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Stereoselective measurement of *E*- and *Z*-doxepin and its *N*-desmethyl and hydroxylated metabolites by gas chromatography–mass spectrometry

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Abstract

A stereoselective method of analysis of the antidepressant drug doxepin (DOX, an 85:15% mixture of *E*–*Z* stereoisomers), its principal metabolites *E*- and *Z*-*N*-desmethyldoxepin (desDOX) and ring-hydroxylated metabolites in microsomal incubation mixtures is described. DOX and its metabolites were extracted from alkalinised incubation mixtures by either: 9:1 hexane–propan-2-ol (method 1) or 1:1 hexane–dichloromethane (method 2), derivatised with trifluoroacetic anhydride and analysed by GC–MS with selected ion monitoring. Both methods were suitable for the analysis of individual desDOX isomers as indicated by correlation coefficients of $\geq$0.999 for calibration curves constructed between 50 and 2500 n*M*, and good within-day precision at 125 n*M* (C.V. $\leq$14%) and 1000 n*M* (C.V. $\leq$8%). Method 1, however, was unsuitable for the analysis of ring-hydroxylated metabolites of DOX, whereas the hydroxylated metabolites of *E*-DOX and *E*-desDOX (generated in situ) were extracted by method 2 with a C.V. of ca. 13%. This is the first assay method that permits the simultaneous measurement of desDOX and hydroxylated metabolites of DOX in microsomal mixtures.

Keywords: Doxepin; Desmethyldoxepin

1. Introduction

Doxepin (DOX) hydrochloride is a widely used tricyclic antidepressant that is administered as an 85:15% mixture of the *E*–*Z* stereoisomers. Its principal oxidative metabolites in human plasma and urine are shown in Fig. 1; DOX can be *N*-demethylated to form *N*-desmethyldoxepin (desDOX), and ring-hydroxylated forms of DOX and desDOX are also formed. The *E*- and *Z*-isomers of DOX have different potencies in the central nervous system; in all animal models of depression *Z*-DOX is more active than *E*-DOX [1]. The metabolite desDOX is also pharmacologically active and has greater sedative properties than the parent drug [1]. DOX and desDOX comprise only approximately 5% of the products excreted in urine [2], and other urinary metabolites are thought to be glucuronide conjugates of hydroxy DOX and desDOX [3], although these have yet to be quantitatively measured. An effective assay, therefore, for the measurement of DOX metabolism

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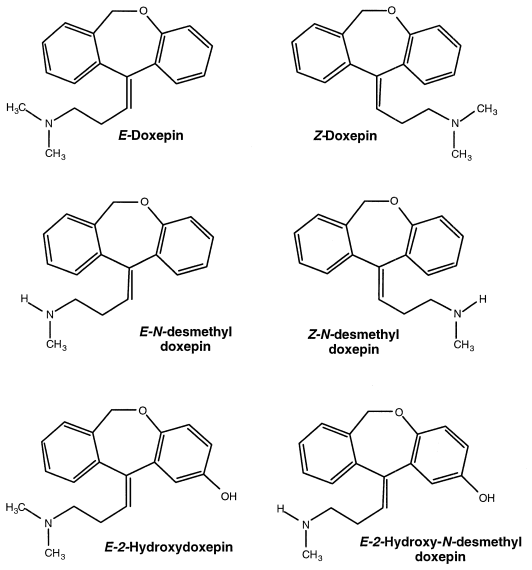

Fig. 1. Structures of *E*- and *Z*-doxepin (DOX) and *N*-desmethylated metabolites. Also shown are the *E*-forms of 2-hydroxydoxepin and 2-hydroxy-*N*-desmethyldoxepin as reported by Shu et al. [3].

in vitro or in vivo, should ideally resolve the isomers of the parent compound, its *N*-demethylated metabolites and ring-hydroxylated metabolites.

Stereoselective methods for DOX isomers utilising HPLC have recently been reported. Yan et al. [4] described the measurement of low plasma concentrations of DOX and desDOX using normal-phase HPLC, although the method was found to be very sensitive to minor variations in proportions of mobile-phase solvents. Another method developed by Adamcyzk et al. [5] employs a novel mobile phase comprised of phosphate buffer, acetonitrile and *n*-nonylamine as a dynamic coating agent for the silica stationary phase. That method, however, requires a lengthy 12 h equilibration time for the column prior to analysis. Both assays require relatively labour-intensive multiple extraction and washing steps for DOX and desDOX analysis in plasma. Shu et al. [3] have reported the only method in which hydroxylated metabolites of DOX have been examined by stereoselective HPLC. The metabolites of DOX were extracted from urine and identified by LC–MS, however the assay was not designed for quantitative determination of DOX metabolism.

GC-based assay methods have also been applied to the stereoselective measurement of DOX and its *N*-demethylated metabolites. Rosseel et al. [6] reported a method in which plasma samples from patients receiving DOX are solvent extracted and the *E*- and *Z*-isomers of DOX and desDOX are measured by GC with nitrogen–phosphorous detection. Midha et al. [7] measured low plasma concentrations of pentafluorobenzoyl derivatives of *E*- and *Z*-desDOX isomers by GC with electron capture detection (ECD), but measurement of the plasma concentrations of the parent drug requires an additional, separate assay by normal-phase HPLC. Ghabrial et al. [2] developed a GC–MS method with superior selectivity using positive-ion chemical ionisation, to assay plasma and urinary DOX and desDOX isomer concentrations. These GC assays have largely been developed for the investigation of DOX in clinical pharmacokinetic studies.

In the present study, we describe a GC–MS method that provides simultaneous stereoselective analysis of DOX, desDOX and its ring-hydroxylated metabolites, and uses a simple extraction method. We demonstrate its application to the measurement of DOX metabolism in an in vitro microsomal system.

2. Experimental

2.1. Chemicals

Trifluoroacetic anhydride (TFAA) was obtained from Alltech (Deerfield, IL, USA) and nortriptyline hydrochloride was obtained from Sigma (St. Louis, MO, USA). The separate stereoisomers of the hydrochloride salts of *E*-doxepin (purity 96.6%), *Z*-doxepin (96.7%) and *E*-*N*-desmethyldoxepin (97.7%) were gifts from Pfizer (Croton, CT, USA) and *Z*-*N*-desmethyldoxepin hydrochloride (purity not stated) was a gift from Dr. Maciej Adamczyk of Abbott Laboratories (Abbott Park, IL, USA). All solvents were of analytical grade and used without further processing. Glass test tubes (10 mL) and GC injection port liners were soaked in 10% dichloromethylsilane (Alltech) in hexane overnight, rinsed in hexane, soaked in methanol for 1 h, and oven dried prior to use. Glucose 6-phosphate dehydrogenase and the disodium salts of glucose 6-phosphate and nicotinamide adenine dinucleotide phos-

phate (NADP) were obtained from Boehringer-Mannheim (Germany).

2.2. Sample preparation — method 1

Immediately after microsomal incubation (vide infra), samples (0.5 mL) were alkalinised with 125 μL of saturated Na_2CO_3 solution and placed on ice to minimise degradation of DOX metabolites. Internal standard nortriptyline (0.15 μg, 0.5 nmol) was added and samples were extracted with 9:1 hexane–propan-2-ol (two times 2 ml) followed by vortex mixing for 2 min. The two phases were separated by centrifugation at 1500 *g* for 5 min. The organic layers were combined and evaporated, not quite to absolute dryness, by a gentle nitrogen stream at 60°C in a heating block (Ratek Instruments, Melbourne, Australia). The residue was taken up in 0.8 mL of hexane with quick vortex mixing, transferred to a 1 mL amber vial and derivatised with 50 μL trifluoroacetic anhydride (TFAA) at 60°C for 60 min. Samples were gently evaporated, not quite to absolute dryness, by a gentle nitrogen stream to remove excess derivatising agent and then redissolved in isooctane (0.8 mL) for analysis by GC–MS with selected ion monitoring.

2.3. Sample preparation — method 2

Immediately after microsomal incubation (vide infra), samples (0.5 mL) were alkalinised with 125 μL of saturated Na_2CO_3 solution and placed on ice. Internal standard nortriptyline (0.15 μg, 0.5 nmol) was added and samples were extracted with 1:1 hexane–dichloromethane (two times 2 mL) followed by vortex mixing for 2 min. The two phases were separated by centrifugation at 1500 *g* for 5 min. The organic layers were combined and evaporated, not quite to absolute dryness, by a gentle nitrogen stream at 60°C in a heating block. The residue was taken up in 0.8 mL of dry ethyl acetate with quick vortex mixing, transferred to a 1 mL amber vial and derivatised with 50 μL of TFAA at 60°C for 60 min. Samples were gently evaporated, not quite to absolute dryness, by a gentle nitrogen stream to remove excess derivatising agent and then redissolved in dry ethyl acetate (0.8 mL) for analysis by GC–MS with selected ion monitoring.

2.4. Calibration standards and assay validation

Stock solutions containing each stereoisomer of DOX HCl at 1.25, 2.5, 5.0, 10, 25 and 50 μ*M* were prepared in 1.15% KCl solution, and desDOX HCl at 0.5, 1.25, 2.5, 5.0, 10 and 25 μ*M* were prepared in 1.15% KCl solution, and stored at −20°C. Calibration curves for *E*- and *Z*-DOX were prepared by dilution of the stock solutions in denatured microsomal mixtures to give final concentrations of 125, 250, 500, 1000, 2500 and 5000 n*M* for each isomer, and calibration curves were also prepared for *E*- and *Z*-desDOX to give final concentrations of 50, 125, 250, 500, 1000 and 2500 n*M* for each isomer. Denatured incubation mixtures consisted of 0.1 *M* sodium/potassium phosphate buffer pH 7.4 and boiled rat liver microsomes (ca. 0.5 mg protein) in a final volume of 0.5 mL. Samples were processed as described above and quantitation of DOX and metabolites was attained by plotting concentration versus peak area units. Calibration curves were fitted to a two-stage quadratic equation.

Using extraction method 1, within-day precision and accuracy of the assay was assessed at 125 and 1000 n*M* of *E*- and *Z*-desDOX. Using extraction method 2, within-day precision and accuracy of the assay was assessed at 250 n*M* of *E*- and *Z*-desDOX, and at 1000 n*M* of *E*- and *Z*-DOX. The between-day variability for method 2 was also measured by calculating the recovery of *E*- and *Z*-desDOX at 250 n*M* over 4 sampling days during a 5 month period.

2.5. Gas chromatography–mass spectrometry

Sample extracts were injected (2 μL) into a Shimadzu QP-2000 gas chromatograph–mass spectrometer (GC–MS, Kyoto, Japan) equipped with a Shimadzu AOC-1400 autosampler and 30 m×0.25 mm column coated with 0.25 μm phase DB-17 (J&W Scientific, CA, USA). Splitless injections were made with a valve wait time of 1 min. The carrier gas was ultra-high-purity helium (Linde Gases, Victoria, Australia) at a linear velocity of 35 cm s^{-1}. The temperature program for the GC–MS was as follows: initial oven temperature 150°C held for 1 min increasing to 270°C at 15°C per min and held for 7 min, with the injection port, interface and ion

source maintained at 270°C. The mass spectrometer was tuned with the ion source temperature at 270°C.

Quantitation of DOX and metabolites was achieved by selected ion monitoring (SIM); the ion pairs monitored (quantitation ion in bold) and retention times of components were: TFA-derivatised *E*-OHDOX (**58.0**, 391.2; 9.70 min), *Z*-DOX (**58.0**, 279.2; 10.60 min), *E*-DOX (**58.0**, 279.2; 10.75 min), diTFA-derivatised *E*-OHdesDOX (**346.2**, 233.2; 11.90 min), TFA-derivatised nortriptyline (**232.2**, 219.2; 12.40 min), TFA-derivatised *Z*-desDOX (**234.2**, 219.2; 12.81 min) and TFA-derivatised *E*-desDOX (**234.2**, 219.2; 13.20 min). Positive ion mass spectra were acquired at 70 eV between 50 and 600 atomic mass units (amu) at 1 s per decade. The mass spectral ions and abundances for all substrates and metabolites monitored for this work were acquired using the Shimadzu QP-2000 GC–MS.

2.6. Metabolism of doxepin in human liver microsomes

The sources of human liver samples, preparation of microsomes and determination of cytochrome P450 content have been previously described [8]. Microsomal incubations (0.5 mL reactions) were carried out in a shaking water bath at 37°C and comprised 0.1 *M* sodium/potassium phosphate buffer pH 7.4 human liver microsomes (0.12 to 0.30 nmol total P450) and an NADPH-regenerating system consisting of 2.5 m*M* $MgCl_2$, 50 m*M* KCl, 1.5 m*M* glucose 6-phosphate, 0.3 m*M* $NADP^+$ and 2.5 IU glucose 6-phosphate dehydrogenase. After a preincubation of 2.5 min, reactions were initiated by the addition of *E*-DOX, *E*-desDOX, *Z*-DOX or *Z*-desDOX (each 10 μ*M* and examined separately) in 1.15% KCl, and were incubated for 15 min. Reactions were terminated by the addition of 125 μL of saturated Na_2CO_3 solution with vortex mixing and placement of the tubes on ice. Control incubations were also performed where incubation mixtures contained all but the NADPH regenerating system. The C.V. of formation of *E*-OHDOX from *E*-DOX, *E*-OHdesDOX from *E*-desDOX, and *Z*-desDOX from *Z*-DOX was determined in six replicate microsomal incubations.

3. Results and discussion

3.1. Chromatography and choice of internal standard

As shown in the total ion chromatogram (Fig. 2) there was complete separation of *E*- and *Z*-isomers of DOX and desDOX, and the internal standard nortriptyline, with the GC conditions described. Amitriptyline (retention time 10.18 min) was also found to be well separated in the chromatogram (data not shown) and could therefore serve as an alternative internal standard. Nortriptyline, however, was selected as the internal standard on the basis of its close structural similarity with the metabolites *E*- and *Z*-desDOX, thus undergoing TFAA derivatisation on the basic nitrogen in a similar fashion to desDOX. Nortriptyline therefore has the advantage as an indicator of the efficiency of derivatisation.

3.2. Calibration curves, precision and accuracy: method 1 vs. method 2

We compared two liquid–liquid extraction methods for recovery of DOX and its metabolites from alkalinised microsomal samples. Hexane and heptane, together with a small percentage of an aliphatic alcohol such as propan-2-ol, have been the commonly used solvent mixtures for extraction of DOX and

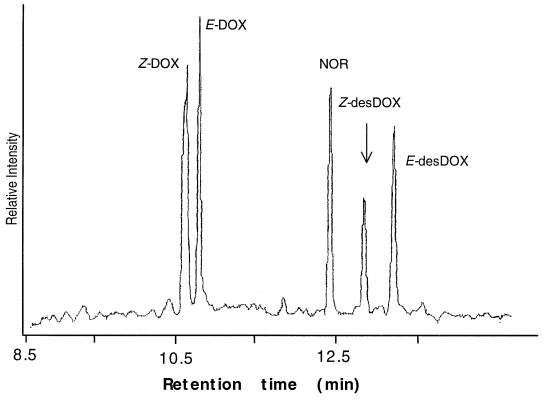

Fig. 2. Total ion chromatogram obtained between 50 and 600 amu of *Z*-DOX, *E*-DOX, and the TFA derivatives of internal standard nortriptyline (NOR), *Z*-desDOX and *E*-desDOX each added to microsomal incubation mixtures at approximately 10 μg mL^{-1}.

Table 1
Mean calibration curve parameters of the equation $y = ax^2 + bx + c$ and correlation coefficients obtained for desDOX stereoisomers extracted from denatured rat liver microsomal incubation mixtures between 0.05 and 2.5 μ*M* and for DOX isomers between 0.125 and 5 μ*M*[a]

	Standard	a ($\times 10^{-6}$)	b	c	r^2
Method 1	*E*-desDOX	5.2	0.564	−18.7	0.9990
	Z-desDOX	6.4	0.474	−11.3	0.9990
Method 2	*E*-DOX	40	2.660	171	0.9990
	E-desDOX	32	0.315	−4.9	0.9996
	Z-DOX	−3	1.802	−28.5	0.9996
	Z-desDOX	31	0.446	−6.7	0.9990

[a] Mean of at least two independent curves for desDOX isomers, single determination for DOX isomers.

its metabolites from plasma [4–6,9–11]. In keeping with this, in method 1, a 9:1 hexane–propan-2-ol mix was used with reconstitution of evaporated samples in *iso*octane. On the other hand, in method 2, we employed solvents of higher polarity and used a 1:1 *hexane–dichloromethane* mix for extraction and *ethyl acetate* for redissolving samples after evaporation. Mean two-stage quadratic equations for extracted *E*- and *Z*-desDOX are compared for methods 1 and 2 in Table 1. *E*- and *Z*-DOX were successfully extracted from denatured microsomal mixtures using method 2 over the concentration range 0.125–5 μ*M* (Table 1). The correlation coefficients obtained for all calibration curves were ≥ 0.999 and the use of two-stage equations allowed superior fit to the data than linear equations due to slight curvature at the highest concentrations. The *a* and *b* coefficients for the calibration curves of *E*- and *Z*-desDOX were similar for methods 1 and 2, suggesting the two extraction methods were equivalent with respect to the recoveries of DOX and desDOX.

The within-day assay precision was good at 125 and 1000 n*M* for *E*- and *Z*-desDOX when extraction method 1 was used, and also good for *E*- and *Z*-DOX and the *N*-demethylated metabolites when method 2 was used (Table 2). Although both methods were very accurate they tended to underestimate the quantity of desDOX in the sample. The assay variability for desDOX isomers is similar to that reported by Yan et al. [4] and Adamczyk et al. [5]. There was little variation in the assay over an approximate 5 month time period of analysis; the between-day C.V. of *E*- and *Z*-desDOX measurements at 250 n*M* was 5.3 and 5.5%, respectively, in four independent measurements.

3.3. Hydroxylated metabolites of DOX

Extraction method 1 was initially developed to analyse DOX and its *N*-demethylated metabolites, however this method was subsequently found to be unsuitable for the hydroxylated metabolites. Method 2, using hexane–dichloromethane as the extraction solvent and ethyl acetate to redissolve the metabolites after evaporation, permitted the hydroxylated metabolites to be analysed. The total ion chromatogram of an extracted human liver microsomal incubation with *E*-DOX (100 μ*M*) shows the formation of the putative hydroxylated metabolite *E*-OHDOX

Table 2
Within-day determination of doxepin and metabolites using extraction methods 1 and 2

	Standard	Added conc. (n*M*)	Determined conc. (n*M*) mean±SD	CV (%)	Bias (%)	*n*
Method 1	*E*-desDOX	125	115±16	14.4	−8.0	8
	E-desDOX	1000	983±59	6.0	−1.7	7
	Z-desDOX	125	129±14	11.0	3.2	8
	Z-desDOX	1000	971±77	8.0	−2.9	7
Method 2	*E*-DOX	1000	1377±95	6.9	37.7	6
	E-desDOX	250	225±13	5.6	−10.0	6
	Z-DOX	1000	1054±59	5.6	5.4	6
	Z-desDOX	250	227±11	5.1	−9.2	6

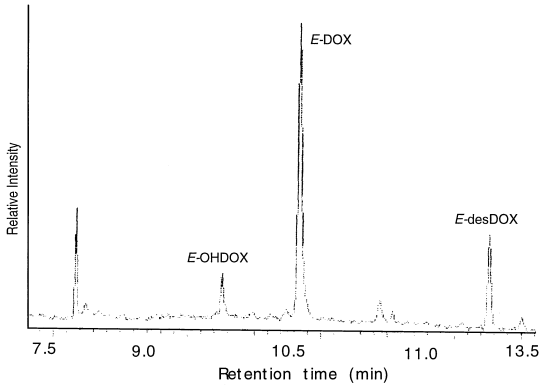

Fig. 3. Total ion chromatogram of extracted microsomal mixture containing *E*-DOX (100 μ*M*) as the substrate, human liver microsomes and a NADPH-regenerating system. The formation of metabolites *E*-OHDOX (9.76 min) and *E*-desDOX (13.20 min) is indicated.

in addition to the *N*-demethylated metabolite *E*-desDOX (Fig. 3). The *E*-OHDOX peak retention time is well separated from DOX and desDOX stereoisomers. The low-polarity extraction and redissolving solvents used in method 1 were much less effective for the hydroxylated metabolites of DOX and yielded highly erratic recovery of the hydroxylated metabolites. This suggests that the hydroxylated DOX metabolites appear to have limited solubility in non-polar solvents such as hexane and *iso*octane. It is very likely that the hydroxylated metabolites of DOX have been overlooked by most other published methods of DOX analysis as these methods mainly employ solvent-extraction steps that use non-polar solvents such as pentane, hexane or heptane [4–6,9–11].

In this study, the hydroxylated metabolites of *E*-DOX and *E*-desDOX were identified from the full-scan mass spectra and retention times obtained from TFAA-derivatised extracts of incubations with human liver microsomes. No metabolites were detected in control incubations, i.e. in the absence of NADPH, and the metabolite peaks did not elute at the same retention times of co-extractives in the incubation mixtures. The mass spectrum of TFAA-derivatised *E*-OHDOX (Fig. 4) is very similar to that of *E*-DOX as the molecular ion is not visible and the sole peak of any significance in the spectrum is *m*/*z* 58, corresponding to the facile loss of $(CH_3)_2NCH_2^+$ from the molecular ion. Unfortunately, there are no

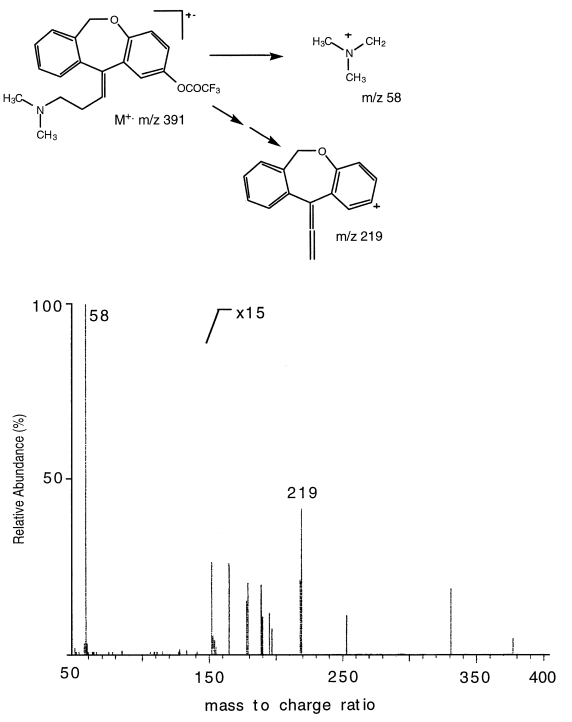

Fig. 4. Mass spectrum (70 eV EI) of TFA-derivatised *E*-OHDOX between 50 and 600 amu (50–400 amu displayed). The metabolite was extracted from a mixture containing human liver microsomes, *E*-DOX (100 μ*M*) as the substrate, and NADPH. The portion of the spectrum above *m*/*z* 150 has been magnified by 15-fold as indicated. The rationale for the fragmentation pattern is shown above the spectrum.

authentic standards of OHDOX and OHdesDOX available to compare retention time and mass spectra and thereby enable unequivocal identification of the putative hydroxylated DOX metabolites in our microsomal studies.

Although the mass spectrum of the acetate derivative of hydroxylated DOX has been reported, it was acquired under different mass spectral conditions. Maurer and Pfeger [12] reported the mass spectrum of an acetate derivative of OHDOX (stereoisomer not specified) that was extracted from human urine. The main mass spectral ions reported were *m*/*z* 337 (10%, $M^{+\cdot}$), 257 (10%), 202 (5%) and 58 (100%). Most of these mass spectral ions are different to the mass spectral ions in our study since Maurer and Pfeger [12] used different derivatising agents, although the *m*/*z* 58 ion is the major ion in both studies.

The mass spectrum (Fig. 5) of *E*-OHdesDOX (which was more complex than that of *E*-OHDOX) assisted its structural elucidation. Maurer and Pfeger [12] reported the major ions for the diacetate derivative of OHdesDOX (stereoisomer not specified) extracted from human urine as m/z 365 (25%, $M^{+\cdot}$), 292 (60%), 250 (70%), 237 (42%), 233 (60%), 99 (21%) and 86 (100%). Other than m/z 233, our mass spectrum and that of Maurer and Pfeger [12] differ due to the different derivatising agents used. The postulated molecule corresponding to m/z 233 contains no TFA derivative (Fig. 5) and therefore it is expected that this ion appears in both spectra.

A measure of the variability of method 2 for determining hydroxylated metabolites of DOX and desDOX was examined using replicate microsomal incubations. The amount of *E*-OHDOX metabolite generated in replicate human liver microsomal incubations with *E*-DOX (at 10 μM) as initial substrate over 15 min was extracted with a within-assay C.V. of 13.8% ($n = 6$). The amount of metabolite generated in each incubation was expressed as area units arising from the peak at 9.7 min. Similarly, *E*-OHdesDOX, retention time 11.90 min, was extracted from replicate microsomal incubations containing *E*-desDOX (10 μM) as initial substrate; the within-assay C.V. was 13.5% ($n = 6$). This demonstrates that method 2 is suitable for the measurement of hydroxylated metabolites generated in microsomal incubations conducted at low substrate concentrations.

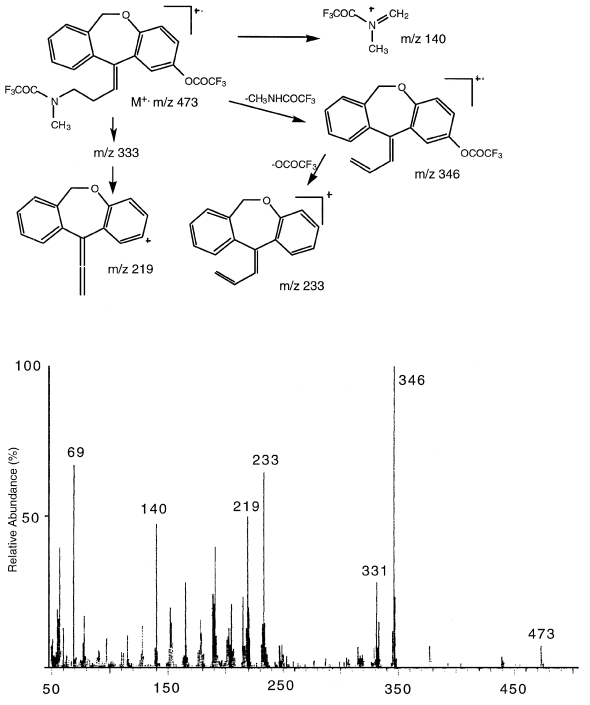

Fig. 5. Mass spectrum (70 eV EI) of the di-TFA derivative of *E*-OHdesDOX acquired between 50 and 600 amu (50–500 amu displayed). The metabolite was extracted from human liver microsomal mixtures containing *E*-desDOX (100 μM) and NADPH. The rationalisation of the fragmentation pattern observed in the mass spectrum is shown above.

An unusual finding of our work is that we could not detect the production of *Z*-hydroxylated metabolites from *Z*-DOX or *Z*-desDOX. This means either the rate of microsomal *Z*-hydroxylation is below the detection limit under the conditions we have employed or our human liver microsome samples for reasons unknown are unable to hydroxylate the *Z*-forms. Consistent with our observations is that no other study to date has reported the production of *Z*-hydroxylated metabolites of DOX.

4. Conclusions

A GC–MS-based method was established and validated for the stereoselective measurement of DOX and its *N*-desmethyl and hydroxylated metabolites. An assay method (method 2) was shown to be suitable for the simultaneous measurement of desDOX stereoisomers, and the hydroxylated metabolites of DOX and desDOX generated in situ in microsomal incubations. The mass spectra of the TFA-derivatised hydroxy metabolites were rationalised for structural information and the major ions were used in selected ion monitoring. The C.V. of analysis of DOX, desDOX and generated *E*-hydroxy metabolites was reasonable (ca. 13%) and demonstrates that the assay may be applied to the kinetic study of these metabolites in vitro.

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