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Quantitative analysis quantitation of 2-*n*-nonyl-1,3-dioxolane by stable-isotope dilution gas chromatography-mass spectrometry

W.Z. Zhong, P.E. Sanders, M.J. Hauer, J.J. Vrbanac*

Drug Metabolism Research, Pharmacia and Upjohn, Kalamazoo, MI, USA

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Abstract

We report here a quantitative methodology developed for determination of SEPA (2-*n*-nonyl-1,3-dioxolane) in human serum. The method employed solid-phase extraction of SEPA and internal standard, [$^{13}C_2$]SEPA, from serum followed by gas chromatography–mass spectrometry analysis using EI monitoring *m/z* 73 and 75. We have investigated the utility of stable isotope dilution gas chromatography–mass spectrometry (GC–MS) for the determination of SEPA concentrations in serum using chemical ionization (positive ion, CI) or electron ionization (EI). The comparison of the specificity and sensitivity between EI and CI indicated that monitoring the *m/z* 73 ion in EI was superior to monitoring either MH^+ or *m/z* 73 using CI. The method was simple, sensitive and accurate, demonstrating a lower limit of quantitation (LLOQ) of 0.25 ng/ml and intra- and inter-assay accuracy and precision of ≤7.5%. © 1998 Elsevier Science B.V.

Keywords: 2-*n*-Nonyl-1,3-dioxolane

1. Introduction

SEPA (2-*n*-nonyl-1,3-dioxolane, Fig. 1) has been reported to enhance the percutaneous absorption of certain drugs [1,2]. Minoxidil is a hypertrichotic agent used topically in treating alopecia androgenetica and it is potent orally in the treatment of severe hypertension. Although minoxidil is nearly completely absorbed in several animal species and in man after oral administration, it is poorly (<3%) absorbed in man after topical administration [3,4]. Thus, a new nonaqueous topical minoxidil formulation containing SEPA is under preclinical and clinical evaluation. A sensitive method for the quantitation of SEPA in serum was required for the characterization of pharmacokinetics of SEPA in animals and in man

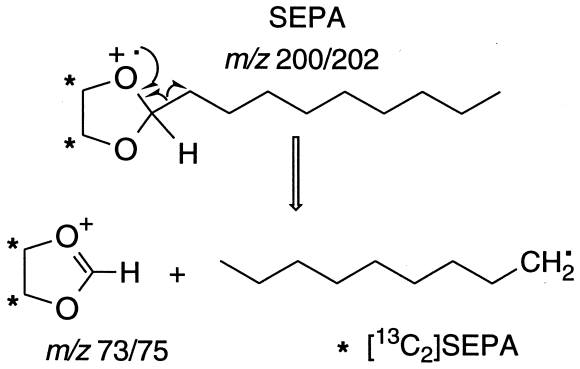

Fig. 1. Structures of Sepa (2-*n*-nonyl-1.3-dioxolane) and the predictable homolytic α-cleavage producing the ion at *m/z* 73(75). The position of ^{13}C in the internal standard is shown by an asterisk.

*Corresponding author.

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following the topical application of the new formulation. SEPA does not have a structure amenable to liquid chromatography (LC)-ultraviolet absorption or LC-fluorescence detection and lacks a functional group(s) for chemical derivatization. However, the structure is ideal for GC-FID analysis. Thus, A GC-FID method with a limit of quantitation of 2.5 ng/ml for determination of SEPA in serum was developed [5]. Although the sensitivity of the GC-FID method was sufficient to support the preclinical pharmacokinetic or toxicokinetic evaluations, a more sensitive method is required for supporting clinical studies in which subjects received much lower doses of topical treatment of SEPA. We have investigated the feasibility of developing a quantitative method for the determination of SEPA concentrations in serum with greater ultimate sensitivity and specificity relative to what has been observed for GC-FID methodology. This report details our investigations into the utility of CI using ammonia and methane reagent gasses and electron ionization (EI). We also have compared a structural analogue internal standard (2-*n*-heptyl-1,3-dioxolane) with a stable isotope internal standard, ([$^{13}C_2$]SEPA (labeled in the 4,5-carbons of the dioxolane ring).

2. Experimental

2.1. Chemicals

SEPA was provided by Pharmacia and Upjohn (Kalamazoo, MI, USA). The internal standard (I.S.) used for SEPA assay, 2-*n*-heptyl-1,3-dioxolane was supplied by the MacroChem (Lexington, MA, USA) and [$^{13}C_2$]SEPA was provided by Drug Metabolism Research, Pharmacia and Upjohn (Kalamazoo, MI, USA). Methanol, hexane and chloroform obtained from Burdick and Jackson (Muskegon, MI, USA) were high purity solvent grade and used without further purification. All water was pretreated with a Milli-Q reagent water system (Millipore, Bedford, MA, USA). Serum used for preparation of standards and quality control samples was obtained by drawing blood from in-house healthy volunteers. The blood was allowed to clot at room temperature for 20 min before centrifugation. Serum was removed to a clean labeled vial and stored at −15 to −25°C.

2.2. Preparation of standards

Stock solution of SEPA or I.S. was prepared by accurately weighing 10 mg of compound into 5 ml methanol and diluting to volume with methanol in a 10-ml volumetric flask to give a concentration of 1000 μg/ml SEPA or I.S. The SEPA stock solution was diluted with methanol to yield 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 working standard solutions for preparation of calibration curve. The I.S. stock solution was diluted with methanol to yield a 0.2 μg/ml I.S. working solution. The stock solutions were stored at −15 to −25°C and the working solutions were stored at 4°C.

Serum standards were prepared by aliquoting 50 μl of each SEPA working standard solution and 25 μl of I.S. working solution (0.2 μg/ml) to 1 ml of blank serum (drug free) in culture tubes. The final standard concentrations for the calibration curve are 0, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 ng/ml.

Quality control (QC) samples were prepared by aliquoting SEPA standards to blank serum to produce concentration pools of 0.25, 5 and 50 ng/ml. QC samples were stored at −15 to −25°C.

Unknown or QC samples were prepared by adding 1 ml of serum sample, 50 μl of methanol, and 25 μl of I.S. working solution into culture tubes.

2.3. Sample preparation

The serum standards, QC samples or unknown serum samples were loaded on phenyl solid-phase extraction (SPE) columns (50 mg/1.0 ml, Varian, Harbor City, CA, USA) which have been prewashed with one column volume of hexane–chloroform (1:1, v/v) followed by one column volume of methanol and one column volume of water with slight vacuum aspiration (approximately 86 kPa). The SPE columns were rinsed with 300 μl of methanol–water (3:7, v/v) followed by two column volumes of water. After the SPE columns were dried with vacuum aspiration (approximately 27 kPa) for approximately 10 s, the compounds of interest were eluted from the column with 100 μl of hexane–chloroform (1:1, v/v) into autosampler vials by manually applying a slow uniform pressure to the top of the column using nitrogen gas. The vials were placed to the autosampler tray, which was maintained at 4°C, and a

2-μl aliquot of the prepared sample was injected into the GC–MS for analysis.

2.4. Gas chromatographic–mass spectrometric instrumentation condition

GC was performed using a Hewlett Packard 5890 GC equipped with a split-splitless injector. A J&W Scientific (Folsom, CA, USA) DB-1 (30 m×0.25 mm I.D., 0.25 μm film thickness) or DB-17 (15 m×0.25 mm I.D., 0.25 μm film thickness) bonded phase wall-coated open tubular (WCOT) fused-silica capillary column was interfaced directly into the ion source of a Nermag R-1010 quadrupole mass spectrometer. Helium (99.995% pure; AGA, Maumee, OH, USA) was used as the carrier gas (=40 cm/s). Tuning of the ion optics was manual. For experiments where m/z 73 and 75 were monitored the instrument resolution was set for a 10% valley between m/z 69 and 70 (CF_3^+ and $^{13}CF_3^+$ of perfluorotributylamine) scanning the instrument over a 10 Da range in 3 s with the pre-filter at 100 kHz and a signal of 60 mV for m/z 70. Calibration of the mass axis and collection of data were under computer control (Teknivent, Vector II, Maryland Heights, MO, USA). In the CI and initial series of EI experiments the DB-1 column was used. Samples were injected at 80°C with the injector operating in the splitless mode at 250°C. After 1 min the injector was placed in the split mode (1:100) and the oven temperature increased from 80°C to 300°C at 20°C/min and held at this temperature for 1 min. For the EI validation experiments the DB-17 column was used. Samples were injected with the injector operating in the splitless mode at 50°C oven temperature (injector still 250°C). After 1 min the injector was placed in the split mode and the oven temperature increased from 50°C to 130°C at 70°C/min, from 130°C to 160°C at 10°C/min, from 160°C to 240°C at 50°C/min and held at this temperature for 1 min.

2.5. Quantification

Peak heights and peak areas were determined using a Vector II data system (Teknivent, Maryland Heights, MO, USA) and these values were manually entered into the Harris computer system. Quantitation was accomplished by peak height ratio of SEPA to internal standard (PHR) using UPACS chromatographic system software (C.H. Mai et al., Pharmacia and Upjohn, Kalamazoo, MI, USA) on the Harris Night Hawk Computer System (Ft. Lauderdale, FL, USA). Standard curves derived from analysis of the serum standards were constructed by a quadratic regression model of $PHR=ax^2+bx+c$ (x= concentration) weighted by $1/x$. The SEPA concentrations of unknown and control samples are calculated by inverse prediction against the calibration curve.

3. Results

3.1. Electron ionization

The structure of SEPA and [^{13}C]SEPA are displayed in Fig. 1. The analyte structure contains two equivalent cyclic ether oxygens that would be expected to participate in a homolytic α-cleavage fragmentation process under electron ionization resulting in an ion of m/z 73 (m/z 75 for [^{13}C]SEPA). This should be a highly favored process. The EI mass spectrum of [^{13}C]SEPA is displayed in Fig. 2a. As expected, the base peak was observed to occur at m/z 75. The isotopic composition (atoms percent purity) of the [$^{13}C_2$]SEPA reference standard calculated by monitoring m/z 75, 74 and 73 was determined to be 95%, 4% and 1% for 4,5-[$^{13}C_2$]SEPA, 4,5-[$^{13}C_1$]SEPA and SEPA, respectively. $M\cdot^+$ was not observed and a weak $M\cdot^+-H\cdot$ was observed at m/z 201. The relative intensities for the EI mass spectrum of SEPA were the same. The majority of the total ion current is represented by m/z 73/75 making this the only ion that can be monitored in the SIM mode of operation that would result in detectable signals in an analytically useful range (e.g., 0.1–1.0 ng injected on-column). Generally, monitoring an ion this low in mass will not impart the necessary specificity to a quantitative GC–MS procedure. A structural analogue of SEPA, 2-*n*-heptyl-1,3-dioxolane, was previously used as an internal standard for investigations into the utility of GC-FID methodology. The EI mass spectrum of 2-*n*-heptyl-1,3-dioxolane was, as expected, dominated by m/z 73. Therefore, CI methodology was investigated for analytical utility.

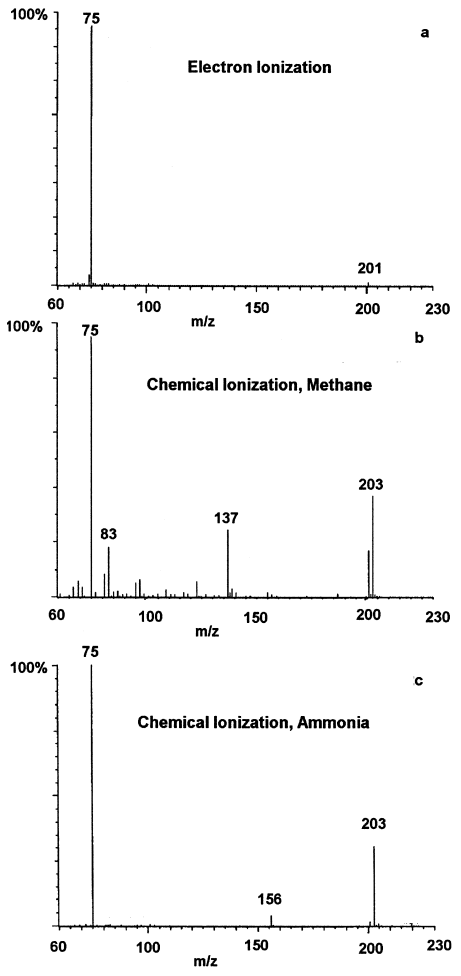

Fig. 2. (a) Chemical ionization mass spectrum of [^{13}C]SEPA obtained using methane as the reagent gas. MH^+, and MH^+-H_2 and have increased by 2 Da and m/z 73 has shifted to m/z 75 Da. (b) Chemical ionization mass spectrum of [^{13}C]SEPA obtained using ammonia as the reagent gas. MH^+, MH^+-H_2 (weak) and the ammonia adduct ions have increased by 2 Da and m/z 73 has shifted to m/z 75 Da. (c) EI mass spectrum of [^{13}C]SEPA. The ion at m/z 73 has shifted two Da to m/z 75 and $M\cdot^+-H\cdot$ has increased from 199 to 201.

3.2. Chemical ionization

The CI mass spectra obtained for [^{13}C]SEPA using methane and ammonia as the reagent gases are displayed in Fig. 2b and c, respectively. The ion at m/z 75 is still observed as the base peak. The protonated molecular ion (pseudo-molecular ion; MH^+) is observed at m/z 203. Fragment ions other than m/z 73 were also observed. MH^+-H_2 ions was observed, although the ion current was much weaker when using ammonia than that observed for methane. The mass spectra for SEPA were quantitatively identical to those obtained for [^{13}C]SEPA. Thus, when using either methane or ammonia the CI mass spectra of SEPA and [^{13}C]SEPA both exhibited m/z 73/75 as the base peak and weaker ion currents at MH^+ and MH^+-H_2.

3.3. Electron ionization and chemical ionization: comparative sensitivity

The relative sensitivity of CI, ammonia and methane, and EI were compared. EI was observed to be approximately 50 fold more sensitive than either CI technique (monitoring m/z 73 and MH^+, respectively). Also, The occurrence of MH^+-H_2 in the CI mass spectrum severely compromised the use of [^{13}C]SEPA as an internal standard. Fortunately, we observed that EI-MS and monitoring the oxonium ion at m/z 73/75 was more selective that CI and monitoring MH^+ for the determination of SEPA in serum plasma.

We developed a quantitative assay using EI and a 15 m DB-17 column in place of the 30 m DB-1 (described in Section 2). The DB-17 was not observed to significantly change the observed interferences from serum in the region where SEPA eluted ($\pm$0.5 min). However, less background at m/z 73 was observed when using the DB-17 column, an observation we admit is difficult to quantify.

3.4. Quantitative analysis of SEPA in human serum using [$^{13}C_2$]SEPA and electron ionization

Human serum samples were spiked with SEPA and [$^{13}C_2$]SEPA and extracted as described above. A typical response observed for a blank sample (e.g., 5 ng of [$^{13}C_2$]SEPA/ml of serum only) is displayed in Fig. 3. The observed peak at m/z 73, by 1% isotopic contamination of the internal standard, is equivalent to 50 pg SEPA/ml. The SIM chromatogram observed for serum alone was flat in this region (data not shown). This peak represents approximately 3 pg of SEPA injected. The data in Fig. 3 demonstrate the

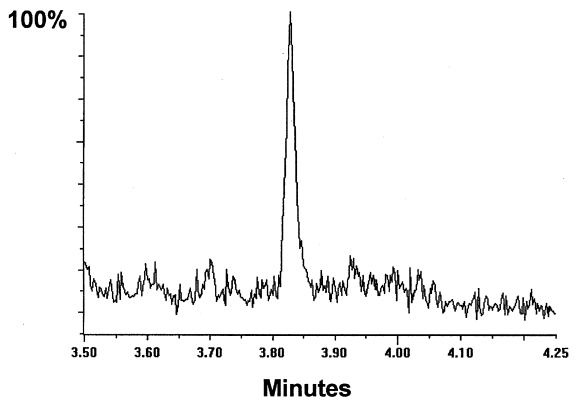

Fig. 3. SIM chromatogram for m/z 73 observed for the blank sample (e.g., 5 ng of [$^{13}C_2$]SEPA/ml of serum only). The observed peak represents 50 pg SEPA/ml of serum (e.g., an approximate 1% contribution at m/z 73 relative to m/z 75.

high degree of analytical specificity afforded by the EI methodology.

3.5. Extraction of serum samples

To improve the assay sensitivity and selectivity, a more concentrated and cleaner sample was needed for GC–MS analysis. Since SEPA is a very volatile compound, any sample preparation procedures involving evaporation will result in low recovery of SEPA. Thus, the solid-phase extraction method was considered for the serum sample preparation. Using 1 ml of serum for extraction, the 50 mg/1.0 ml phenyl (PH) SPE column was found to be sufficient to retain the compounds and also provided the possibility of using a minimum volume of elution solvent (100 μl). A 100 μl of hexane–chloroform (1:1, v/v) was finally chosen as the elution solvent, giving a absolute extraction recovery of 80–95% for both SEPA and I.S., and a 10:1 concentrated extract for GC–MS analysis. A 300-μl aliquot of methanol–water (3:7, v/v) was sufficient to wash out most serum proteins retained on the SPE columns, as a result generating much cleaner chromatograms.

3.6. Method validation

The method validation was carried out with four analytical runs each including a set of fortified serum standards (0–100 ng/ml) and the low (0.25 ng/ml), medium (5 ng/ml) and high (50 ng/ml) quality control samples analyzed in triplicate [6,7]. For the best fit of these standard curves, a polynomial regression model of $PHR=ax^2+bx+c$ ($x=$ concentration) weighted by $1/x$ was used for SEPA quantitation. The correlation coefficients of these four standard curves (0.25–100 ng/ml) were 0.999 or greater. The C.V. of the slopes from each standard curve was 6%. The intra- and inter-assay accuracy and precision calculated from the QC data were all ≤7.5% (Table 1). The lower limit of quantitation (LLOQ) was 0.25 ng/ml if a concentration range of 0.25–100 ng/ml was used for the calibration curve. The assay LLOQ sensitivity can be further improved if a lower concentration range of calibration curve and lower internal standard concentration are used.

3.7. Quantitative analysis of SEPA in human serum following topical administration

The applicability of this method has been demonstrated by analysing human serum samples collected on treatment Day 21 from subjects who received topical treatments for 21 days with a minoxidil formulation containing 5% SEPA. Fig. 4 displays the SEPA serum concentration–time data for two repre-

Table 1
Intra- and inter-assay accuracy and precision for the determination of SEPA in human serum using the GC–MS method

SEPA added (ng/ml)	Intra-assay ($n=3$)			Inter-assay ($n=12$)		
	Mean (ng/ml)	C.V. (%)	Bias (%)	Mean (ng/ml)	C.V. (%)	Bias (%)
0.25	0.268	5.0	+7.0	0.25	15	0
5	5.32	1.6	+7.5	5.12	5	+3.0
50	48.8	2.3	−1.4	47.6	2.7	−3.9

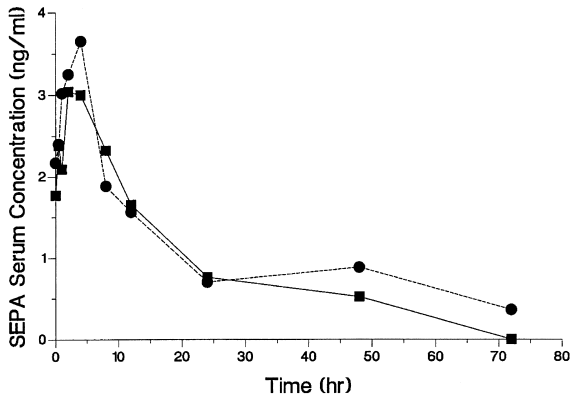

Fig. 4. SEPA serum concentration–time profiles on treatment Day 21 for Subject No. 35 (■) and Subject No. 36 (●) following topical administration of a minoxidil solution containing 5% SEPA for 21 days.

sentative subjects (Subjects 35 and 36). These results indicated that SEPA concentrations can be measured at least 48 h after the last treatment using the GC–MS method, suggesting that this method has sufficient sensitivity for evaluating clinical pharmacokinetics of SEPA. Peak serum concentrations were observed to be between 2 and 4 ng/ml.

4. Discussion

The superior sensitivity analytical utility of EI when monitoring a low mass ion compared to CI and monitoring MH^+ (as well as the greater specificity of monitoring m/z 73 compared to monitoring MH^+ when using CI) was not anticipated by the authors and was not immediately identified. In general, the complexity of the ion chromatograms observed between, as an arbitrary example, m/z 30 and 90 when using GC–EI-MS analysis of a simple extract of mammalian plasma is much greater relative to that observed for m/z 200 and above, when using either EI or CI. The reason for the increased specificity for this low mass fragment ion in the present situation may, directly contradicting dogma relate at least in part to two different phenomenon. First, the formation of m/z 73 when using EI is highly favored for SEPA (represents the vast majority of the total ion current). Second, biological material that would form m/z 73 (e.g., organic acids, esters [8]) would most likely be eliminated in the 1:1 water–methanol wash in the sample preparation.

In these studies it was also noted that great care must be taken to maintain a low chemical background (e.g., low column bleed) when using the EI method ($(CH_3)_3Si^+$, m/z 73, and $HO^+=Si(CH_3)_2$, m/z 75, are potential background contributors, as is the A+2 ion from m/z 71 derived from aliphatic hydrocarbons). Also, an artificial increase in the amounts of SEPA observed at the low end of the standard curve was not easily characterized as resulting from contamination of the SPE apparatus (i.e., the ultimate instrumental sensitivity of approximately 50 fmole allows the detection of contamination that usually would be well below the limit of detection of the vast majority of analytical methodologies). Another unanticipated problem was the apparent increased concentration of SEPA in a few of the low-end stock solutions, presumable due to evaporation of solvent.

In summary, the GC–MS method using EI and 4,5-[$^{13}C_2$]SEPA as an internal standard provides a highly specific and sensitive quantitative assay for SEPA in human serum. The method was observed to exhibit superior analytical utility relative to GC–CI-MS. The LLOQ was determined to be 0.25 ng SEPA/ml serum. Based upon the clean chromatogram displayed in Fig. 3, the ultimate LLOQ should in theory be less than 50 pg SEPA/ml serum (e.g., addition of less internal standard). The LLOQ in serum is very surprising in that a simple SPE procedure is coupled with a GC–EI-MS method that monitors ions of low mass (m/z 73 and 75). The authors are unaware of any GC–EI-MS technique used to candidate endogenous or exogenous organic compounds (with a molecular mass of a few hundred Daltons) in serum with a LLOQ of less than 0.25 ng/ml that uses a simple SPE clean-up and monitors low mass ions.

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