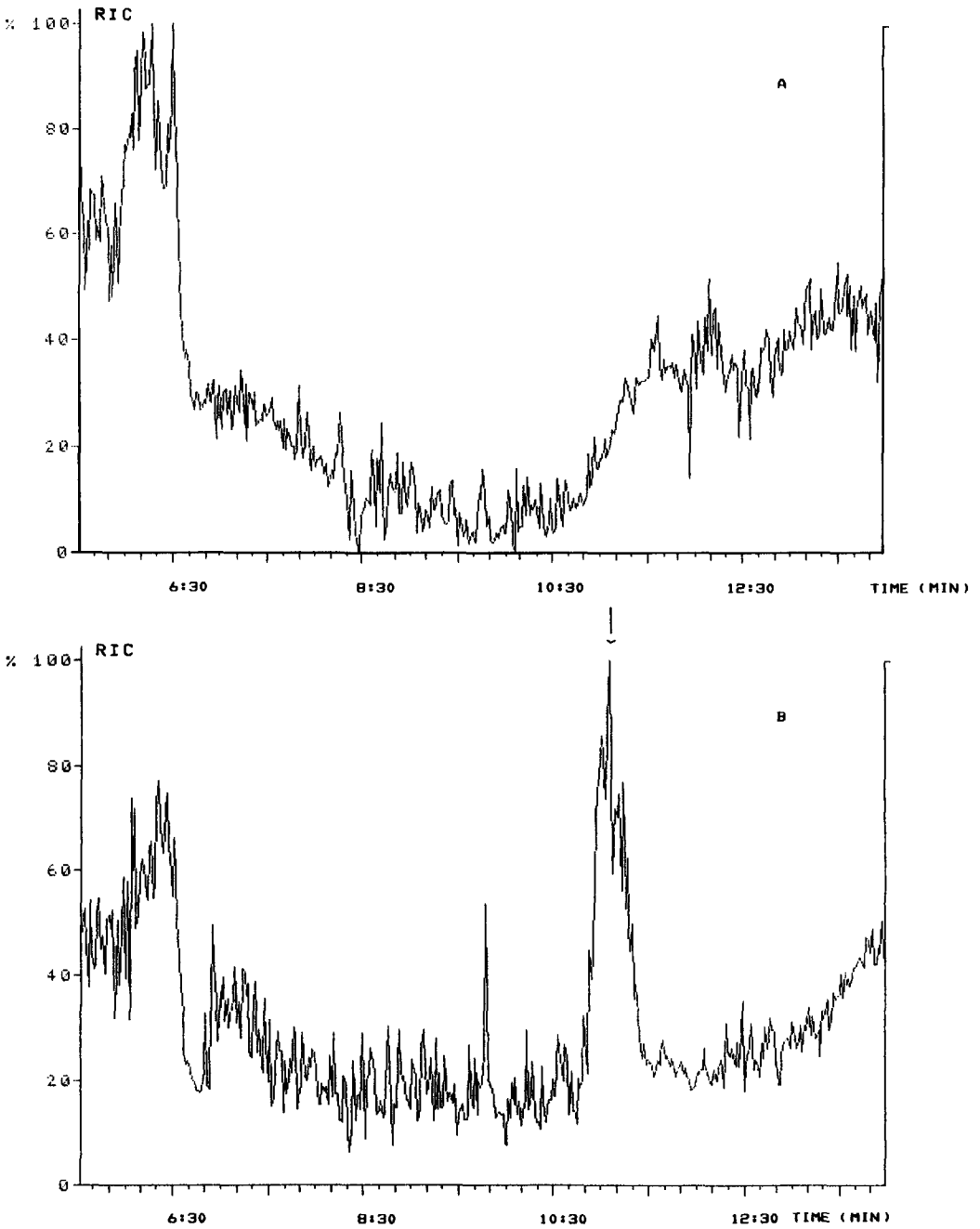

Fig. 2. Total ion current chromatograms of human plasma extracts. (A) Blank plasma sample; (B) spiked plasma sample containing 52.5 μg/ml EM-ES and 37.5 μg/ml EM-[$^2H_5$]-ES (apparent retention time 11 min).

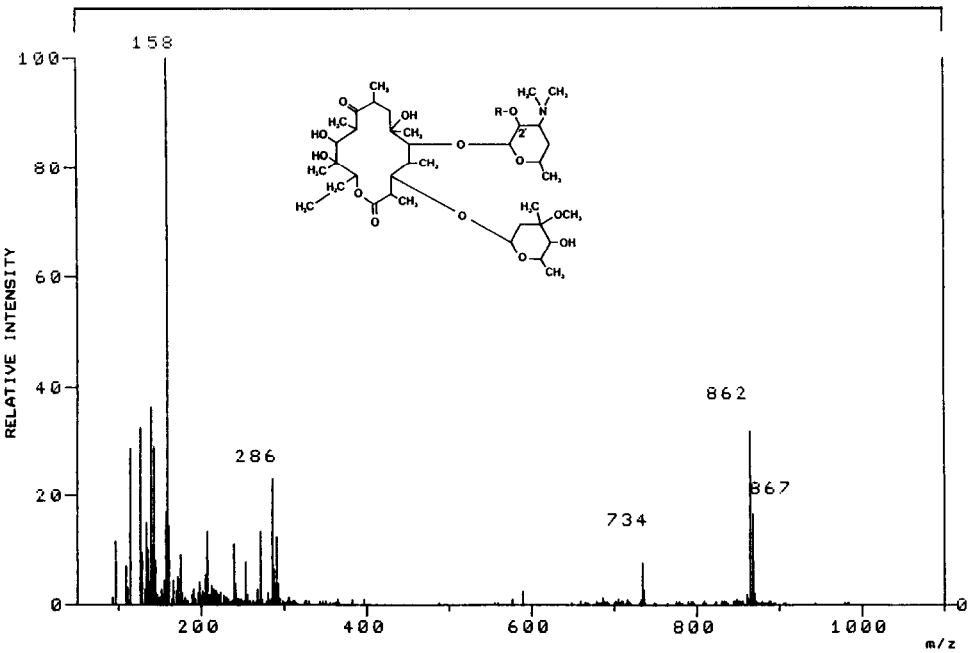

Fig. 3. Background-subtracted PSS CF-FAB mass spectrum of EM-ES [R = $CO(CH_2)_2COOCH_2CH_3$] (MW 861) and EM-[$^2H_5$]-ES [R = $CO(CH_2)_2COOC^2H_2C^2H_3$] (MW 866) taken from the analysis shown in Fig. 2B.

background noise is observed, due to the change in solvent composition after valve switching. The PSS CF-FAB mass spectrum of EM-ES ($[M + H]^+$, at *m/z* 862) and EM-[$^2H_5$]-ES ($[M + H]^+$, at *m/z* 867) taken from the chromatogram in Fig. 2B is given in Fig. 3. The peaks at *m/z* 158, 286 and 291 are due to the D-desosamine group (the N-containing sugar ring) [20], the D-desosamine group with the ethylsuccinyl and the [$^2H_5$]ethylsuccinyl side-chain, respectively. The peak at *m/z* 734 can be explained as a fragment peak, formed after the loss of the ethylsuccinyl or [$^2H_5$]ethylsuccinyl side-chain from the parent compound or the internal standard, although it may also result from hydrolysis of the EM-ES during the storage, pretreatment and/or analysis of the plasma samples [7]. As the protonated molecules of EM and EM-ES are separated in MS detection, the determination of EM-ES is not affected.

The injection of a new sample can be performed every 11 min. The total run time for one analysis is 13 min, including the separation on the analytical column, heart cutting, enrichment on the trapping column, desorption from the trapping column and transfer to the CF-FAB target. In Fig. 4 the multiple ion monitoring chromatograms of protonated EM-ES (at *m/z* 862) and the protonated internal standard are shown. In Fig. 4 the timing of switching of valves 1 and 2 (V1 and V2) and injections (inj) of the samples onto the HPLC column are also shown.

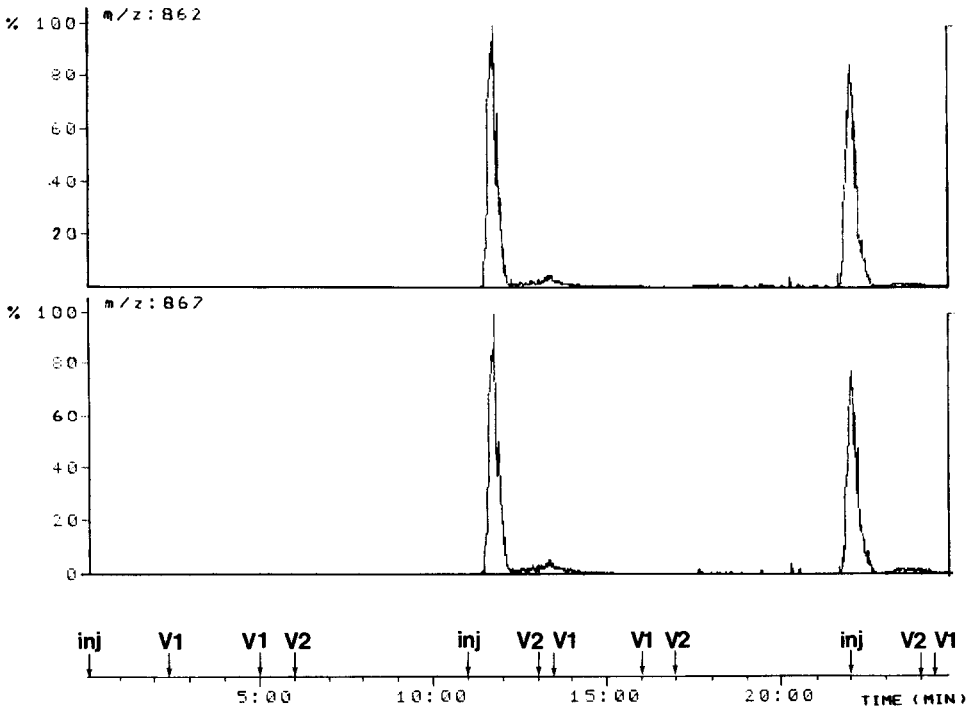

Fig. 4. Multiple ion monitoring chromatograms of protonated EM-ES (upper trace, *m/z* 862) and protonated EM-[$^2H_5$]-ES (lower trace, *m/z* 867). Plasma spiked with 5.25 μg/ml EM-ES and 3.75 μg/ml EM-[$^2H_5$]-ES.

Using a desorption flow-rate of 15 μl/min, twelve samples can be analysed in 2 h, after which the wick has to be replaced. Preliminary experiments using the PSS CF-FAB LC–MS system together with a higher desorption flow-rate and a splitter indicated that an increased sensitivity of the method and an increased sample throughput can be achieved [19]. When a desorption flow-rate of 230 μl/min is applied, the residence time of EM-ES is reduced from 5.5 min to 50 s. Therefore, the total analysis time is reduced from 13 min to *ca.* 7 min, making the method more attractive for routine analysis.

Analytical data

The samples used for recovery experiments were prepared by adding the analyte before extraction and the internal standard in acetonitrile at the end of evaporation. The reference sample was prepared by adding both the analyte and the internal standard at the end of evaporation. The recovery was calculated by comparing the peak-area ratios of the analyte and the internal standard. The recovery of the extraction method was found to be 77 ± 4% [recovery ± standard deviation (S.D.), $n = 4$] at the 5.25 μg/ml level.

TABLE I

ACCURACY AND WITHIN-RUN PRECISION FOR THE DETERMINATION OF EM-ES IN HUMAN PLASMA BY MEANS OF PSS CF-FAB LC–MS (n = 5)

Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	R.S.D. (%)
0.105	0.146 ± 0.005	6.4
0.525	0.52 ± 0.03	6.1
5.25	4.8 ± 0.3	5.8

The within-run precision and the accuracy of the method were studied by analysing spiked plasma samples containing 0.105, 0.525 and 5.25 μg/ml of EM-ES. Table I summarizes the relative standard deviations (R.S.D.s) of the ratio of the peak areas of the analyte and the internal standard. The accuracy of the method was determined by calculating the concentration found for the ratio of the peak areas of the analyte and the internal standard from the corresponding calibration graph constructed on the same day. The day-to-day precision was determined by analysing a spiked plasma sample containing 1.05 μg/ml EM-ES. The concentration found was 0.93 ± 0.11 μg/ml (mean ± S.D.). The R.S.D. of the concentrations found in spiked plasma samples was 12% (n = 6) for determinations on different days. The day-to-day reproducibility and accuracy for the calibration samples for the determination of EM-ES in human plasma by means of PSS CF-FAB LC–MS are given in Table II.

The linearity (ratio of peak area of analyte to internal standard *versus* concentration) of the method was checked in the range 0.105–10.5 μg/ml EM-ES in plasma, which corresponds to typical concentration levels found in plasma in

TABLE II

DAY-TO-DAY REPRODUCIBILITY AND ACCURACY FOR CALIBRATION SAMPLES FOR THE DETERMINATION OF EM-ES IN HUMAN PLASMA BY MEANS OF PSS CF-FAB LC–MS

Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	n	R.S.D. (%)	Absolute error in the concentration found (%)
0.105	0.21 ± 0.08	6	36	+100
0.210	0.24 ± 0 04	5	16	+ 14
0.525	0.53 ± 0.12	6	22	+ 10
1.05	0.93 ± 0.08	6	8.1	− 11
2.10	1.82 ± 0.15	5	8.2	− 13
5.25	5.7 ± 0.4	6	7.3	+ 8
10.5	10.5 ± 0.12	6	1.1	0

pharmacokinetic studies [7]. A calibration sample without EM-ES and containing only the internal standard was included in the calibration each day. The calibration graphs are linear (mean correlation coefficient $r = 0.999$) over the range 0.105–10.5 μg/ml. The mean calibration graph can be described by the equation $y = -0.050 + 0.468x$, where y = peak-area ratio and x = concentration.

The determination limit is 0.1 μg/ml for EM-ES, which corresponds to *ca.* 40 ng introduced into the mass spectrometer. At this concentration level the method is still reasonably precise and accurate (see Tables I and II). The sensitivity of the method can be further improved by increasing the injection volume.

In comparison with previously applied HPLC methods [1–6], the specificity of the method for the determination of EM-MS is considerably improved. On-line analysis and automation possibilities are the main advantages over the conventional FAB MS method [7]. The PSS method for the determination of EM-ES in plasma is more reliable and reproducible and consumes less sample than the previous CF-FAB LC-MS method using a conventional HPLC column and a splitter [8].

The PSS CF-FAB LC – MS method has proved to be applicable to the determination of EM-ES in plasma. However, our main intention was to demonstrate the more general applicability of the PSS CF-FAB LC–MS method for the quantitative target compound analysis.

CONCLUSIONS

The PSS CF-FAB LC–MS method is precise and accurate, which is essential in quantitative bioanalysis. The method described is sufficiently sensitive for the determination of EM-ES in plasma. The method can be easily applied to the determination of EM-ES also in other biological samples, *e.g.*, tissues, having a complex matrix background. Using the PSS system together with CF-FAB LC–MS, many problems encountered in interfacing LC and MS systems are overcome. A favourable mobile phase can be used for the HPLC separation and for the MS detection. The required flow-rate reduction from 1.0 ml/min in the HPLC system to 15 μl/min going into the mass spectrometer can be achieved without splitting. The post-column addition of water to increase the retention of compounds on the trapping column extends the range of applicability. In addition to quantitative applications, PSS CF-FAB LC–MS can also be used for the identification of trace compounds, *e.g.*, metabolites or synthetic impurities, after enrichment on the trapping column [21].

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