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Determination of doxepin and desmethyldoxepin in human plasma using liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive method for the simultaneous determination of doxepin and its active metabolite desmethyldoxepin in plasma was established, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The samples were extracted with hexane–isoamyl alcohol, separated on a Phenomenex Luna C_{18} 5 μm, 150×2.1 mm column with a mobile phase consisting of methanol–water–formic acid (600:400:0.5, v/v) at a flow-rate of 0.25 ml/min. Detection was achieved by a Perkin-Elmer API 2000 mass spectrometer at unit resolution in multiple reaction monitoring mode monitoring the transition of the protonated molecular ions *m/z* 280.2, 266.2 and 250.1 to the product ions *m/z* 107.1, 107.1 and 191.0 for analyte, metabolite and internal standard (benzoctamine-HCl), respectively. TurboIonSpray ionisation was used for ion production. The mean recovery for doxepin and desmethyldoxepin was 90% and 75%, respectively, with a lower limit of quantification at 0.320 ng/ml and 0.178 ng/ml for the analyte and its metabolite, respectively, using 0.5 ml plasma for extraction. This is the first assay method described for the simultaneous determination of doxepin and desmethyldoxepin in plasma using LC–MS–MS. The method is sensitive enough to be used in drug bioavailablity studies with doxepin.

Keywords: Doxepin; Desmethyldoxepin

1. Introduction

Doxepin hydrochloride is a mixture of geometric *cis*- and *trans*-isomers (usually in a *cis*:*trans* ratio of 15:85) of 3-{dibenz[*b*,*e*]oxepin-11(6H)ylidene}-*N*,*N*-dimethylpropylamine hydrochloride [1,2] (Fig. 1). Doxepin is a member of the tricyclic antidepressants. Like many other tricyclic antidepressants, doxepin provides effective treatment for depression and it also has anti-anxiety and anti-histamine properties [2,3].

Doxepin is readily absorbed from the gastro-intestinal tract, and is extensively demethylated by first-pass metabolism in the liver to its primary active metabolite desmethyldoxepin (Fig. 1). Doxepin and desmethyldoxepin are widely distributed throughout the body (9–33 l/kg) and are extensively bound to the plasma and tissue protein. Doxepin has been established to have a plasma half-life ranging from 8 to 24 h, while the half-life of desmethyldoxepin is longer [4].

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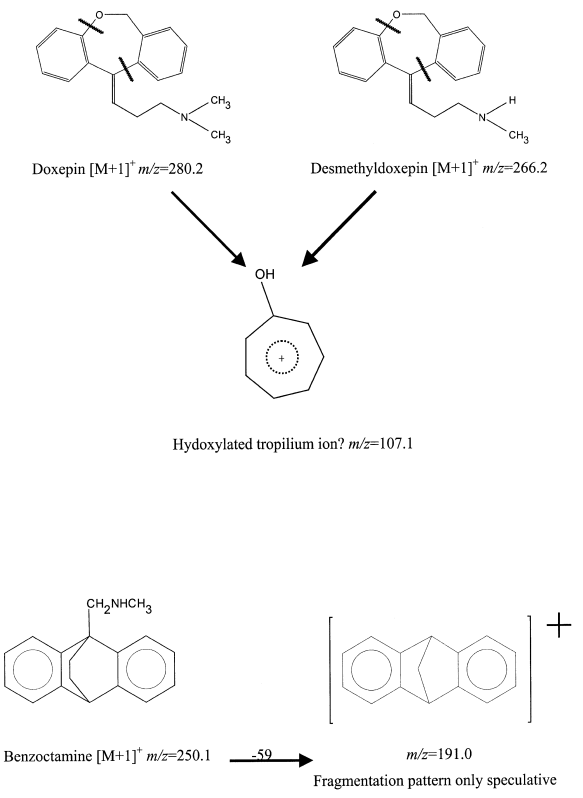

Fig. 1. Molecular structures and MS fragmentation of doxepin, desmethyldoxepin and benzoctamine internal standard to their product ions.

In the treatment of depression, doxepin is usually given orally as the hydrochloride in doses equivalent to 25 mg doxepin, although doses of up to 300 mg daily may be required [2]. The dosage in this study consisted of a single oral dose of 100 mg doxepin per treatment phase.

Most references centred on conventional high-performance liquid chromatography (HPLC) methods for the determination of doxepin and desmethyldoxepin in plasma. Many of these studies involved the simultaneous determination of doxepin and desmethyldoxepin with other antidepressants in human serum such as alprazolam [5], fluoxetine, amitriptyline, imipramine as well as their active metabolites norfluoxetine, nortriptyline and desipramine [6]. Different detection modes were employed which include cathodic-stripping voltammetry [7], UV detection at 254 nm [5] and 220 nm [6]. Capillary gas chromatography with nitrogen-selective detection also proved successful [8]. To construct a pharmacokinetic profile after a single oral dose of doxepin a sensitive procedure in the lower ng/ml levels in biological fluids was developed which involved the use of a mass-selective detector with tandem mass spectrometry (MS–MS) capabilities in tandem with liquid chromatography (LC) using a Perkin-Elmer API 2000 MS–MS detector.

2. Experimental

2.1. Materials and chemicals

A Phenomenex Luna C_{18} 5 μm, 150×2.1 mm column (Phenomenex, Torrance, CA, USA) was used for separation at a flow-rate of 0.25 ml/min and injecting 10 μl onto the column. The mobile phase was delivered by a Hewlett-Packard Series 1050 pump and the samples injected by a Hewlett-Packard Series 1100 autosampler (Hewlett-Packard, Palo Alto, CA, USA). Detection was performed by a Perkin-Elmer Sciex API-2000 detector (Perkin-Elmer Sciex, Ontario, Canada) using TurboIonSpray ionisation (ESI) for ion production.

Isoamyl alcohol (Pro-Analysi grade) was obtained from Merck (Darmstadt, Germany); formic acid (high purity) from BDH (Poole, UK); hexane and methanol (Burdick and Jackson, high purity) were obtained from Baxter (USA), and sodium hydroxide (analytical-reagent grade) from Fluka (Buchs, Switzerland). All chemicals were used as received. Water was purified by RO 20SA reverse osmosis and a Milli-Q polishing system (Millipore, Bedford, MA, USA).

Doxepin-HCl, $C_{19}H_{21}NO$-HCl, was supplied by Sifavitor and desmethyldoxepin-HCl, $C_{18}H_{19}NO$-HCl, was obtained from Teva Pharmaceutical Industries. Benzoctamine-HCl internal standard was taken from the FARMOVS Research Centre internal pure substance reference material library (Fig. 1).

2.2. Extraction procedure

Doxepin and desmethyldoxepin standard solutions were made up in methanol and used immediately to spike plasma and discarded thereafter. Calibration standards and quality control standards were pre-

pared in normal human plasma by spiking a pool of normal plasma which was then serially diluted with normal blank plasma to attain the desired concentrations (0.32–81.1 ng/ml for doxepin and 0.18–45.1 ng/ml for desmethyldoxepin). The calibration standards and quality control standards were aliquoted into tubes and stored under the same conditions as the trial samples; approximately −20°C in the same freezer as the subjects' samples.

To 0.5 ml plasma in a 10-ml amber glass ampoule was added 50 μl benzoctamine internal standard solution (9.52 ng/ml in methanol), 250 μl of 2 *M* sodium hydroxide and 3 ml of a 2% solution of isoamyl alcohol in hexane. The sample was vortex-mixed for 2 min and centrifuged at 3300 *g* for 5 min at 10°C.

The aqueous phase was frozen at −30°C on a Fryka Polar cooling plate (Kältetechnik, Esslingen, Germany) and the organic phase decanted into a clean 5-ml amber glass ampoule containing 200 μl of a 1% formic acid solution. After vortex-mixing for 2 min, the aqueous phase was again frozen at −30°C and the organic phase discarded. The aqueous phase was transferred to an autosampler vial and 10 μl was injected onto the HPLC column. To prevent any possible degradation of the analyte, extractions were performed under sodium vapour illumination and amber vials were used when samples were placed on the autosampler tray.

2.3. Liquid chromatography

All chromatographic solvents were sparged with helium before use. Chromatography was carried out at ambient temperature at a flow-rate of 0.25 ml/min with methanol–water–0.05% formic acid (600:400:1, v/v) as mobile phase.

2.4. Mass spectrometry

Electrospray ionisation was performed in the positive ion mode with nitrogen as the nebulizing, turbospray and curtain gas with the optimum values set at 50: 80: 50 (respective arbitrary values). The turboionspray temperature was set at 400°C. The instrument response was optimised for doxepin, desmethyldoxepin and benzoctamine by infusing a constant flow of a solution of the drugs dissolved in mobile phase into the stream of mobile phase eluting from the column. The optimum voltages for the mass dependent parameters were: RO2 (V): −39, −42, −42; RO3 (V): −44, −47, −47; IO3 (V): −51, −54, −54 (for analyte, metabolite and internal standard, respectively). The pause time was set at 5 ms and the dwell time at 200 ms.

The Perkin-Elmer Sciex API 2000 LC–MS–MS system was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions m/z 280.2, 266.2 and 250.1 to the product ions m/z 107.1, 107.1 and 191.0 (for the doxepin, desmethyldoxepin and benzoctamine internal standard, respectively). TurboIonSpray ionisation (ESI) was used for ion production. As opposed to single ion monitoring where lack of chromatographic resolution would prevent the monitoring of the same ion for doxepin and desmethyldoxepin, the power of MS–MS in this particular scan mode allowed such monitoring since the ion with m/z 107.1 was the product ion originating from two different parent ions with m/z 280.2 and 266.2, isolated during the first MS stage (see Fig. 1). The instrument was interfaced to an Apple Macintosh computer running Perkin-Elmer MassChrom version 1.1 with MacQuan version 1.6 software.

2.5. Validation

The method was validated by analysing plasma quality control samples five times at six different concentrations, i.e., 71.2, 39.5, 19.3, 1.53, 1.15, 0.760 and 0.378 ng/ml for doxepin and 39.0, 21.7, 10.6, 1.43, 1.07, 0.710 and 0.314 ng/ml for desmethyldoxepin to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing nine different concentrations spanning the concentration range (81.1–0.320 ng/ml for doxepin and 45.1–0.178 ng/ml for desmethyldoxepin). Calibration graphs were constructed using a weighted linear regression ($1/\text{concentration}^2$) of the drug/internal standard peak-area ratios of the product ions for doxepin and desmethyldoxepin, versus nominal drug concentrations.

Specificity was determined by analysing six different sources of ''blank'' plasma without the addition

Table 1
Intra-day quality control results of doxepin in human plasma as obtained during the validation with and without internal standard (I.S.)

Nominal concentration (ng/ml)	Doxepin (n=5) (mean concentration found, ng/ml)		RSD (%)		Accuracy (% nominal)	
	With I.S.	Without I.S.	With I.S.	Without I.S.	With I.S.	Without I.S.
0.38	0.33	0.33	4.3	10.4	84.8	83.4
0.76	0.78	0.78	2.8	5.8	102.1	102.8
1.15	1.17	1.15	7.8	13.1	101.9	100.2
1.53	1.55	1.51	4.6	8.5	101.4	98.7
19.3	20.0	20.2	4.2	4.5	103.5	104.4
39.5	39.4	39.4	2.8	5.9	99.8	105.9
71.2	69.8	69.1	3.4	4.8	96.9	104.8

of the internal standard to determine possible interference with the analyte, metabolite and the internal standard.

The matrix effect (coeluting, undetected endogenous matrix compounds that may influence the analyte ionisation [10]) was investigated by extracting ''blank'' biological fluids from six different sources, reconstituting the final extract in mobile phase containing a known amount of the analyte, analysing the reconstituted extracts and then comparing the peak areas of the analytes.

Absolute recoveries of analyte, metabolite and internal standard were determined in triplicate in normal plasma by extracting drug free plasma samples spiked with doxepin and desmethyldoxepin. Recoveries were calculated by comparison of the analyte peak-areas of the extracted samples with those of the unextracted system performance verification standard mixtures representing 100% recovery. The recoveries were calculated using the system performance verification standard since no difference in ionisation between extracted samples and pure solutions was observed.

3. Results and discussion

The mean absolute recovery of doxepin determined in triplicate at 71.2, 19.3 and 0.76 ng/ml was 92%, while for desmethyldoxepin, the mean recovery at concentrations of 39.0, 10.6 and 0.71 ng/ml was 76%. The recovery for benzoctamine internal standard was calculated at 90%. The lower limit of quantitation (LLOQ) is defined as that concentration of doxepin and desmethyldoxepin which can still be determined with acceptable precision (RSD<20%) and accuracy (bias<20%) and was found to be 0.32 and 0.18 ng/ml for doxepin and its metabolite, respectively. Results from the intra-day validation assays indicate a valid calibration range of 0.32–81.1 ng/ml for doxepin and 0.18–45.1 ng/ml for the desmethyldoxepin metabolite. The intra- and inter-

Table 2
Intra-day quality control results of desmethyldoxepin in human plasma as obtained during the validation with and without internal standard (I.S.)

Nominal concentration (ng/ml)	Desmethyldoxepin (n=5) (mean concentration found, ng/ml)		RSD (%)		Accuracy (% nominal)	
	With I.S.	Without I.S.	With I.S.	Without I.S.	With I.S.	Without I.S.
0.35	0.35	0.34	10.4	6.7	100.0	97.1
0.71	0.73	0.73	5.4	3.2	102.7	103.0
1.07	1.11	1.09	5.9	6.5	103.6	101.8
1.43	1.42	1.38	3.2	7.4	99.2	96.7
10.6	10.3	10.4	3.8	4.5	97.0	98.2
21.7	21.1	21.1	4.2	5.2	97.3	97.2
39.0	37.5	37.3	3.8	5.3	96.2	95.4

Table 3
Inter-day quality control results of doxepin in human plasma as obtained during the clinical study batches with and without internal standard (I.S.)

Nominal concentration (ng/ml)	Doxepin (n=12) (mean concentration found, ng/ml)		RSD (%)		Accuracy (% nominal)	
	With I.S.	Without I.S.	With I.S.	Without I.S.	With I.S.	Without I.S.
0.76	0.73	0.75	8.3	8.0	96.5	99.0
1.53	1.56	1.59	4.6	11.5	102.2	104.2
19.3	20.0	20.1	5.0	6.9	103.8	104.0
39.5	39.5	39.5	5.0	7.4	99.9	100.0

day assay method performance statistics are presented in Tables 1–4.

Plasma samples containing doxepin and desmethyldoxepin were stored at −20°C until they were analysed. No evidence of doxepin or its metabolite degradation could be found and it has been reported that doxepin samples are stable at −20°C for at least 3 months [9].

On-instrument stability was inferred from special stability samples which were prepared and included in the validation batch. No significant degradation could be detected in the cooled samples left on the autosampler for at least 7 h (Fig. 2).

Due to the high specificity of MS–MS detection, no interfering or late eluting peaks were found when chromatographing blank plasma extracts from six different sources.

Several extraction procedures were tested which include solid-phase and liquid–liquid extraction methods using different organic solvents. Different concentrations of acetic acid, formic acid and ammonium acetate were also tested for optimum ionisation of the analytes, and a liquid–liquid back-extraction procedure of the analyte and metabolite from hexane into a 1% formic acid solution proved to be successful. This procedure did not only save solvent evaporation time, but also resulted in the introduction of much cleaner extracts with high recovery rates into the ionization source. The extraction yield was further improved by the addition of 2% isoamyl alcohol to the hexane.

The extraction, chromatography and ionisation of the analyte and metabolite were reproducible enough for the method to be used with or without an internal standard (see Tables 1–4).

Retention times were 1.76, 1.87 and 1.94 min for benzoctamine internal standard, doxepin and desmethyldoxepin metabolite, respectively. A total chromatography run time of 3 min was allowed, which made it possible to analyse batches of up to 300 samples per day.

In testing the matrix effects, no matrix interference with the doxepin, desmethyldoxepin or benzoctamine could be detected with any of these extractions. Fig. 3A shows representative chromatograms obtained at concentrations of 81.1 ng/ml doxepin and 45.1 ng/ml desmethyldoxepin, while Fig. 3B shows the chromatograms of doxepin and desmethyldoxepin at

Table 4
Inter-day quality control results of desmethyldoxepin in human plasma as obtained during the clinical study batches with and without internal standard (I.S.)

Nominal concentration (ng/ml)	Desmethyldoxepin (n=12) (mean concentration found, ng/ml)		RSD (%)		Accuracy (% nominal)	
	With I.S.	Without I.S.	With I.S.	Without I.S.	With I.S.	Without I.S.
0.35	0.32	0.30	6.8	16.7	90.3	84.1
0.71	0.73	0.71	10.3	9.3	102.5	100.6
1.43	1.45	1.46	9.4	11.3	101.1	102.0
10.6	10.7	10.8	7.8	9.6	100.7	101.6
21.7	21.5	21.7	7.5	8.4	99.1	99.8

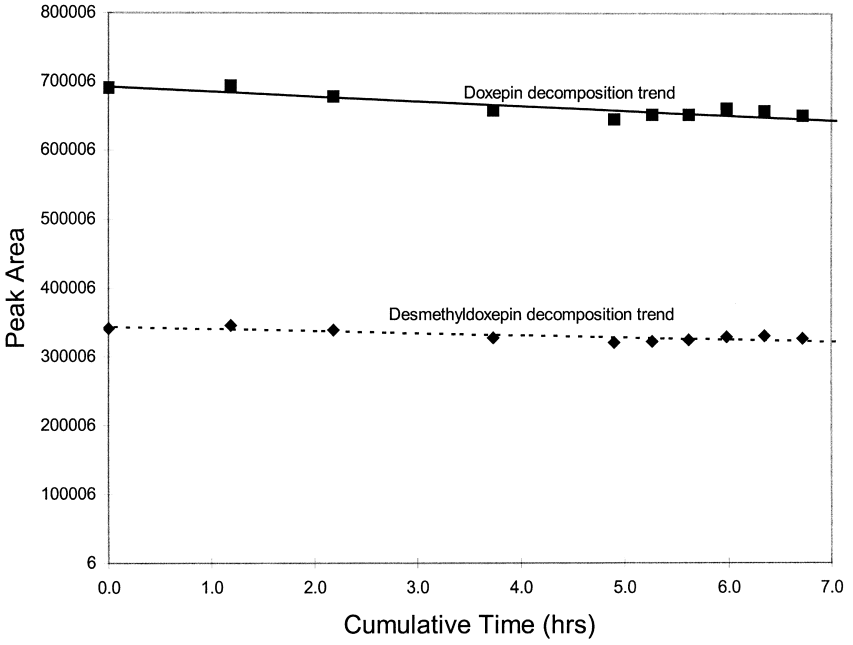

Fig. 2. On-instrument stability as depicted when samples containing 71.2 ng/ml doxepin (A) and 39.0 ng/ml desmethyldoxepin were left on the autosampler for about 7 h.

concentrations of 0.32 ng/ml and 0.18 ng/ml, respectively (the LLOQs). Fig. 3C depicts chromatograms of a blank plasma extract to show the absence of any interference at the relevant retention times of 1.76, 1.87 and 1.94.

During a biostudy with doxepin over a 2-week period, the following statistics of the slope of the linear calibration line were obtained: doxepin with internal standard: 0.01159 to 0.01254 (mean= 0.01216; RSD=3.6%); doxepin without internal standard: 11 467 to 14 094 (mean=12 814; RSD= 8.0%); desmethyldoxepin with internal standard: 0.01017 to 0.01210 (mean=0.01143; RSD=7.4%) and desmethyldoxepin without internal standard: 10 919 to 13 437 (mean=11 956; RSD=9.6%).

The method was employed to analyse plasma samples containing doxepin and desmethyldoxepin obtained after a single oral dose of 100 mg doxepin per treatment phase in 20 healthy volunteers. Concentration vs. time profiles were constructed for up to 96 h. The maximum doxepin plasma concentrations obtained varied between 6.79 and 18.57 ng/ml and between 6.33 and 17.85 ng/ml for the desmethyldoxepin metabolite. Fig. 4 shows the average doxepin and desmethyldoxepin pharmacokinetic profiles in a study with 18 trial subjects after each one received a 100 mg oral dose of doxepin.

4. Conclusion

A highly sensitive and selective method for the quantification of doxepin and desmethyldoxepin in human plasma has been developed and validated. Plasma concentrations of doxepin could be quantified from 0.32 ng/ml to 81.1 ng/ml, and between 0.18 ng/ml and 45.1 ng/ml for the metabolite, making it applicable to pharmacokinetic studies where 96 h concentration vs. time profiles after a single oral 100 mg dose of the drug are required. If needed, a considerably lower LLOQ can be achieved by injecting a bigger volume of the extract. This is the first chromatographic method described for the simultaneous determination of doxepin and desmethyldoxepin in plasma using liquid chromatography in tandem with mass spectrometry.

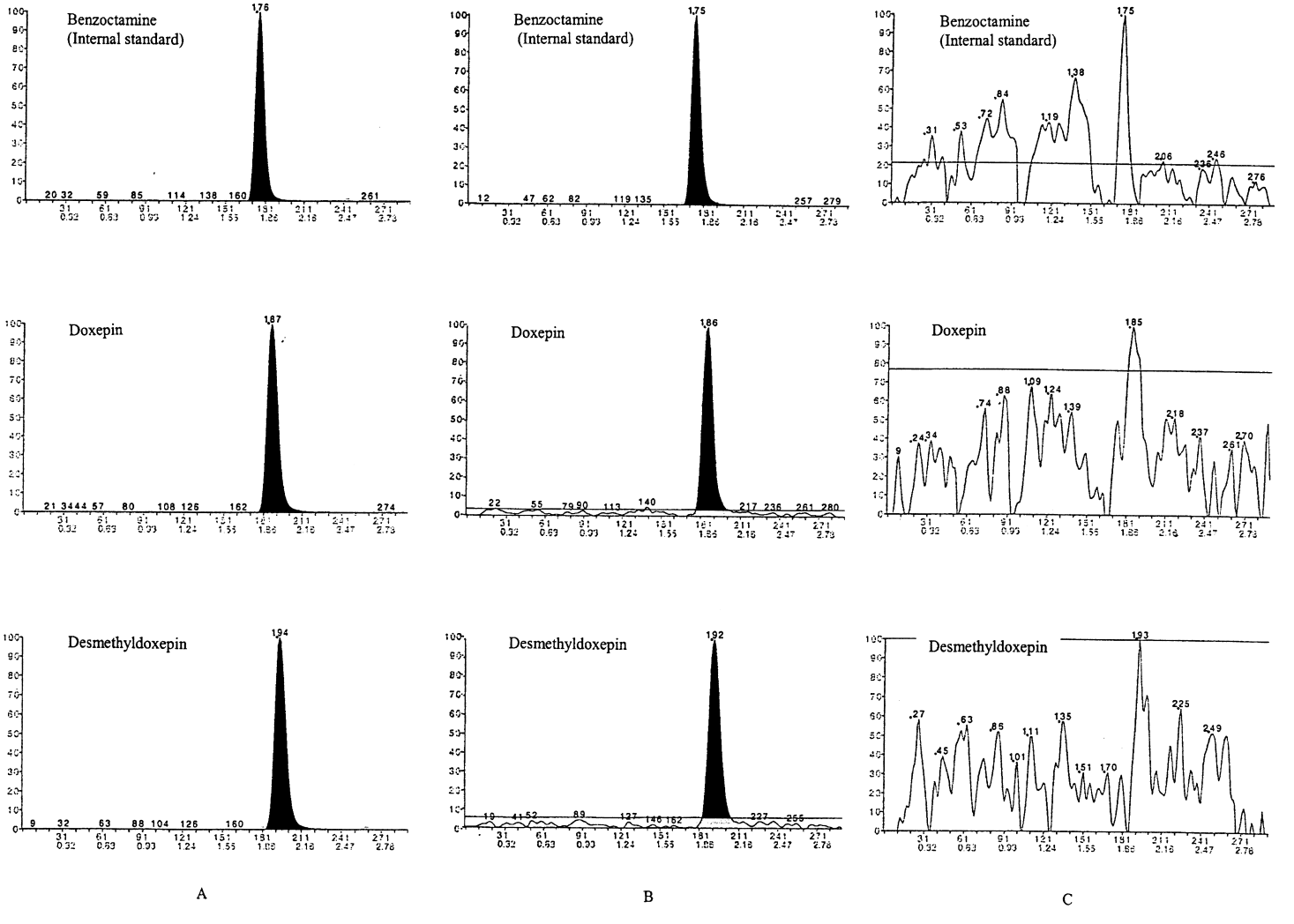

Fig. 3. Chromatograms of calibration standards containing the following concentrations in plasma: (A) 93.1 ng/ml benzoctamide; 81.1 ng/ml doxepin and 45.1 ng/ml desmethyldoxepin, (B) 93.1 ng/ml benzoctamide; 0.32 ng/ml doxepin and 0.18 ng/ml desmethyldoxepin, (C) blank extracts to show absence of interference at 1.76, 1.87 and 1.94 min.

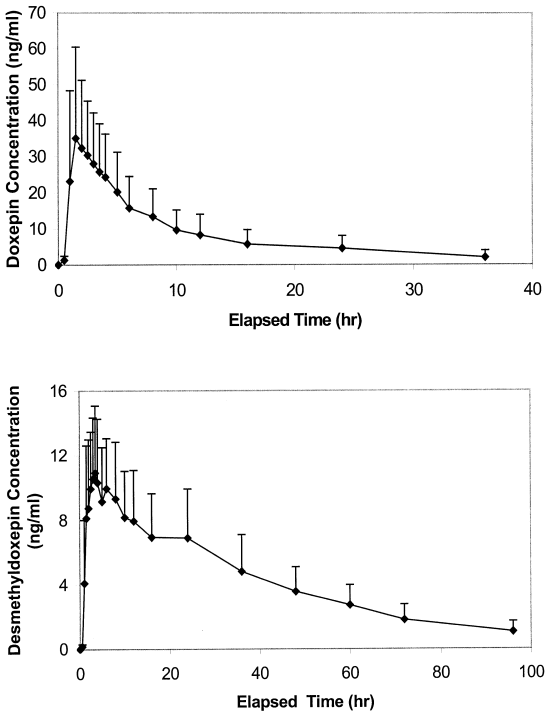

Fig. 4. Representative doxepin and desmethyldoxepin plasma concentrations vs. time profiles as obtained after a single 100 mg oral dose.

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