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Determination of the vitamin D analog EB 1089 (seocalcitol) in human and pig serum using liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatographic–tandem mass spectrometric assay in human and pig serum has been developed for quantitative analysis of EB 1089 (seocalcitol). EB 1089 is a novel vitamin D analog under development for the treatment of cancer. The analyte was extracted from serum after protein precipitation using an automated solid-phase extraction procedure involving both a reversed-phase and normal-phase procedure on a single C_{18} cartridge. The analytical chromatography was performed using a Symmetri C_8 50×2.1 mm, 3.5 μm column. The mobile phase was a linear gradient from 75% to 99% methanol with a constant concentration of 2 m*M* ammonium acetate. EB 1089 and the internal standard [d_6]-EB 1089 were detected by using MS–MS. The ion source was operated in the positive electrospray ionisation (ESI) mode. The assay is specific, sensitive, and has a capacity of more than 100 samples per day, with a limit of quantitation of 10 pg ml^{-1} for a 1.0-ml sample aliquot. It is now used for routine analysis in connection with pharmacokinetic studies in humans and toxicokinetic studies in pigs.

Keywords: EB 1089; Seocalcitol; Vitamin D

1. Introduction

EB 1089 [1(*S*),3(*R*)-dihydroxy-20(*R*)-(5′ethyl-5′-hydroxy-hepta-1′(*E*), 3′(*E*)-dien-1′-yl)-9,10-seco-pregna-5(*Z*),7(*E*),10(19)-triene] (seocalcitol) is an analog of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], the physiologically active form of vitamin D_3. 1α,25$(OH)_2D_3$ plays a crucial role in the regulation of calcium homeostasis [1] and in cell growth regulation [2]. The effects of 1α,25$(OH)_2D_3$ are believed to be mediated via a specific intracellular receptor found in many cells and tissues, and are not restricted to those involved in the regulation of calcium metabolism [3]. The clinical usefulness of 1α,25$(OH)_2D_3$ is primarily limited by its effect on the calcium homeostasis, with the risk of inducing hypercalcemia and soft-tissue calcifications. However, a synthetic analog, EB 1089 with strong antiproliferative effects — but with reduced effects on calcium metabolism, has been developed [4–7]. At present, EB 1089 enters clinical phase III for the treatment of various solid tumours.

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EB 1089 is orally administered to humans in doses from 1 to 25 μg per individual [8]. Consequently, a highly sensitive assay in the picogram range is required in order to study pharmacokinetics in humans, but also to investigate toxicokinetics in animals.

A highly sensitive assay for another analog F_6-1α,25$(OH)_2D_3$ in human serum using GC–high-resolution selected ion monitoring (HRMS) has been reported [9]. However, the assay involves a complex purification procedure including solid-phase extraction (SPE) and HPLC before the derivatization and GC–MS, which does not make it attractive for routine analysis. Others have developed a very sensitive radio receptor assay (RRA) for the determination of dihydroxyvitamin D_2 metabolites, calcipotriol and 1α,25$(OH)_2D_3$ in plasma [10,11]. To achieve high specificity, this assay also requires a complex extraction procedure including both SPE and HPLC to separate the analogs from metabolites and from endogenous 1α,25$(OH)_2D_3$ prior to the RRA. The RRA is based on the bovine thymus vitamin D receptor, and therefore, the assay is only useful for analogs with similar affinity for the vitamin D receptor as in the case of 1α,25$(OH)_2D_3$, which was not the case with EB 1089 [5]. Alternatively, similar extraction procedures have been combined with a radioimmunoassay (RIA) [12]. In this RIA assay, the fact that EB 1089 has a high cross-reactivity against the available antibodies being raised against the 1α,25$(OH)_2D_3$ structure is utilized. Unfortunately, the robustness for an EB 1089 RIA assay has not been satisfactory. Liquid chromatography methods with fluorimetric, electrochemical or MS–MS detection for measurement of 1α,25$(OH)_2D_3$ or its analogs in serum or plasma have not demonstrated the required sensitivity [13–20]. However, this report describes a highly sensitive and specific LC–ESI–MS–MS assay using an automated SPE clean-up procedure for determination of the vitamin D analog EB 1089 in serum from pigs and humans with a capacity of running more than 100 samples per day.

2. Experimental

2.1. Materials and chemicals

EB 1089, [26a, 27a-d_6]-EB 1089 (used as internal standard, I.S.), the four diastereoisomeric forms of 26-hydroxy EB 1089, and the two 26a-hydroxy epimers of EB 1089 (the six hydroxy derivatives of EB 1089 are likely metabolites) (Fig. 1) were synthesised at Leo Pharmaceutical Products (Ballerup, Denmark) [4,21]. All other chemicals and reagents were commercially available. All solvents were HPLC grade, ammonium acetate was Analytical Reagent grade and nitrogen gas was 99.999% pure.

2.2. Preparation of standards and QC samples (calibration curves and precision/accuracy)

Stock solutions of 100 μg ml^{-1} of EB 1089 in 2-propanol were prepared. The stock solution was diluted with methanol to a working solution (40 ng ml^{-1}). Similarly, a working solution for [d_6]-EB 1089 (internal standard) (400 ng ml^{-1}) was prepared. The working solutions for EB 1089 and internal standard were diluted with methanol–1 *M* ammonium acetate–water (500:2:500) to a series of calibration standards. To perform a standard curve, 1.00 ml of serum (from a pool from pigs or humans) was spiked with 50 μl of calibration standards. The concentration of EB 1089 in the calibration samples was 0, 10, 20, 40, 60, 80 and 100 pg ml^{-1}, respectively, and the concentration of the internal standard was approximately 1000 pg ml^{-1}. QC samples were prepared correspondingly at 10, 50 and 100 pg ml^{-1}. Both the calibration standard and the QC samples were prepared on the same day as the extraction procedure, except, however, for the stability samples.

2.3. Preparation of blanks

Serum from three male and three female healthy subjects, and from three male and three female minipigs were used for selectivity. The samples were handled as above (Section 2.2) but with and without internal standard.

2.4. Preparation of recovery samples

One ml of blank serum was precipitated with two volumes of acetonitrile. Thereafter, the precipitated samples were handled in the same way as the

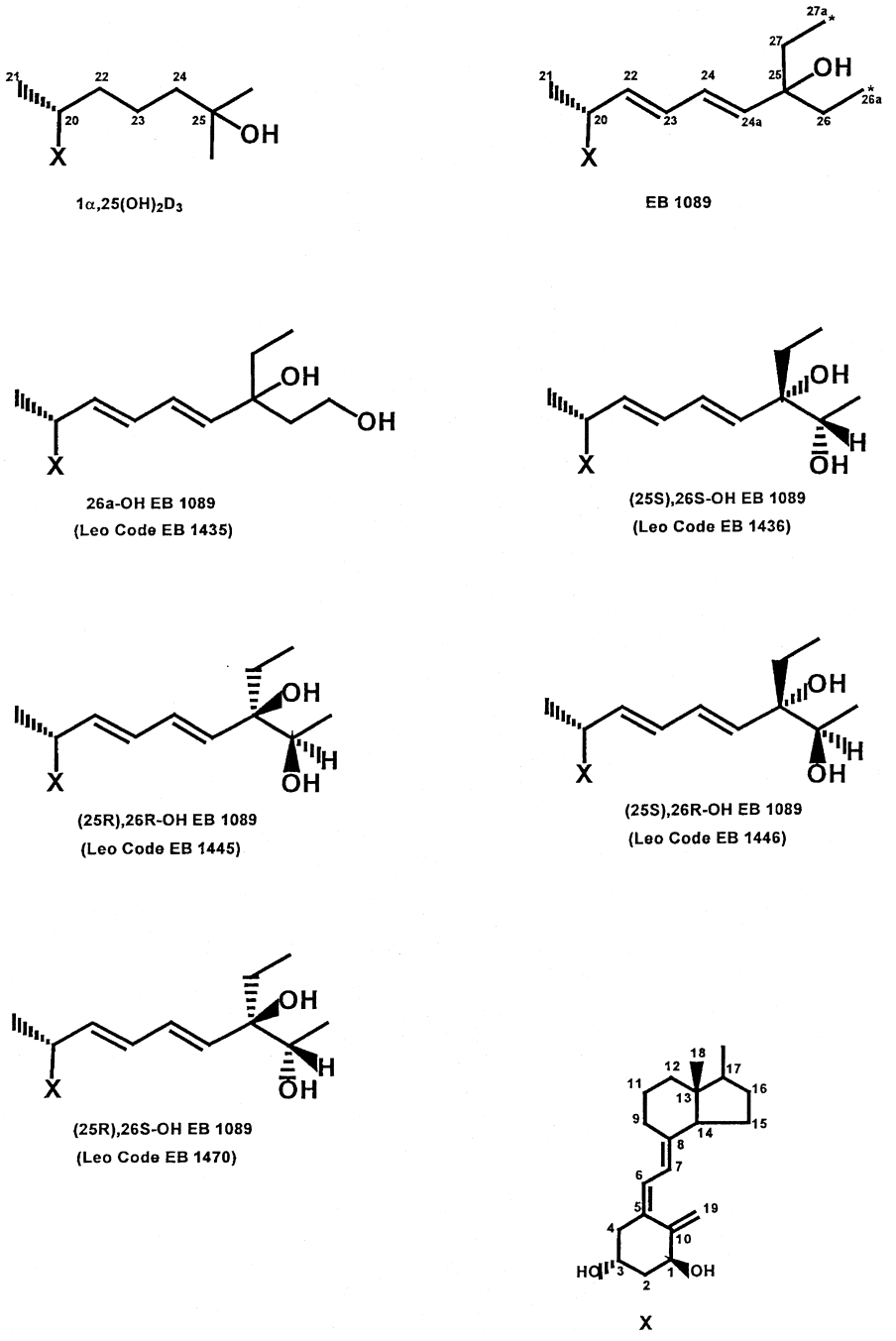

Fig. 1. The chemical structure of $1\alpha,25(OH)_2D_3$, EB 1089 and EB 1089 derivatives. X represents the vitamin D ring system. The asterisks show the deuterium position in the internal standard d_6-EB 1089.

standards until reconstitution. The samples were reconstituted in calibration standards containing 10, 50 or 100 pg ml^{-1} serum of EB 1089 and 1000 pg ml^{-1} serum of internal standard.

2.5. Preparation of stability samples

2.5.1. Freeze–thaw cycle

The stability following three freeze–thaw cycles was determined by using samples identical to the QC samples at three levels. Two samples at each level were analysed immediately. Two samples at each level were frozen at −18°C for 24 h and thawed unassisted at room temperature. When the samples were completely thawed, they were returned to the original freezer and kept frozen for 24 h. The cycle of thawing and freezing was repeated two more times, and the samples were analysed after the third cycle.

2.5.2. Room-temperature stability

The effect after 5 days' storage at room temperature (protected, however, from light) was investigated by using samples identical to the QC samples at three levels. Two samples at each level were analysed immediately. Two samples at each level were stored at room temperature for 5 days and then frozen at −80°C until analysis.

2.5.3. Autosampler stability

The effect of 24 h storage in the autosampler was investigated by using samples identical to the QC samples. The samples at each level were analysed in duplicate at time 0 and time 24 h.

2.6. Extraction procedures

The spiked calibration standards and QC samples were protein precipitated with two volumes of acetonitrile. After centrifugation the supernatant was mixed with one volume water. The mixture was loaded to an Isolute™ MF C_{18} SPE column (100 mg, 1 ml) (IST, Mid-Glamorgan, UK) that had been successively preconditioned with heptane, 2-propanol, methanol and acetonitrile–water (33:67). Following the loading of the sample, the column was successively washed with acetonitrile–water (33:67), methanol–water (70:30) and heptane. Finally, EB 1089 and [d_6]-EB 1089 were eluted with heptane–2-propanol (93:7). The SPE procedure was performed using an ASPEC™ XL4 sample processor with a 404 Syringe Pump and 724 Software package (Gilson, Middleton, WI, USA). The organic solvent was evaporated under a stream of N_2 at 40°C using a TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA). The residues were reconstituted in 200 μl of methanol–1 *M* ammonium acetate–water (500:2:500).

2.7. Liquid chromatography–tandem mass spectrometry

The reversed-phase HPLC system consisted of a Waters Alliance 2690 Separation Module with cooling on the autosampler, online vacuum degassing and a column oven (Waters, Milford, MA, USA). The analytical column was a Symmetri C_8 50×2.1 mm (I.D.) (3.5-μm particle size) (Waters) and kept at 30°C. The mobile phase was a linear gradient from 75% to 99% methanol with a constant concentration of 2 m*M* ammonium acetate with a flow-rate of 0.3 ml min^{-1}. The injection volume was 150 μl.

The mass detector was a PE/Sciex API 3000 Mass Spectrometer using a PE/Sciex TurboIonSpray ion source (Concord, Ontario, Canada). The ion source was operated in the positive electrospray ionisation (ESI) mode at 250°C with a setting of 12 for the nebulizer gas and a gas flow of 6 l min^{-1} for the auxiliary gas. The curtain gas setting was 10, and the orifice, ring and Q0 settings were 20, 180 and −3.5 V, respectively. The mass spectrometer was operated in the selected reaction monitoring (SRM) mode using collision-induced dissociation (CID) with nitrogen as the collision gas, a collision gas setting of 2 and a collision energy of 10.5 V. The system was equipped with a diversion valve and only HPLC eluent entered the mass spectrometer from 3.6 to 5.8 min after the injection. The retention time for EB 1089 and the internal standard was approximately 5.3 min.

Optimisation of the mass spectrometer parameters was done by infusing a standard solution of EB 1089 directly into the mass spectrometer at 10 μl min^{-1}. The ion source parameters were optimised by infusion of 10 μl min^{-1} of an EB 1089 standard solution into the HPLC eluent (75% MeOH with a total

concentration of 2 m*M* ammonium acetate) flowing at 0.29 ml min^{-1} just before the ion source.

2.8. Calculation of validation parameters

The method was validated over the concentration range 10–100 pg ml^{-1}. The calibration curve was based on six spiked samples in singlet and quality control (QC) samples in duplicate at three levels in three separate runs. A fixed amount of 1000 pg ml^{-1} of d_6-EB 1089 was used as internal standard for all samples. Calibration curves were constructed using 1/concentration weighted linear regression (peak height ratios vs. the concentrations). Peak heights, regression parameters, concentrations and intra-run accuracy and precision were calculated using TurboQuan™ Ver. 1.0.

3. Results and discussion

The objective of this work was to develop and validate a sensitive, specific and robust assay with a relatively high capacity for the determination of EB 1089 in serum from humans and minipigs, suitable for clinical and preclinical pharmacokinetic studies. On the basis of a previous SPE procedure used in our laboratory [22] we found that a simple reversed-phase procedure did not provide a satisfactory purification of the serum. However, we obtained the necessary purification by using a 'phase-switching procedure,' which included both a reversed-phase and a normal-phase extraction procedure on a single C_{18} cartridge [23]. By using this procedure giving a clean extract, the risk of ion suppression was also reduced. In the chromatographic part of the assay, a narrow-bore column was chosen to meet the demand of a low flow of mobile phase going into the mass spectrometer. The chromatography has also been developed to separate known metabolites of EB 1089, i.e. 26- and 26a-hydroxy EB 1089 (Fig. 1) [21]. However, a better separation is required, if each metabolite should be individually quantified. Their retention times are given in Table 1. The maximal capacity both on the SPE procedure and on LC–MS–MS is 144 samples per day.

Table 1
Retention time and transition of EB 1089, 26-hydroxy and 26a-hydroxy EB 1089

Compound	Retention time (min)	MRM transition
EB 1089	5.27	472→437
26a-OH EB 1089 (a)	2.94	488→453
26a-OH EB 1089 (b)	3.31	488→453
(25*S*),26*S*-OH EB 1089	3.36	488→453
(25*R*),26*R*-OH EB 1089	2.98	488→453
(25*S*),26*R*-OH EB 1089	3.80	488→453
(25*R*),26*S*-OH EB 1089	3.29	488→453

3.1. MS–MS and selectivity

Tandem mass spectrometry has been chosen to enhance mass spectrometric selectivity.

The ESI mass spectra of EB 1089 and d_6-EB 1089 are shown in Fig. 2. The most intense ions formed from EB 1089 and d_6-EB 1089 were m/z 472 and m/z 478, respectively, corresponding to the ammonium adduct ion ($[M+NH_4]^+$). The differences in the ESI-spectra of EB 1089 and d_6-EB 1089, respectively [e.g. m/z 355.6 in Fig. 2(A) and m/z 315.4 in Fig. 2(B)] may be due to the differences in the profile of the impurities of the two compounds manufactured at slightly different methods. The product ion mass spectra of EB 1089 and d_6-EB 1089 are shown in Fig. 3. The base peak was m/z 437 and m/z 443 for EB 1089 and d_6-EB 1089, respectively, corresponding to a neutral loss of 35 mass units, e.g. water (H_2O) and ammonia (NH_3). The adduct ions were relatively fragile and dissociation occurred easily. From Fig. 2 it can be seen that some dissociation occurs in spite of the fact that no collision gas is present. Thus, the energy in the skimmer zone, the collision energy and collision gas setting have an optimal setting that is different from the average generic settings.

A neutral loss of 35 is usually not considered to be specific. However, by using the 'phase-switching' SPE clean-up procedure combined with reversed-phase HPLC a very high degree of selectivity is achieved. Consequently, the transitions m/z 472→434 and 478→440 could be used as the MRM settings.

In addition, all the 26-hydroxy and 26a-hydroxy derivatives of EB 1089 also formed ammonium

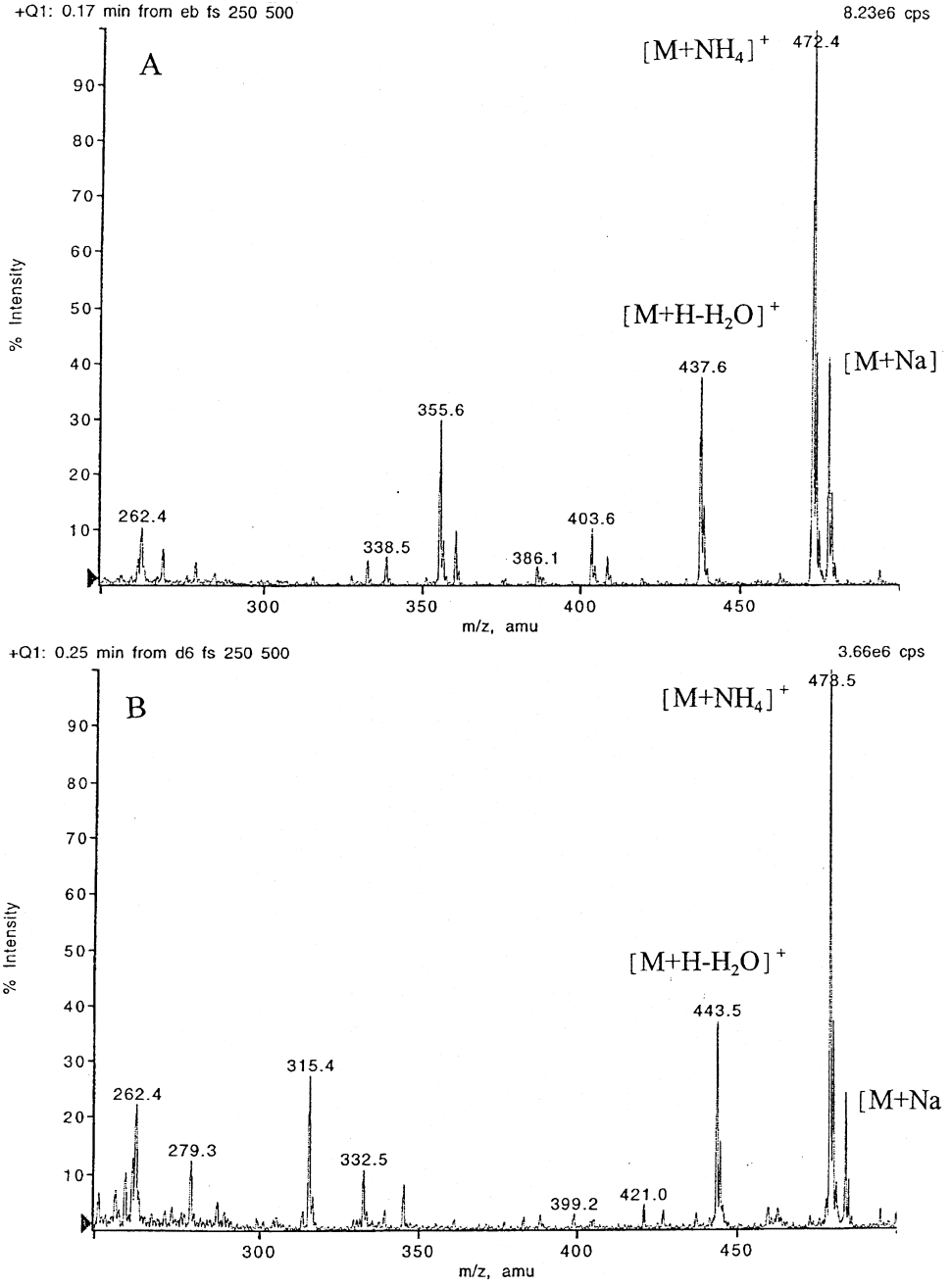

Fig. 2. (A) Full-scan positive ESI ion mass spectrum (MS) of EB 1089 ([M+NH_4]+m/z of 472.3). (B) Full-scan positive ESI ion mass spectrum (MS) of d_6-EB 1089 ([M+NH_4]+m/z of 478.3).

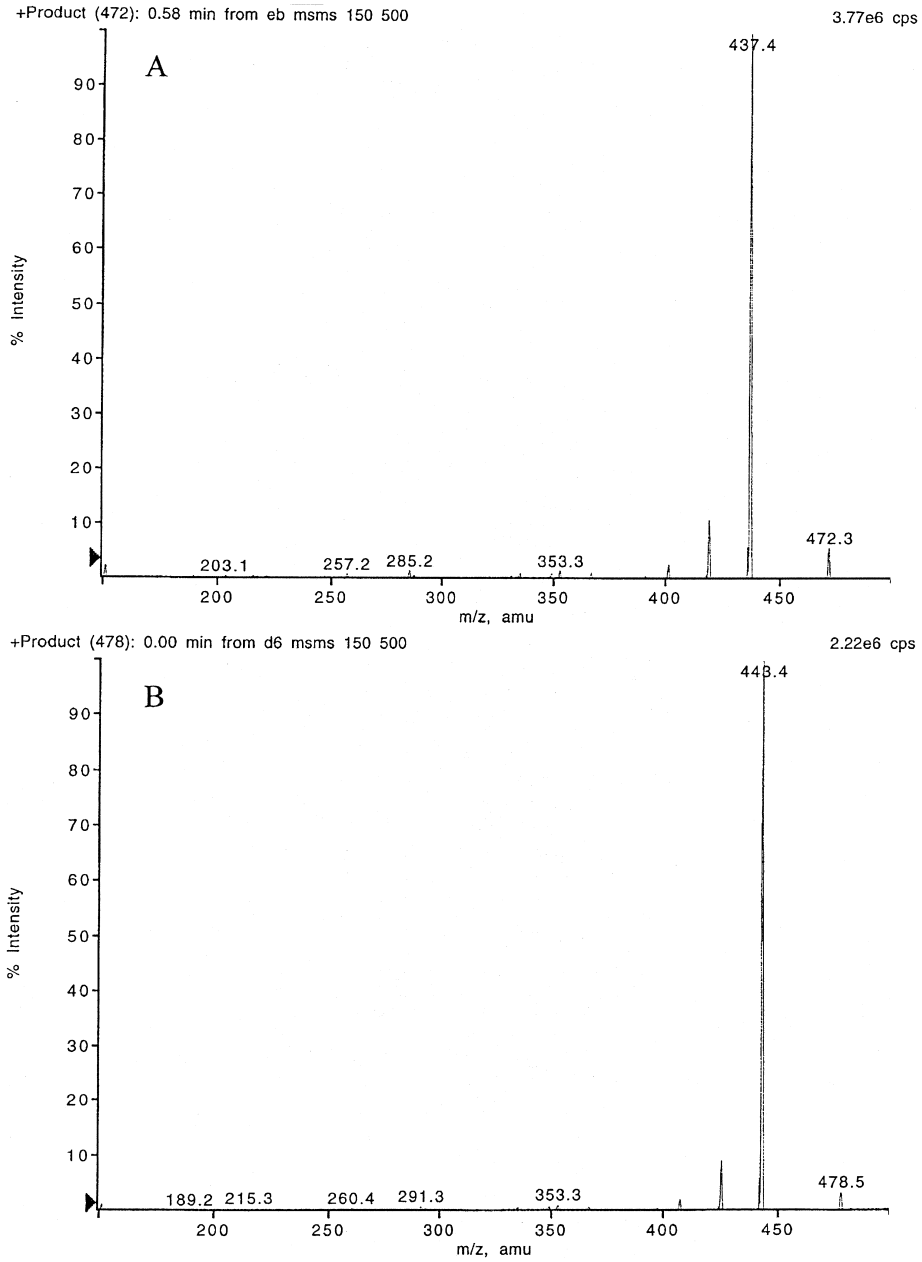

Fig. 3. (A) Product ion mass spectrum (MS–MS) of the EB 1089 ($[M+NH_4]+m/z$ of 472.3). (B) Product ion mass spectrum (MS–MS) of d_6-EB 1089 ($[M+NH_4]+m/z$ of 478.3).

adducts and a neutral loss of m/z 35 after CID (Table 1). These observations indicate that the loss does not occur in the side-chain of the molecules, but rather somewhere in the ring system. Typical SRM ion chromatograms of extracted blank human and pig serum and similar samples with the addition of 10 pg ml^{-1} of EB 1089 and of 1000 pg ml^{-1} of d_6-EB 1089 are shown in Figs. 4 and 5. No peaks interfered with the EB 1089 signal in the blank serum samples when running individual serum samples from healthy

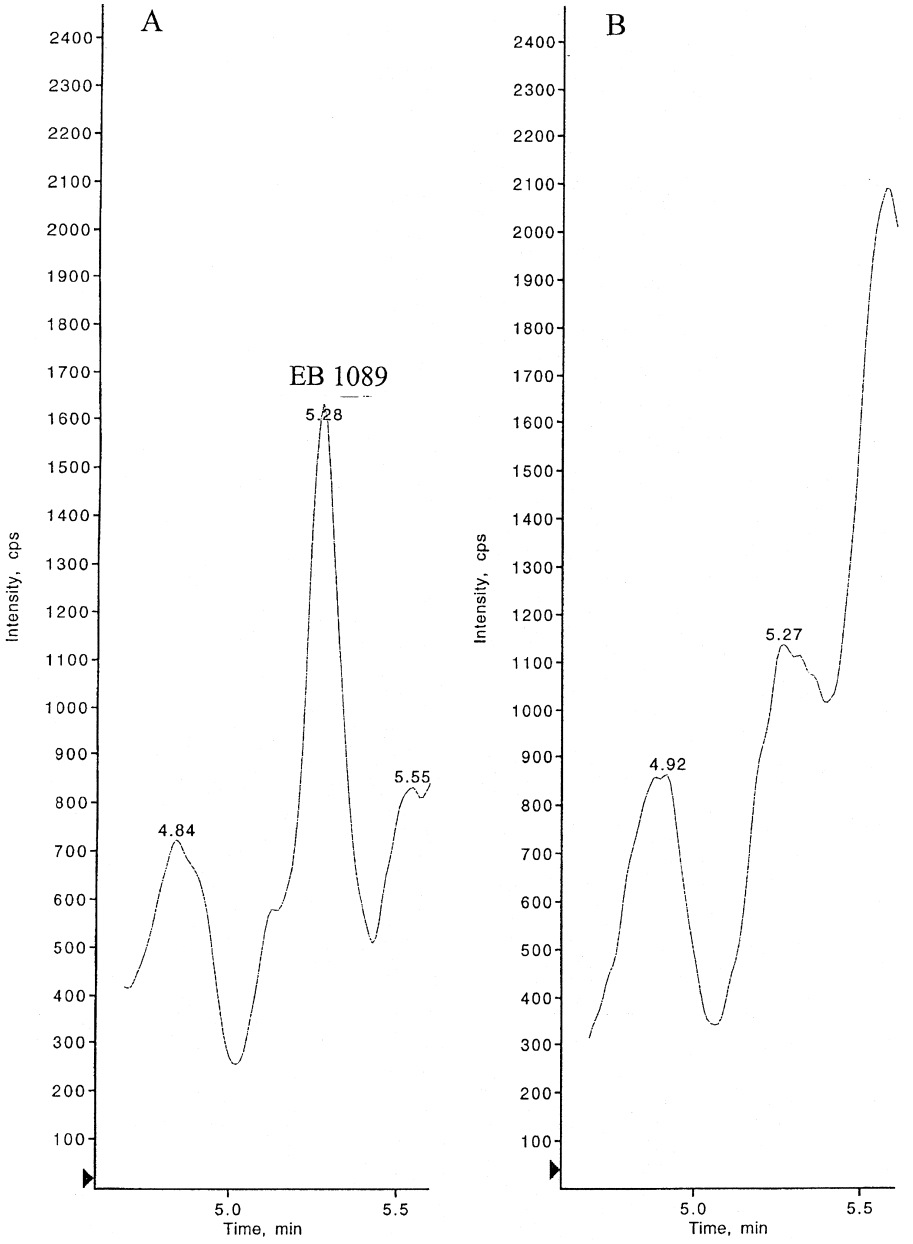

Fig. 4. (A) Selected reaction monitoring (SRM) ion chromatogram of human serum spiked with 10 pg ml^{-1} of EB 1089 (LLOQ). Transitions: m/z 472.3→437.3 for EB 1089. (B) Selected reaction monitoring (SRM) ion chromatogram of blank human serum. Transitions: m/z 472.3→437.3 for EB 1089.

subjects or minipigs. However, a more distinguished peak from the blank serum interfered with a signal from the internal standard. Thus, the concentration of the internal standard has been set to 1000 pg ml^{-1} in order to ignore this interfering signal, without causing any problems with ion suppression. The signal from the internal standard is about 30-fold higher than the signal from the interfering signal.

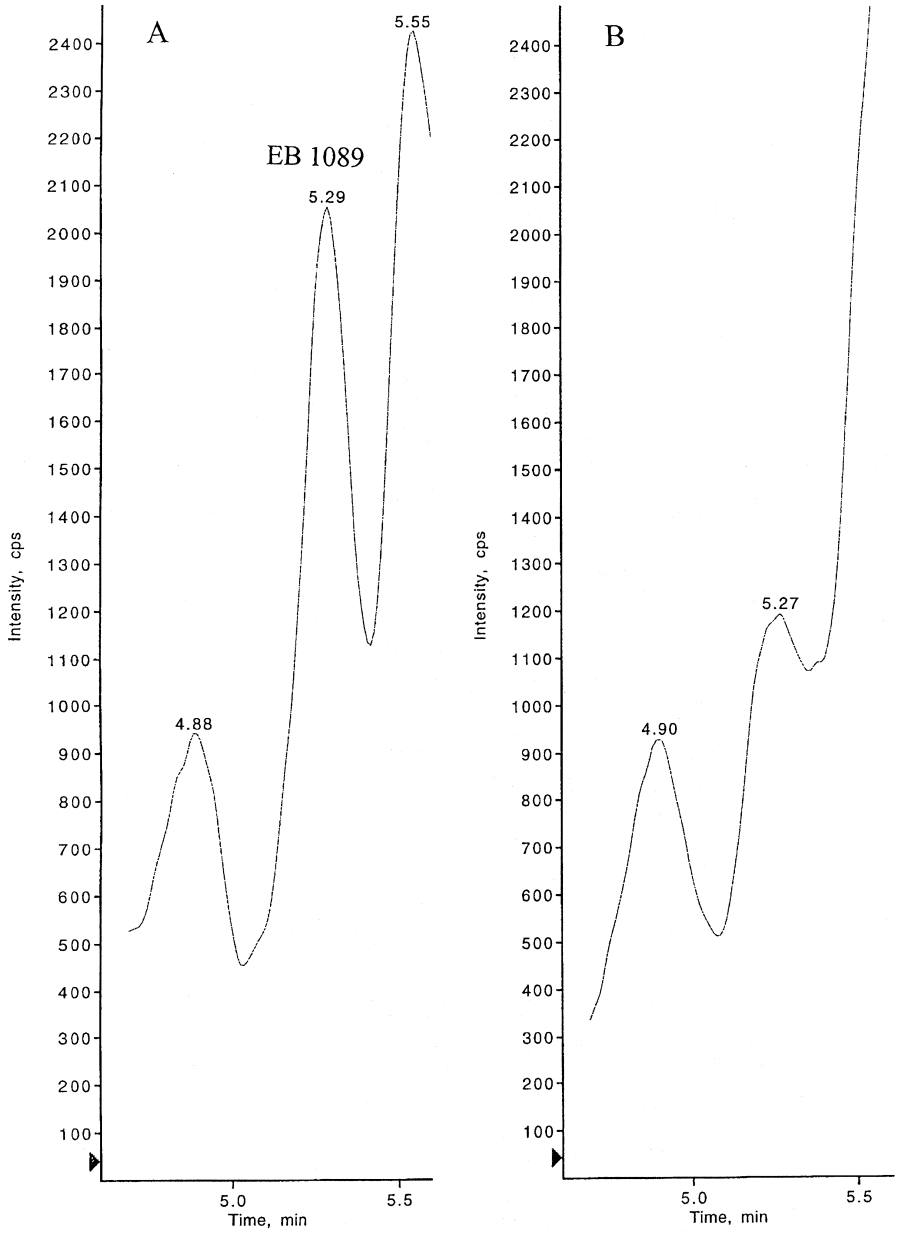

Fig. 5. (A) Selected reaction monitoring (SRM) ion chromatogram of pig serum spiked with 10 pg ml^{-1} of EB 1089 (LLOQ). Transitions: m/z 472.3→437.3 for EB 1089. (B) Selected reaction monitoring (SRM) ion chromatogram of blank pig serum. Transitions: m/z 472.3→437.3 for EB 1089.

3.2. Linearity

The response function for the calibration curve covering the concentration 10–100 pg ml^{-1} was determined with every run within the validation. The best calibration curves were obtained using 1/concentration weighted linear regression (peak height ratio of the analyte vs. the I.S., plotted against the concentration). The slopes, intercepts, coefficients of regression appear in Table 2 and the residuals are

Table 2
Calibration curve equations using 1/concentration weighing of EB1089 in human and pig serum

Species	Slope	Intercept	Correlation coefficient (r)
Human	0.00071	0.01208	0.9994
	0.00088	0.00394	0.9990
	0.00100	0.00424	0.9979
Pig[a]	0.00958	0.0331	0.9981
	0.00896	0.0341	0.9994
	0.00879	0.0164	0.9999

[a] The concentration of the internal standard was 100 pg ml^{-1}.

stated in Table 3. The highest concentration in the standard curve has been chosen to be 100 pg ml^{-1} because all serum samples are expected to contain less than 100 pg ml^{-1}.

3.3. Precision and accuracy

The precision and accuracy were investigated for two conditions: within run (intra-run) and between run (inter-run). Six determinations at three levels covering the analytical range were used to determine the intra-run precision and accuracy. Between the runs, parameters were assessed from duplicate samples at each level on three separate days. The precision is calculated as C.V. (%) (=SD/mean×100%), and the accuracy is calculated as the percentage of nominal concentration (measured conc./nominal conc.×100%). The intra- and inter-run precision and accuracy are given in Tables 4 and 5, respectively. The repeatability (intra-run) and reproducibility (inter-run) were less than 15% at all levels

Table 3
Percentage residuals of the calibration curve of EB 1089 in human and pig serum

Nominal concentration (pg ml^{-1})	Pig			Human		
	Run 1 (%)	Run 2 (%)	Run 3 (%)	Run 1 (%)	Run 2 (%)	Run 3 (%)
10	−5.1	−3.4	−1.2	1.8	3.6	−2.0
20	2.7	0.9	1.3	−5.0	−7.2	−1.3
40	7.4	1.8	−0.7	3.8	1.3	−0.4
60	−2.5	3.3	1.5	0.9	4.0	6.5
80	−4.9	0.6	−0.9	1	1.0	2.6
100	2.7	−2.9	Ex[a]	−1.2	−2.7	−5.3

[a] Ex, excluded to optimise linear regression.

Table 4
Intra-run precision and accuracy (n=6) of EB1089 in human and pig serum

Species	Nominal concentration					
	10 pg ml^{-1}		50 pg ml^{-1}		100 pg ml^{-1}	
	Precision C.V. (%)	Accuracy (%)	Precision C.V. (%)	Accuracy (%)	Precision C.V. (%)	Accuracy (%)
Human	4.3	86	3.1	91	4.5	98
Pig	18	94	1.5	99	1.8	100

Table 5
Inter-run precision and accuracy (n=6) of EB1089 in human and pig serum

Species	Nominal concentration					
	10 pg ml^{-1}		50 pg ml^{-1}		100 pg ml^{-1}	
	Precision C.V. (%)	Accuracy (%)	Precision C.V. (%)	Accuracy (%)	Precision C.V. (%)	Accuracy (%)
Human	14	88	2.6	100	4.9	98
Pig	17	85	6.7	97	6.8	99

except at 10 pg ml^{-1} in pig serum where the repeatability and reproducibility were between 15% and 20%. The accuracy was within 85–115% at all levels.

3.4. Lower limit of quantitation (LLOQ)

The lower limit of quantitation is defined as the lowest quantified level with precision and accuracy of ±20%. From the results listed in Tables 4 and 5 it can be seen that 10 pg ml^{-1} of EB 1089 in human and pig serum fulfils the definition of LLOQ.

3.5. Extraction recovery

The extraction recovery was determined by analysis of six samples at three levels by comparison of the response from spiked samples and from extracted blank samples fortified with EB 1089 and internal standard prior to analysis. Recovery is given as the ratio between the mean height of a spiked sample vs. the mean height of a blank serum sample fortified with analytes and corrected for the dilution (0.86) of spiked samples. The mean ($n=6$) recoveries are stated in Table 6. The data suggest the recoveries of EB 1089 to be independent of the concentration in the tested ranges from human and pig serum.

3.6. Stability

The stability of EB 1089 under various conditions was investigated. A stock solution of 100 μg ml^{-1} of EB 1089 in 2-propanol was demonstrated to be stable for at least 4 months when stored at −18°C. A working solution of 40 ng ml^{-1} of EB 1089 in methanol was found to be stable for at least 1 month at −18°C. The stability of EB 1089 was also investigated in human and pig serum at three concentration levels. After three freeze–thaw cycles, the concentrations were within the range 85–115% of the nominal concentration at the two highest concentration and within the range 80–120% of the nominal concentration at the LLOQ concentration (Table 7). These data indicate that EB 1089 was stable up to three freeze–thaw cycles. In contrast, EB 1089 was not stable after 5 days' storage at room temperature, as the concentrations of EB 1089 in serum were outside the above ranges of acceptance. The extended stability of EB 1089 in serum stored at −18°C and −80°C is ongoing. Data are not yet available. Finally, the stability of EB 1089 in extracted and reconstituted serum samples stored in the autosampler at 8°C for 24 h was demonstrated, as the concentrations compared between 0 and 24 h storage were within the above ranges of acceptance (Table 8).

Table 7
Freeze–thaw stability of EB 1089 in human and pig serum

Species	Percentage of nominal concentration after three freeze–thaw cycles		
	10 pg ml^{-1}	50 pg ml^{-1}	100 pg ml^{-1}
Human	105	92	95
Pig	117	110	97

Table 8
Twenty-four hour autosampler stability of EB 1089 in human and pig serum in reconstituted samples

Species	Percentage of nominal concentration after 24 h in autosampler		
	10 pg ml^{-1}	50 pg ml^{-1}	100 pg ml^{-1}
Human	96	92	94
Pig	102	103	106

Table 6
Percentage recovery of EB 1089 and d_6-EB 1089 from human and pig serum[a]

Species	Recovery (%)			
	EB 1089			d_6-EB 1089
	10 pg ml^{-1}	50 pg ml^{-1}	100 pg ml^{-1}	1000 pg ml^{-1}
Human	83 (5.5/9.4)	75 (3.4/2.7)	71 (4.7/4.2)	88 (11.3/6.0)
Pig	90 (13.7/4.4)	78 (3.8/2.8)	76 (7.1/2.0)	86 (4.5/2.9)

[a] The numbers in brackets are C.V. (%) of the peak height found in spiked and fortified samples, respectively.

4. Conclusion

A liquid chromatography–tandem mass spectrometry assay for the determination of EB 1089 in human and pig serum has been developed and validated with a lower limit of quantitation of 10 pg ml^{-1}. The assay is specific, sensitive and has a capacity of more than 100 samples per day. Until now, the exposure to EB 1089 in humans or minipigs has indirectly been based on the level of serum calcium. Using this new LC–MS–MS assay, we have made a direct investigation of the exposure to EB 1089 in minipigs to support toxicity studies in this species. Moreover, we are presently measuring the concentration of EB 1089 in human serum collected from various phase II and III studies in order to evaluate exposure. Finally, we plan to carry out a complete pharmacokinetic study in humans.

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