Anti-Cancer Drugs 2001, **12,** pp. 653–666

Clinical report

Search for metabolites of ecteinascidin 743, a novel, marine-derived, anti-cancer agent, in man

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Ecteinascidin 743 (ET-743) is a potent anti-tumoral agent of a marine origin. It is currently being tested in phase II clinical trials using a 3-weekly 24-h i.v. infusion of 1500 μg/m^2 and 3-h infusions of 1650 μg/m^2. Knowledge of the metabolism of ET-743 is, however, still scarce. In the present study, a qualitative chromatographic discovery of metabolites of ET-743 in man is reported. ET-743 and its demethylated analog ET-729 were incubated at 37°C in the presence of enzyme systems, pooled human microsomes, pooled human plasma and uridine 5′-diphosphoglucuronyltransferase, respectively, in appropriate media. Reaction products were investigated chromatographically using photodiode array and ion spray-mass spectrometric detection (LC-MS). The main reaction products in microsomal incubations of ET-743 resulted from a remarkable breakdown of the molecule. In plasma the drugs were deacetylated, and the transferase did actually yield a glucuronide of both ET-743 and ET-729. In contrast, screening of urine, plasma and bile, collected from patients treated with ET-743 at the highest dose levels, using a sensitive LC-MS assay, did not result in detection of ET-729 and metabolites which were generated *in vitro*. The urinary excretion of ET-743 in man was lower than 0.7% of the administered dose for a 24-h infusion. [© 2001 Lippincott Williams & Wilkins.]

Key words: Ecteinascidin 729, ecteinascidin 743, high-performance liquid chromatography, metabolism, microsomes.

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Introduction

The ecteinascidins (ETs) form a group of active anti-tumor tris(tetrahydroisoquinolines), isolated from the Caribbean tunicate *Ecteinascidia turbinata* and were identified 10 years ago.[1] Most of these compounds showed potent pre-clinical cytotoxic activities,[2,3] e.g. in leukemia, melanoma, and lung and colon carcinoma models. Because of the promising pre-clinical results of all ETs, the most abundant and very potent compound in the extracts of the tunicate, ecteinascidin-743 (ET-743, Figure 1), was selected for further exploration as an anti-tumor drug.[4]

ET-743 is a minor groove alkylator and reacts preferentially with guanine-rich DNA sequences, particularly to the guanine 2-amino group.[5] Binding and DNA recognition is facilitated by the units A and B and the *N*-methyl group of the ET-743 molecule.[6] As a specific feature for a minor groove alkylator, ET-743 bends DNA into the major groove.[7] Overall, the exact mechanism of anti-tumor activity of ET-743 remains unclear and several other possible explanations for the efficacy of ET-743 have been reported, e.g. the modification of the interaction of some DNA binding proteins with DNA,[8,9] induction of topoisomerase I mediated protein-linked DNA breaks[10] and disorganization of the microtubule network.[11]

After selection of ET-743 from the ETs for further pre-clinical investigations, specific *in vivo* anti-tumor activity was observed against human tumor xenografts from melanoma, non-small cell lung and ovarian cancer,[12,13] and other human xenograft cancer models.[4]

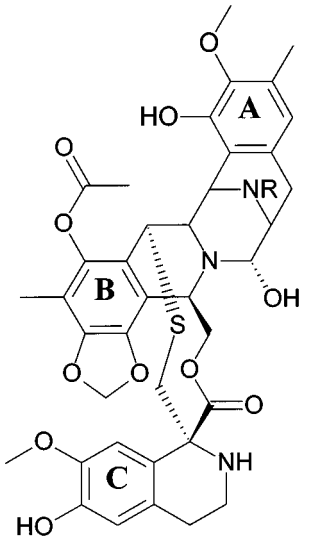

Figure 1. Chemical structure of ET-743 (R = CH_3) and ET-729 (R = H).

Furthermore, ET-743 showed pre-clinical schedule-dependent activity resulting in the preference for long-term[14,15] and intermittent[12] exposure to ET-743 for clinical evaluations.

Phase I clinical studies were therefore started using a 3-weekly repetition of a 1- (later changed into a 3-), 24- or 72-h i.v. infusion or five daily 1-h i.v. infusions. The studies of the 3-[16] and 24-h[17] and daily $\times 5$[18] ET-743 schedule against solid tumors have been finished, and resulted in recommended doses of 1650, 1500 and 5×325 $\mu g/m^2$, respectively. In addition to one complete remission (melanoma) in the 3-h study, several partial responses have been observed in the phase I program. Dose-limiting toxicities in the different regimens were myelosuppression and severe fatigue for the different regimens. Further, transient elevation of hepatic transaminases, not dose-limiting, occurred very frequently in all studies. Presently, the recommended doses of 1500 $\mu g/m^2$ for the 24-h infusion and 1650 $\mu g/m^2$ for the 3-h infusion are applied in several, still ongoing, phase II clinical studies. Promising preliminary results have been reported recently for the 24-h infusion, indicating evidence of clinical activities against heavily pretreated advanced/metastatic breast cancer[19] and different types of soft tissue sarcomas.[20,21]

ET-743 shows linear pharmacokinetics with a large interpatient variability, a high volume of distribution of 3.9 ± 1.9 m^3 and a long terminal half-life of 89 ± 41 h in the 24-h infusion at a dose level of 1500 $\mu g/m^2$ ($n = 25$).[22]

Data about ET-743 metabolism are very limited. The first potential metabolites of ET-743 (Figure 2A–C) have recently been discovered in a study using incubations with a human lymphoblast-expressed CYP3A4 isoform.[23] *In vitro*, the male-dominant CYP3A2 appears the major CYP catalyzing ET-743 metabolism in rats. Further, metabolism in rats was induced by pre-treatment with dexamethasone and phenobarbital, but not by 3-methylcholanthrene.[24] In addition to ET-743 concentrations, metabolite concentrations *in vivo* will be very low due to the relatively low administered dosages of the drug.

In the current study, a four-step approach was followed for the discovery of human metabolites of ET-743. First, potential metabolites were generated using several incubations with human microsomes, human plasma and uridine 5′-diphosphoglucuronyl transferase (UDPGT). Second, the discovered compounds were identified using liquid chromatography (LC)-UV and LC-mass spectroscopy (MS)(-MS) as far as possible. Third, the MS-MS detection was optimized for the most prominent potential metabolites using purified samples obtained by semi-preparative LC. Fourth, human samples (urine, plasma and bile) of treated patients were screened for the presence of these potential metabolites of ET-743. In addition, the first results of measurements of ET-743 in human urine and bile will be presented, as well as ET-743 plasma pharmacokinetics in a patient with Gilbert's syndrome.

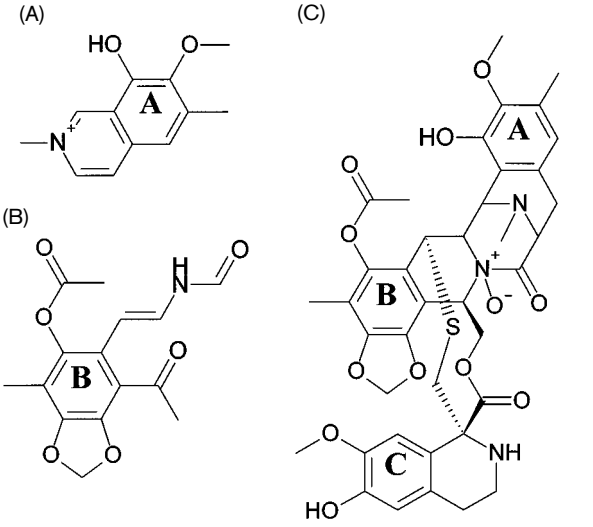

Figure 2. Structures of recently discovered potential metabolites of ET-743.[23] (A) ETM-204 (ET743S1 in this study), (B) ETM-305 (ET743S2 in this study) and (C) ETM-775.

Materials and methods

Chemicals

ET-743 and ET-729 (Figure 1) were kindly provided by PharmaMar (Madrid, Spain). Methanol [absolute for high-performance liquid chromotography (HPLC)] was provided by Biosolve (Valkenswaard, The Netherlands) and water was home-purified by reversed osmosis on a multi-laboratory scale. Pooled human liver microsomes (20 mg/ml protein in 250 mM sucrose; 420 pmol/mg CYP of total protein; equal fractions of 10 individuals; lot 7) were supplied by Gentest (Woburn, MA) and blank, drug-free human plasma was obtained from the Stichting Rode Kruis Bloedbank (Utrecht, The Netherlands). All other chemicals were of analytical grade from Sigma (St Louis, MO).

A 100-μl volume of 15 u/ml glucose-6-phosphate dehydrogenase in 2% (w/v) sodium hydrogencarbonate, 100 μl of 5 mg/ml $NADP^+$ sodium salt, 100 μl of 39 mg/ml disodium D-glucose-6-phosphate dihydrate and 700 μl water were mixed to obtain the $NADP^+$ regenerating system (NRS). Stock solutions of ET-743 and ET-729 (1 or 2 mg/ml) were prepared in methanol.

Equipment

Chromatographic analyses were performed on two different configurations.

In HPLC system A, two LC 10AD liquid chromatographic pumps (Shimadzu, Kyoto, Japan) were used for high-pressure gradient formation and were coupled to an U6K injector (Waters Chromatography, Milford, MA) and a Hewlett Packard (HP) series 1100 Photodiode array detector (HP, Wilmington, DE). The column was thermostated in a water bath with the temperature being controlled by a thermomix 1420 heating device (B Braun, Melsungen, Germany). Chromatographic and spectrophotometric data were recorded and processed on a HP Vectra XA personal computer (Delfgauw, The Netherlands), equipped with the HP Chemstation chromatographic data system, version A.05.02.

In HPLC system B, a Perkin-Elmer 200 series pump and an ISS 200 autosampler (Perkin-Elmer, Norwalk, CT) were used and the column temperature was controlled by a Model 7971 column heater (Jones Chromatography, Lakewood, CO). The eluent flow was split (1:4) into the API 365 triple quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada) equipped with a TurbulonSpray sample inlet for the electrospray ionization.

Fluorescence spectra were recorded using a SFM-25 spectrofluorometer (Kontron, Zürich, Switzerland).

Chromatographic conditions

Partial-loop injections (50 μl) were made on a Symmetry C_{18} column (100×4.6 mm i.d., 3.5 μm; Waters, Bedford, MA) with a Symmetry C_{18} precolumn (20×3.8 mm i.d., 5 μm; Waters). The column temperature was kept at $40 \pm 2^{\circ}$C. The eluent comprised 10 mM ammonium acetate and 0.04% formic acid in a mixture of methanol and water. Two different gradient programs were used (Table 1). The analytical chromatographic system was also used to fractionate incubated mixtures in order to obtain purified degradation products and metabolites of ET-743.

Procedures

Microsomal studies. An aliquot of 100 μl of an aqueous dilution of an ET stock solution [typically 100 μg/ml in 5% (v/v) methanol] or a purified degradation product of ET-743 were pipetted into a polypropylene micro tube on ice and 50 μl of the 0.5 M potassium phosphate buffer (pH 7.4) and 100 μl of the NRS solution were added, respectively. After brief vortex-mixing, the tubes were conditioned at 37°C for 2 min. Next, 10 μl of the microsomal suspension was added, the tube was vortex-mixed again and incubated further at 37°C (typically for 4–6 h). The reaction was terminated by adding 250 or 300 μl of methanol and briefly vortex-mixed; the sample was centrifuged for 5 min at 1.3×10^3 g for removal of proteins. The supernatant was then injected for HPLC analysis. Control experiments were

Table 1. Gradient profiles

	Gradient A		Gradient B	
	Conditions (% methanol)	Duration (min)	Conditions (% methanol)	Duration (min)
Starting conditions	5	10	5	0
Gradient profile	linear	15	linear	20
Finishing conditions	60	10	80	5

performed without substrate and without the microsomal suspension.

Plasma incubations For plasma incubations, 20 μl of a 1 mg/ml ET stock solution was vortex-mixed in a conical glass tube with 480 μl pooled drug-free human plasma, originating from four healthy individuals and incubated at 37°C for 24 h. After incubation, 100 μl of 25 mM phosphate (pH 7.0) was added and the sample was extracted using 2 ml chloroform. After shaking manually for 25 s and centrifugation for 5 min at 4.3×10^3 *g*, the organic phase was transferred into a clean tube and evaporated at ambient temperature under a nitrogen flow. The residue was reconstituted in 0.5 ml of a mixture of methanol and 0.2 M ammonium acetate (pH 5.0) (2:3, v/v).

Glucuronidation. Attempts to form glucuronides of both ETs were undertaken by pipetting equal volumes (20–50 μl) of 0.1 M magnesium dichloride, 15 mg/ml UDPGT in 0.5 M Tris buffer (pH 7.4), 15 mg/ml UDPG in water and 200 μg/ml ET-743 or ET-729 in water/methanol (80/20, v/v) into a polypropylene micro tube. After brief vortex-mixing, the mixture was incubated at 37°C for typically 5 h. The reaction was terminated by adding an equal volume of methanol and briefly vortex-mixed. Proteins were removed by centrifugation for 5 min at 1.3×10^3 *g* and the supernatant was injected for HPLC analysis. Blank experiments were performed without enzyme, UDPG and ETs, respectively.

Semi-preparative fractionation of degradation products and metabolites of ET-743. Several portions of 100 μl of an appropriate incubation mixture were injected into chromatographic system A in separate gradient runs. Eluate fractions from degradation products and potential metabolites of ET-743 were collected. Fractions were pooled for each peak and evaporated at 40°C using a nitrogen flow until a volume of about 50 or 100 μl remained. These purified, concentrated samples were used for microsomal incubations (the 50-μl samples, diluted to 250 μl with water) or identification using LC-MS(-MS) (the 100-μl samples). The eluate fractions used for optimization of the ionization parameters of the MS detector were not concentrated by evaporation.

Screening of clinical samples for ET-743 metabolites. Plasma, urine and bile were investigated from patients treated with ET-743 at 1500 or 1800 μg/m^2 during a 24-h infusion ($n = 4$) or at 1650 μg/m^2 during a 3-h infusion ($n = 3$). All patients gave written informed consent. Bile was available from one patient (1500 μg/m^2; 24-h infusion) and urine and plasma from all subjects. Plasma and bile samples were taken before treatment, and at the end and 2 h after the end of infusion. Urine samples were collected pre-dose and twice after the start of the course during 24-h intervals. All samples were submitted to two different pre-treatment procedures. From all samples, 450 μl was extracted with 6.75 ml chloroform, followed by evaporation of the organic extract (40°C under nitrogen flow), and reconstitution in 200–250 μl of a mixture of 0.04% (v/v) formic acid, 5% (v/v) methanol and 95% (v/v) water (eluent A). Alternatively, proteins were removed from 450-μl plasma samples using 900 μl methanol at ambient temperature, the solvent was then evaporated at 40°C using a nitrogen flow and the residue was reconstituted in 200–250 μl of eluent A. Urine and bile samples (100 μl) were alternatively injected after dilution with 400 μl of the eluent. MS settings were optimized for each of the most prominent potential metabolites, using the fractionated samples. These settings were combined with the known optimal settings for ET-743 and ET-729[25] for the screening of the clinical samples.

Quantification of ET-743 in clinical samples. ET-743 has been measured in urine of several patients receiving 1500 μg/m^2 during a 24-h infusion or 1650 μg/m^2 during a 3-h infusion and bile was available from one patient (1500 μg/m^2; 24-h infusion). In addition, plasma samples from a patient (first course 900 μg/m^2, second course 1200 μg/m^2; 24-h infusion) suffering from Gilbert's syndrome were analyzed and the results were compared with data from patients without this syndrome. The validated chromatographic assay used has been described previously[25] and comprises LC-MS(-MS) with solid-phase extraction as a pre-treatment procedure for plasma samples, the limit of quantification is 10 pg/ml). The urine samples were diluted 1:10 (v/v) and the bile samples 1:6 (v/v) with drug-free human plasma and both treated further as plasma samples.[25]

Results

Microsomal studies

Conversion of ET-743 after 6-h incubation in the NRS solution was 30–40% without and 60–70% in the presence of the microsomal enzymes. Percentages were calculated by comparison of the UV response at 254 nm to the response of a non-incubated sample. Microsomal incubation of ET-743 resulted in the formation of eight products [ET742M1, M2, M3, M5, M6, S1 (Figure 2A), S2 (Figure 2B) and S3 ('M' from

metabolite and 'S' from stability)], of which three (ET743S1, S2 and S3) are also formed without the presence of the hepatic phase I enzymes (Figure 3A). UV spectra of all these compounds are shown in Figure 4, and all chromatographic, spectrophotometric and mass spectrometric data are summarized in Table 2.

The two main degradation products of ET-743, ET743S1 and ET743S2, were also formed in an aqueous solution (not buffered) when kept at ambient temperature during overnight storage. Both compounds show substantially different UV spectra (Figure 4A, B and D and Table 2). Maxima in the fluorescence spectrum of ET743S1 were 448 and 530 nm for excitation and emission, respectively, at pH 7.4. The UV spectrum of the minor degradation product ET743S3 shows partial correspondence with ET743S1 at the maxima at 208 and 380 nm; furthermore, the ET-743 UV maximum at 287 nm seems to remain in this product (Figure 4A, B and D and Table 2). The mass spectrum of ET743S3 shows three

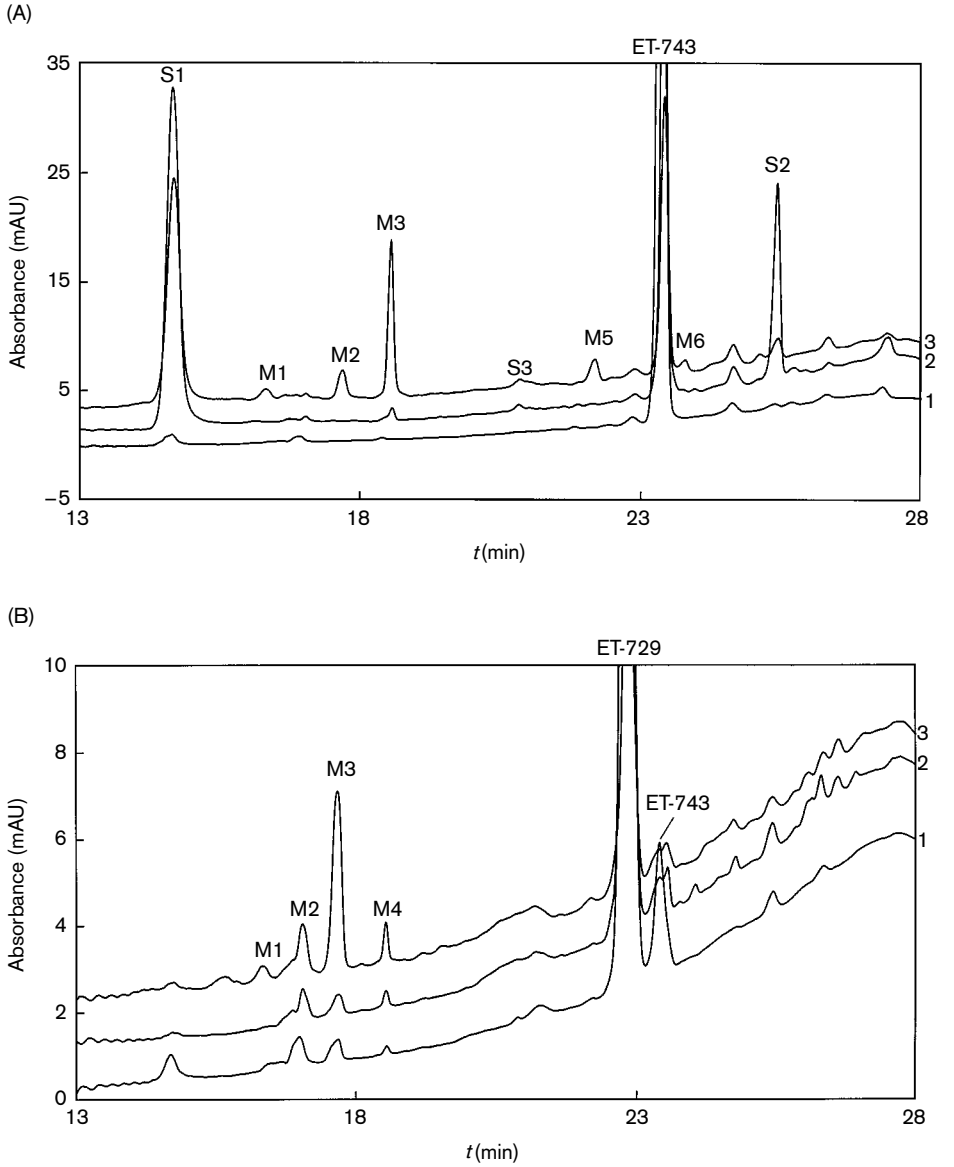

Figure 3. Chromatograms at 254 nm using gradient A of: (A) ET-743 reference solution (1), ET-743 after incubation using the microsomal procedure but without addition of microsomes (2) and ET-743 after incubation with microsomes (3); (B) ET-729 reference solution (1), ET-729 after incubation using the microsomal procedure but without addition of microsomes (2) and ET-729 after incubation with microsomes (3). In both figures the prefixes 'ET743' and 'ET729' from the names of the metabolites have been omitted.

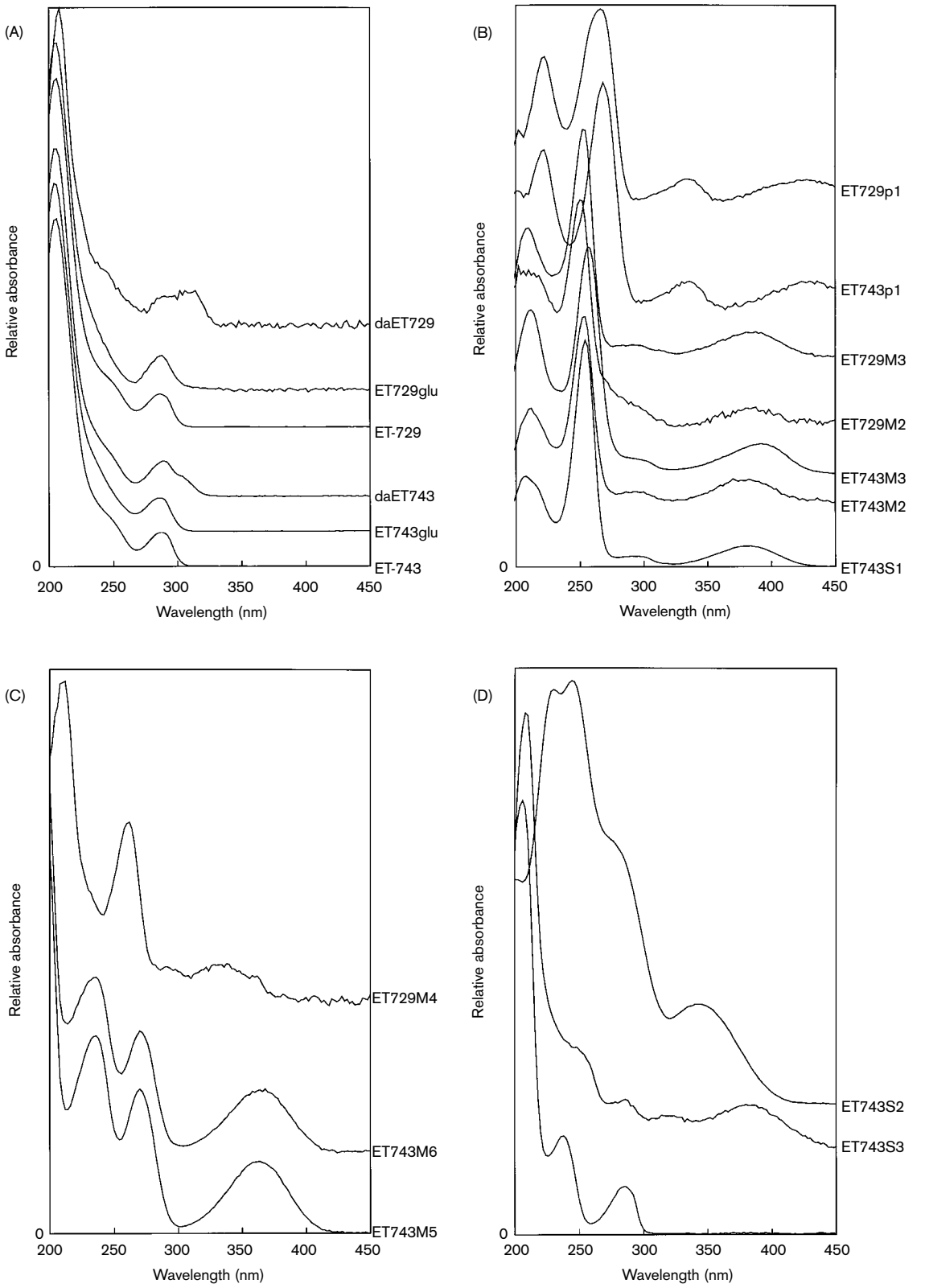

Figure 4. UV spectra of ET-743, ET-729 and their metabolites, and degradation products found. (A) Parent compounds, glucuronidated ETs (glu) and deacetylated ETs (da). (B) S1 degradation product of ET-743, and the M2, M3 and p1 metabolites of both ETs. (C) M5 and M6 metabolites of ET743S2, and M4 metabolite of ET-729. (D) M1 metabolite (of both ET-743 and ET-729), and S2 and S3 degradation products of ET-743.

Table 2. Chromatographic, spectroscopic and MS (positive ions) results of ET-743, ET-729 and their degradation and reaction products

Component	t_R[a] (min)		UV	MS (single quadrupole)	MS-MS (triple quadrupole)	
	A[a]	B[a]	Absorbance maxima (nm)	Primary spectrum	Selected ion	Secondary (product ion) spectrum
ET-743	23.3	13.5	**205**; 287	**744**; *762*; 776; 784		
ET743M1[b]	16.3	7.4	**206**; 238; 286	236		
ET743M2	17.7	8.1	212; **253**; 376	203; 218; 551		
ET743M3	18.6	8.6	**212**; **256**; 392	315; 419	419	371; 386; **389**
ET743M5	22.2	12.6	**198**; 236; 270; 364	**242**; **262**; 302; 318		
ET743M6	23.8	14.2	**198**; 236; 270; 368	**234**; 262	262	131; 164; 189; 207
ET743S1	14.7	6.1	208; **254**; 382	189; *204*	189	118; 132; 146; **160**
					204	118; 132; 146; **161**; 189
ET743S2	25.4	15.8	**230**; **244**; 342	219; 288; *306*; 328; **344**		
ET743S3	20.8	10.9	**209**; 380	507; 525; 539; **698**; *716*; **730**	730	204; **234**; 249; 477
ET743p1			222; **268**; 334; 430			
daET743		11.7	**205**; 288	**702.4**; *720.4*	702.4	218.6; 401.9; 433.2; 453.0; 487.3
ET743glu		10.5		**920.3**; *938.1*; 952.4	920.3	744.4; 495.3; 218.1
ET-729	22.9	13.2	**205**; 286	**730**; *748*; 762; 770; 784		
ET729M1[b]	16.3	7.4	**206**; 238; 286			
ET729M2	17.0		210; **250**; 385			
ET729M3	17.7		210; **250**; 385	**187**; 202		
ET729M4	18.6		**211**; 262	700		
ET729p1		7.6	222; **268**; 334; 430			
daET729		11.3	**208**; 314	**461**; *689*		
ET729glu		10.3		**906.5**; *924.5*; 938.5		

[a]Retention times for chromatographic system A for both gradient A and gradient B.
[b]ET743M1 and ET729M1 are the same compounds.
Bold = prominent signals. Italic = molecular ions.

prominent peaks (Table 2), each 46 a.m.u. lower compared to the three ET-743 peaks. These MS peaks of ET-743 have previously been ascribed to the parent ($m/z = 762$ a.m.u., $[M+H]^+$), the dissociated parent ($m/z = 744$ a.m.u., $[M+H-H_2O]^+$) and the (chemically) methoxylated parent ($m/z = 776$ a.m.u., $[\{M-OH+OCH_3\}+H]^+$) ions.[25] The methoxylation is caused by the methanol in the eluent. Both ET743S1 and ET743S2 show mass spectra that are not comparable with ET-743.

ET743M1 showed a UV spectrum with one extra maximum at 238 nm in addition to the two known ET UV maxima at 205 and 287 nm (Figure 4A and D and Table 2). ET743M2 showed a UV spectrum almost identical to ET743S1. The spectrum of ET743M3 also corresponds significantly with these spectra, but shows maxima at slightly higher wavelengths (Figure 4B and Table 2). Metabolites ET743M5 and ET743M6 show almost identical UV spectra without any overlaps with ET-743, its degradation products and its other metabolites. Figure 4(C) depicts the almost identical spectra of ET743M5 and ET743M6, the typical wavelength maxima are reported in Table 2.

The two major non-enzymatic degradation products of ET-743 (ET743S1 and ET743S2) were also tested for their accessibility for metabolic reactions by the microsomal system after their chromatographic fractionation and purification. ET743S1 appeared to be stable under these conditions while ET743S2 was converted for approximately 99%. Several ($n \geq 5$) metabolites of ET743S2 were formed of which the two most prominent compounds had the same chromatographic and UV spectroscopic properties as ET743M5 and ET743M6.

Typically, no non-enzymatic degradation was observed for ET-729, the des-methyl analog of ET-743 (Figure 1). The chromatographic pattern of its enzymatically generated metabolites (Figure 3B) was partially comparable to the pattern for ET-743 (Figure 3). The M1 metabolites of both ETs (ET743M1 and ET729M1) show identical chromatographic and spectrophotometric properties (Figure 3 and 4D, and Table 2). ET729M2 and ET729M3 show almost identical UV spectra compared to ET743M2 and ET743M3 (Figure 4B and Table 2). Further, the retention times of the M2 and M3 metabolites are shorter for ET-729 than for ET-

743 (Figure 3). Only ET729M4 had a unique UV spectrum (Figure 4C, Table 2) not comparable with any of the ET-743 related compounds. A small amount of ET-743 was observed in the standard solution of ET-729; however, because the most prominent product of ET-743, ET743S1, was not detectable with UV absorbance detection after the ET-729 incubations, this contamination was neglected.

Plasma incubations

Plasma incubations resulted in the formation two major products of ET-743 (Figure 5). One showed almost identical UV spectra compared to the parent (Figure 4A and Table 2) and its MS spectrum showed a clear analogy with ET-743 with a mass difference of −42 a.m.u. (M_r = 719; Figure 6 and Table 2) corresponding with deacetyl-ET-743 (daET743). After incubation overnight, approximately 50% of the parent compound was recovered. However, the yield of the deacetylated compound, based on UV responses at 254 nm, which were presumed to be equal for both compounds, was about 6%. Based on identical UV spectrometric and chromatographic data, the other major product was found to be ET743S1 (a degradation product also seen in the microsomal experiments). Additionally, a minor product, ET743p1 could only (poorly) be separated from endogenous plasma compounds using isocratic HPLC with 35% methanol in the eluent and was not observed in the chromatogram using gradient B (Figure 5). The UV spectrum shows some overlap with ET743S1 and can be distinguished by the shift of the absorbance maxima to higher wavelengths (Figure 3B and Table 2).

Identical incubations with ET-729 in plasma resulted in the formation of a small amount of a metabolite that has also a UV (Figure 4A) and mass spectrum (Table 2) indicative for a deacetylated product (Figure 5). Additionally, two major products were observed (Figure 5) and, based on chromatographic and spectrophotometric data, identified as ET729M2 and ET729M3, two metabolites already discovered during the microsomal experiments. Finally, a very small peak, ET729p1, could be observed (Figure 5) with a UV spectrum comparable to the ET743p1 spectrum (Figure 4B and Table 2).

Glucuronidation

Glucuronidation experiments with both ET's resulted in the formation of one metabolite for each ET (Figure 7). Both products showed UV spectra almost identical to those of their parent compounds (Figure 4A and Table 2). Furthermore, the corresponding MS spectra revealed a clear analogy with the parent ET compounds with a mass difference of +176 a.m.u. (M_r = 937 for the ET-743 metabolite and M_r = 923 for the ET-729 metabolite; Figure 6 and Table 2). Both products were therefore presumed to be glucuronides of ET-743 (ET743glu) and ET-729 (ET729glu), respectively. Based on the UV responses at 254 nm, which were presumed to be equal for both compounds, approximately 10–15% of ET-743 is glucuronidated after incubation for 5 h under the tested conditions.

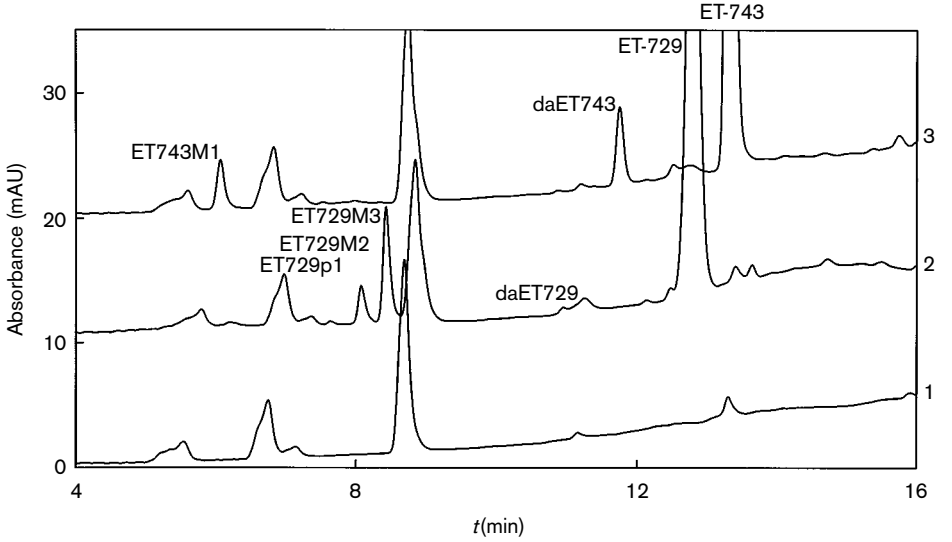

Figure 5. Chromatograms at 254 nm using gradient B of a blank ET-free solution (1), ET-729 (2) and ET-743 (3) after incubation in plasma.

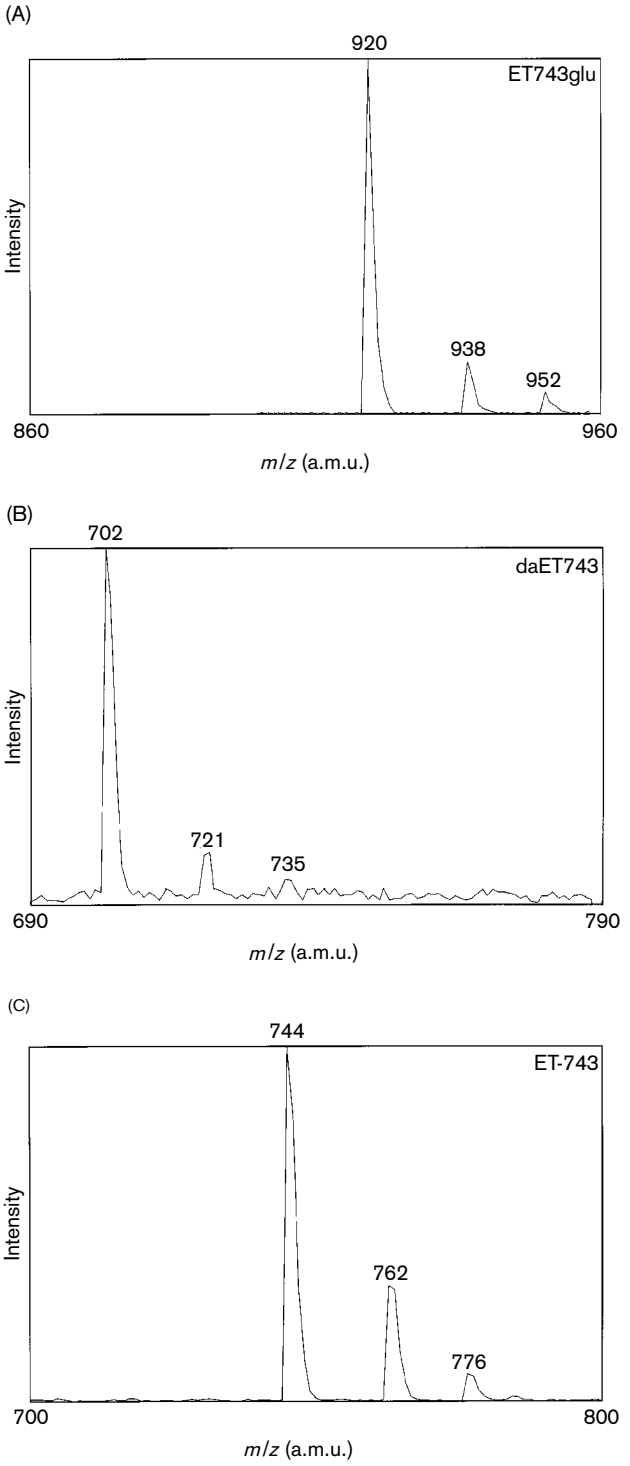

Figure 6. MS spectra (using a single quadrupole) of (A) ET743glu, (B) daET743 and (C) ET-743.

Screening of clinical samples

Prior to the screening of the clinical samples with LC-MS(-MS) and starting with the mass spectrometric settings for ET-743 and ET-729[25] the MS-MS settings were optimized individually for ET743S1, ET743M3, daET743 and ET743glu using continuous infusion of the purified compounds. Only for ET743M3, no high

abundant ion was found, and all urine, bile and plasma samples were therefore only screened for the presence of the three other potential metabolites (ET743S1, daET743 and ET743glu) in addition to ET-743 (Figure 8) and ET-729 using the ion masses listed in Table 3. All these five compounds were observed to be (partially) extracted using chloroform, the glucuronide excluded. No (traces of) ET743S1, daET743, ET743glu and ET-729 were discovered in the clinical samples after both different sample pre-treatment procedures

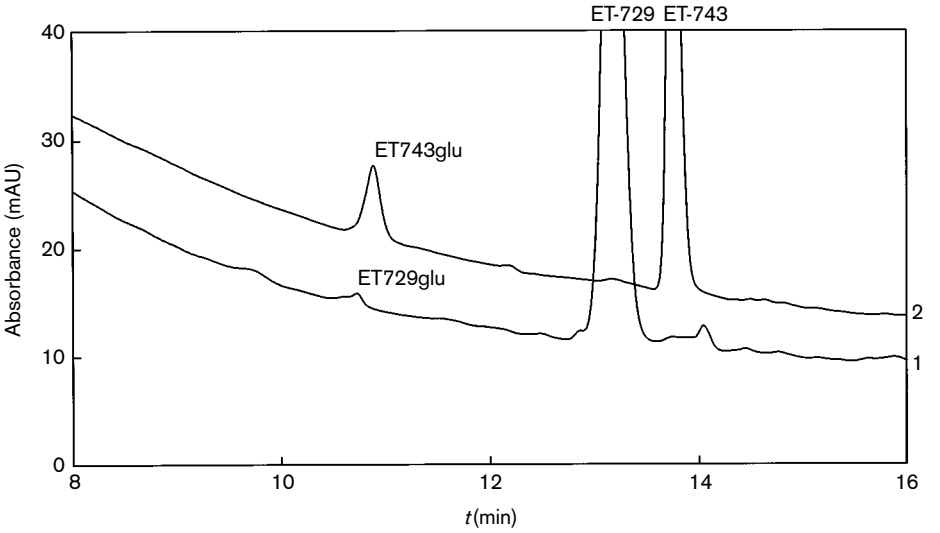

Figure 7. Chromatograms at 254 nm using gradient B of ET-729 (1) and ET-743 (2) after incubation in the glucuronidation system.

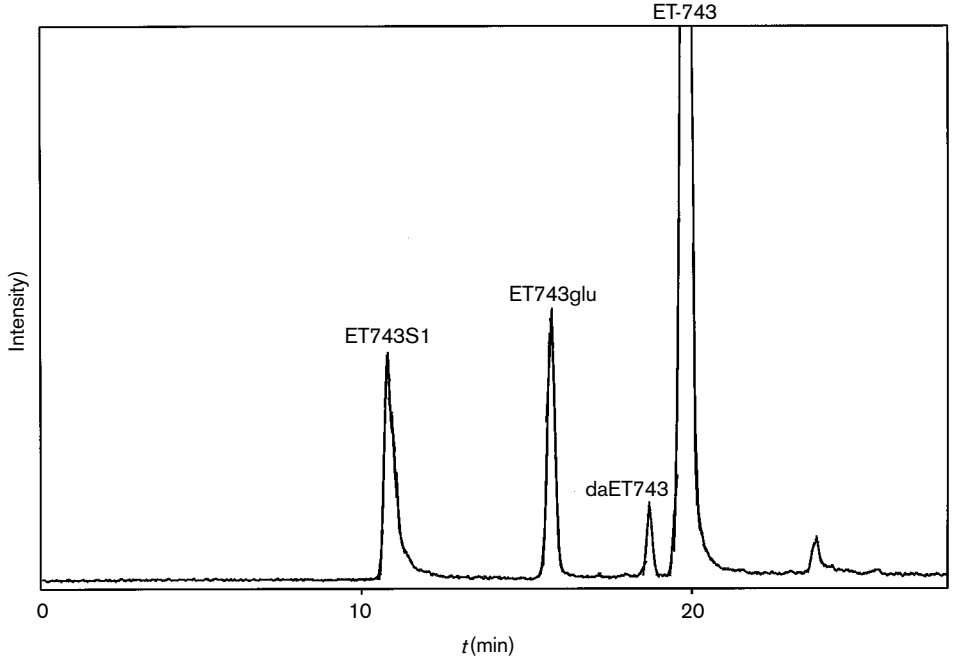

Figure 8. MS-MS chromatogram using gradient B of a standard mixture containing ET743S1, ET743glu, daET743 and ET-743. The response is the sum of the ions listed in Table 3. The retention times in the (low-pressure gradient) LC-MS(-MS) chromatogram are longer compared to the (high-pressure gradient) LC-UV data due to the higher gradient lag from the LC equipment coupled to the mass spectrometer.

Table 3. Masses detected in the first and third quadrupoles, selected for optimum sensitivity, for LC-MS(-MS) of clinical samples using gradient B

Component	Retention time (min)[a]	Mass (*m/z*)	
		First quadrupole	Third quadrupole
ET743S1	11	204	189
ET743glu	16	920	495
ET-729	18	730	479
daET743	19	702	433
ET-743	20	744	495

[a]See remark in the legend of Figure 8.

(chloroform extraction and protein precipitation with methanol).

Quantification of ET-743 in clinical samples

The urinary ET-743 excretion in patients receiving 1650 μg/m^2 in a 3-h infusion ($n = 5$) ranged from 0.14–0.36% (mean $\pm$ SD = 0.24 $\pm$ 0.11%) from the administered dose during the first 24 h. The excretion in patients receiving 1500 μg/m^2 ET-743 in a 24-h infusion ($n = 10$) ranged from 0.03 to 0.61% (0.27 $\pm$ 0.18%) of the total administered dose during the infusion and from 0.00 to 0.19% (0.06 $\pm$ 0.06%) during the 24 h afterwards. The total 48-h urinary excretion ranged from 0.03 to 0.69% (0.32 $\pm$ 0.21%). The complete pharmacokinetic results of ET-743 in plasma for the patients of this 24-h regimen in a phase I study have been reported previously.[22] Concentrations of ET-743 found in the bile samples were 0.09, 0.46 and $<$ 0.06 ng/ml, respectively, during the 24-h infusion, 0.50 $\pm$ 0.07 ng/ml ($n = 4$) during the first hour after infusion, and 0.22 $\pm$ 0.08 ng/ml ($n = 7$) from 1 to 71 h after infusion (here n stands for the number of bile samples from one patient). Remarkably, at 71 h after the end of the infusion 0.24 ng/ml ET-743 still remained present in bile. For the patient with Gilbert's syndrome, the area under the plasma concentration–time curve ($0 \rightarrow \infty$) ($AUC_{0 \rightarrow \infty}$) was 21 h·$\mu$g/l in the first course (900 μg/m^2; 24-h infusion) and 23 h·μg/l in the second course (1200 μg/m^2); the plasma half-life was 49 and 40 h during course 1 and 2, respectively. No dose-limiting toxic effects were observed in this patient during these two courses nor during an additional third course (1500 μg/m^2). Analogous pharmacokinetic data from patients without this syndrome are: $AUC_{0 \rightarrow \infty} = 36 \pm 16$ h·μg/l and plasma half-life = 64 $\pm$ 58 h ($n = 3$) for a first 900 μg/m^2 course and 37 $\pm$ 23 h·μg/l and 26 $\pm$ 12 h ($n = 4$) for a second 1200 μg/m^2 course.[22]

Discussion

The presented investigation was an attempt to discover and identify metabolites of the novel marine anti-cancer agent ET-743. Knowledge on metabolic reactions of drugs is very important to explain toxicological and anti-cancer effects, and drug–drug interactions. Recently, three potential metabolites of ET-743 have been discovered and identified[23] after incubation of ET743 with a human lymphoblast-expressed CYP3A4 isoform. In this study three other enzyme systems were tested, each with their different (metabolic) reaction product(s). We used the presented four-step approach in an attempt to detect *in vitro* discovered metabolites in bodily fluids from patients treated with ET-743 in phase I studies.

Microsomal studies

The enzymatic contribution of the tested liver microsomal proteins to the direct conversion of ET-743 is approximately 50% relative to the total conversion. Two main chemical reaction products are formed during the (microsomal) incubations: ET743S1 and ET743S2. The proposed structure for ET743S1 is shown in Figure 2(A). The MS data of this compound (ETM204) correspond with the patented data published recently.[23] In addition, the spectrophotometric data are in accordance with the assignment of a heteroaromatic ion (2,6dimethyl-8hydroxy-7methyloxyisoquinolinium). Additionally, the structure of ETM305 is the proposed structure for ET743S2 (Figure 2B). The UV spectra of both compounds are identical (Figure 4D).[23] Further, the five peaks in the new MS data of this compound (Table 2) are also observed in patented data published recently.[23] From these five peaks only *m/z* 344 could not be explained.[23]

The remarkable structural breakdown of ET-743 into ET743S1 and ET743S2, a third fragment could not be observed, is remarkable. For example, the ET743S1 ion was also observed during the structure elucidation of ET-743 using fast atom bombardment-MS-MS.[1] We clearly showed that the ETM204 (here: ET743S1) and ETM305 (here: ET743S2) 'metabolites' are formed by spontaneous degradation. However, an enzymatic contribution to its formation, as reported by the original discoverers who did not perform an enzyme-free control experiment, cannot be totally ruled out. Another metabolite of ET743, ETM 775 (Figure 2C),[23] recently discovered in incubations with a human lymphoblast-expressed CYP3A4 isoform has not been observed in this study.

ET743M5 and ET743M6 are undoubtedly metabolites of ET743S2. They are not formed directly from ET-

743. According to the mass spectrometric data, both metabolites (ET743M5 and ET743M6) are probably smaller molecules than their parent compound ET743S2 (Table 2). Their structures have not been elucidated because the observed mass spectra could not be correlated with fragments of their parent molecule.

To explain the mass loss of −46 a.m.u. of ET743S3 compared to ET-743, an ET-like structure for this product would be expected. However, the UV spectrum differs considerably from ET-743 and the −46 a.m.u. mass loss could not be explained.

The spectrophotometric similarity between ET743S1, M2 and M3 indicates the presence of the same isoquinoline ring structure in these compounds. The mass spectrometric data of the enzymatically formed metabolites in the microsomal system (ET743M2 and ET743M3) are rather unclear. The relatively low masses measured for these metabolites indicate cleavage of the ET parent in at least two significant parts. Because the M2 and M3 metabolites of both ET-729 and ET-743 have all corresponding UV spectra and because the retention times of both ET-729 metabolites are shorter compared to their ET-743 analogs, ET743M2 and ET743M3 probably both contain the *N*-methyl group, differentiating ET-743 from ET-729 (Figure 1).

Identical UV spectra and retention times indicate that ET743M1 and ET729M1 are the same compounds.

Plasma incubations

The main reaction product observed in the plasma incubation could be identified as the deacetylated ET-743. The mass spectrum clearly showed a spectrum comparable to ET-743 with a difference of −42 a.m.u. In the secondary spectrum the ion at $m/z = 453$ corresponds with the main fragment of ET-743 in a collision induced mass spectrum at $m/z = 495$.[25] Further, no alternative explanation for the −42 a.m.u. mass loss could be found. Identical UV spectra and a reduction of the retention time of this metabolite compared to ET-743 also support the proposed deacetyl structure. Additionally, the deacetylated product of ET-729 and the ET-729 parent compound also show identical UV spectra and the same mass difference in the primary MS spectra. The poor chromatographic resolution and low yield of both ET743p1 and ET729p1 from endogenous plasma compounds hindered elucidation of these metabolites. Typically, the formation of other metabolites (ET743S1, ET729M2 and ET729M3) in plasma, already observed after the microsomal incubations, is different for both ETs during plasma incubations.

Glucuronidation

The molecular masses of the glucuronidated products of both ET-743 and ET-729 could be found because their mass spectra were similar to the primary mass spectra of the leading compounds, showing a mass shift of +176 a.m.u. The product ion spectrum of the glucuronidated ET-743 was almost identical to the ET-743 spectrum, probably because of the loss of the glucuronide in the second quadrupole. Comparison of retention times and UV spectra of this metabolite with ET-743 also supports the proposed glucuronide formation; because both glucuronides show a comparable small deviation in their UV spectra compared to the (identical) spectra of the parent compounds. The site of the ET molecules for glucuronidation has still to be elucidated. Since *O*-glucuronidation is more likely than *N*-glucuronidation and the phenolic functions are the most reactive, glucuronic acid conjugation will probably take place at one of the phenolic hydroxyl groups of ET-743 (Figure 1).

Clinical samples

A significant difference was observed between the observed metabolite profile *in vitro* in the three investigated incubations and the profile in bodily fluids of patients. In the urine, bile and plasma samples ET743S1, daET743, ET743glu and ET-729 could not be detected. The outlined approach, *in vitro* synthesis of the potential metabolites, followed by optimization of the selective and sensitive MS-MS detection and attempts to detect these compounds in clinical samples, is in our opinion the most adequate method to discover metabolites of ET-743 *in vivo*. However, concentrations of metabolites *in vivo* may very well be extremely low and unidentifiable. These low concentrations result from the low ET-743 dose and concentrations (maximum concentration in plasma is approximately 2 ng/ml for a 24-h infusion and 20 ng/ml for a 3-h infusion). Additionally, conversion rates can be slow, like in the *in vitro* experiments, and volumes of distribution can be large as has also been observed for the parent compound. Another complication is that the metabolism in the investigated patients can significantly differ from the metabolism in the *in vitro* experiments due to the high ratio (above 40 000) of the ET-743 concentrations in both situations. As shown now, the concentration of ET-743 in bile can be very low (less than 1 ng/ml) compared to the microsomal experiments (40 μg/ml). Finally, the possibility of the formation of other metabolites also remains and the mass balance of ET-743 in human has not yet been elucidated.

Individuals showing Gilbert's syndrome have a reduced (about 30% of normal[26,27]) hepatic bilirubin UDPGT activity (UGT1A1 measured from liver tissue). Because no elevated AUC or half-life and no toxic effects were observed in the presented patient with this syndrome, an indication was obtained that glucuronidation is not a significant route of detoxification in ET-743 therapy.

In summary, several potential metabolites of ET-743 have been discovered *in vitro*. Four compounds, 2,6-dimethyl-8-hydroxy-7-methyloxyisoquinolinium ion (ET743S1), a second fragment of ET-743 (ET743S2), deacetyl-ET-743 and a glucuronide of ET-743 could be identified. Using LC-MS(-MS), ET743S1, daET743, ET743glu and ET-729 could not be observed in urine, serum or bile samples from patients treated with ET-743 i.v. Further investigations in our institute will incorporate mass balance studies in patients using ^{14}C-labeled ET-743.

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(Received 15 May 2001; accepted 16 June 2001)