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Application of solid-phase extraction in the method development for determination of SEPA, an acronym for soft enhancement of percutaneous absorption, in human, rat, and rabbit serum using GC–FID method[1]

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Abstract

A new nonaqueous topical minoxidil formulation containing SEPA (2-*n*-nonyl-1,3-dioxolane) for enhancement of percutaneous absorption was under evaluation. SEPA does not have chromophore for either ultraviolet or fluorescence detection using liquid chromatography and has no functional groups for derivatization. Therefore, a direct gas-chromatographic method with flame-ionization detection (GC–FID) was developed. Owing to the limited detection response of the FID detection, it needs a selective and concentrated extract for GC–FID analysis to improve the assay sensitivity to meet the requirement for pharmacokinetic evaluation after topical application. In addition, SEPA is a very volatile compound. Any extraction procedures involving evaporation will result in a poor recovery. The application of solid-phase extraction (SPE) makes it possible to achieve a selective and a 10-fold concentrated extract with an absolute extraction recovery of approximately 90%, which greatly improved the assay sensitivity. This method involved the extraction of SEPA and the internal standard (2-*n*-heptyl-1,3-dioxolane) from serum (0.1–1 ml) with 100 μl of hexane-chloroform (1:1, v:v) using a 50 mg 1.0 ml^{-1} phenyl SPE column (Varian, Harbor City, CA, USA), followed by direct GC–FID analysis on a fused-silica column chemically bonded with cross-linked methyl silicone gum phase (Hewlett Packard Ultra-1, 12 m × 0.2 mm × 0.33 μm, Avondale, PA, USA). The assay demonstrated a lower limit of quantitation of 2.5 ng ml^{-1} and a linear range of 2.5 to 250 ng ml^{-1} with intra- and inter-assay precision and accuracy of $\leq 10\%$.

Keywords: SEPA; Gas-chromatography; Solid-phase extraction; Human serum; Rat serum; Rabbit serum

1. Introduction

A new nonaqueous topical minoxidil formulation containing SEPA (2-*n*-nonyl-1,3-dioxolane, PNU-100325, Fig. 1) was under development for topical treatment of alopecia androgenetica. Al-

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though 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 [1,2]. SEPA, an acronym for soft enhancement of percutaneous absorption [3,4], was therefore, used in the formulation development. A study to determine if SEPA would augment the scalp hair growth effects of topical minoxidil in the balding stumptail macaque indicated that minoxidil-SEPA treatments produced significantly greater cumulative hair weight over the entire 16-week study compared to either of the Rogaine TS (without SEPA) treatments [5]. Clinical evaluation also showed that percutaneous minoxidil absorption increased 1.8–2.4 fold for the minoxidil-SEPA formulation relative to Rogaine (unpublished data). Thus, extensive characterization of pharmacokinetics and toxicology of the minoxidil-SEPA formulation in animals and in man was carried out to meet the requirement of registration. To support the pharmacokinetic and toxicokinetic evaluation of SEPA after topical treatments, a sensitive bioanalytical method is required. However, the chemical structure of SEPA does not have chromophore for either ultraviolet or fluorescence detection using liquid chromatography and has no functional groups for derivatization as well. Therefore, the effort was focused on developing a gas-chromatographic method with flame-ionization detection (GC–FID) or mass-spectrometric detection (GC–MS). In this report, the application of solid-phase extraction in the GC–FID method development for determination of SEPA in human, rat, and rabbit serum are described. The development of a GC–MS method for SEPA was reported elsewhere [6].

2. Experimental

2.1. Chemicals and reagents

SEPA was provided by Pharmacia and Upjohn (Kalamazoo, MI, USA). The internal standard (IS) used for SEPA assay, 2-*n*-heptyl-1,3-dioxolane (Fig. 2), was supplied by MacroChem (Lexington, MA, 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 pre-treated with a Milli-Q reagent water system (Millipore, Bedford, MA, USA).

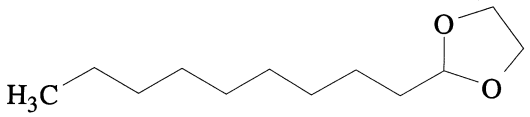

Fig. 1. Chemical structure of SEPA (2-*n*-nonyl-1,3-dioxolane).

2.2. Instrumental parameters

A Hewlett Packard Model 5890A gas chromatograph (Hewlett Packard, Avondale, PA, USA) was equipped with a split/splitless injector, flame-ionization detector (FID) and a model 7673 autosampler. A fused-silica column chemically bonded with cross-linked methyl silicone gum phase (Hewlett Packard Ultra-1, 12 m $\times$ 0.2 mm $\times$ 0.33 μm, Avondale, PA, USA) was used for the analysis. The carrier gas was helium (electronic grade, AGA, Maumee, OH, USA) adjusted to deliver a column flow-rate of 1 ml min^{-1} at the initial oven temperature. The hydrogen and air flow rates were set at 30 and 400 ml min^{-1}, respectively, for the detector with a make-up gas (helium, electronic grade) flow-rate of 30 ml min^{-1}. The injection inlet and detector temperature were maintained at 250°C. For the analysis, the column temperature was programmed from 80 to 185°C at a rate of 20°C min^{-1} and held at 185°C for 1 min, and thereafter to 220°C at a rate of 35°C min^{-1} with a 2-min final holding time. The splitless inlet purge delay was set at 1 min.

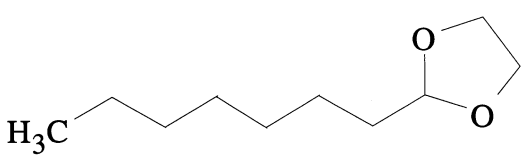

Fig. 2. Chemical structure of the internal standard (2-*n*-heptyl-1,3-dioxolane).

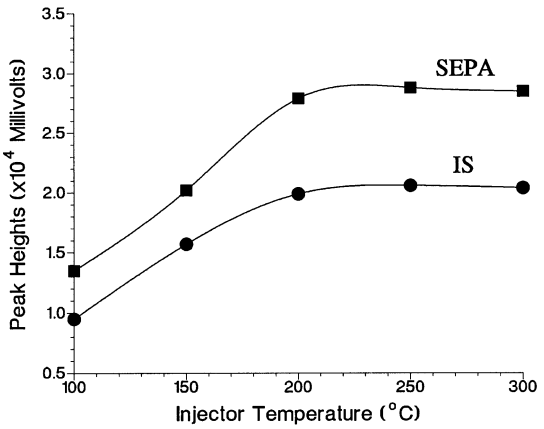

Fig. 3. Effect of injection temperature on the peak heights of SEPA (■) and IS (●).

2.3. Preparation of standards

Stock solution of SEPA or IS 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^{-1} SEPA or IS. The SEPA stock solution was diluted with methanol to yield 5, 2, 1, 0.5, 0.2, 0.1, and 0.05 μg ml^{-1} working standard solutions for the preparation of calibration curve. The IS stock solution was diluted with methanol to yield a 5 μg ml^{-1} IS 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 IS working solution (5 μg ml^{-1}) to 1 ml of blank serum (drug free) in culture tubes. If a blank serum of <1 ml was used for the preparation of serum standards, additional water should be added to make a total volume of approximately 1 ml. This will produce a concentration series of 0, 2.5, 5, 10, 25, 50, 100, and 250 ng per serum sample.

Quality control (QC) samples were prepared by aliquoting SEPA standards to blank serum to produce concentration pools of 5, 50, and 200 ng ml^{-1} for human serum and 50, 500, and 2000 ng ml^{-1} for rat or rabbit serum. 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 IS working solution into culture tubes. If less than 1 ml of serum sample was used for extraction, additional water should be added to make a total volume of approximately 1 ml.

2.4. 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^{-1}, Varian, Harbor City, CA, USA) which have been prewashed with one column volume of hexane–chloroform (1:1, v:v), followed consequently by one column volume of methanol and water with slight vacuum aspiration (approximately 86 pka). 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 pka) 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 (about 0.2 kg cm^{-2}). The vials were placed to the autosampler tray, which was maintained at 0–4°C, and a 1-μl aliquot of the prepared sample was injected for gas chromatographic analysis.

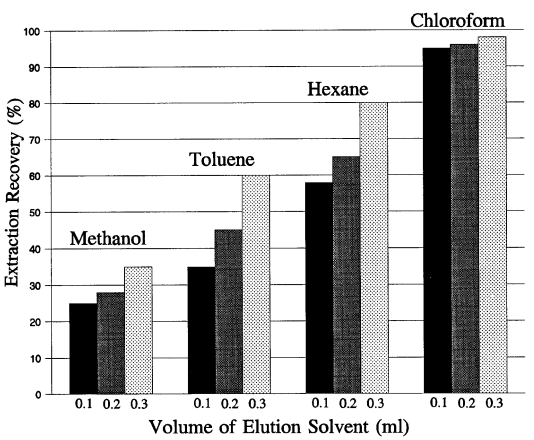

Fig. 4. Effect of the elution solvent and the volume of elution solvent on the extraction recovery of SEPA.

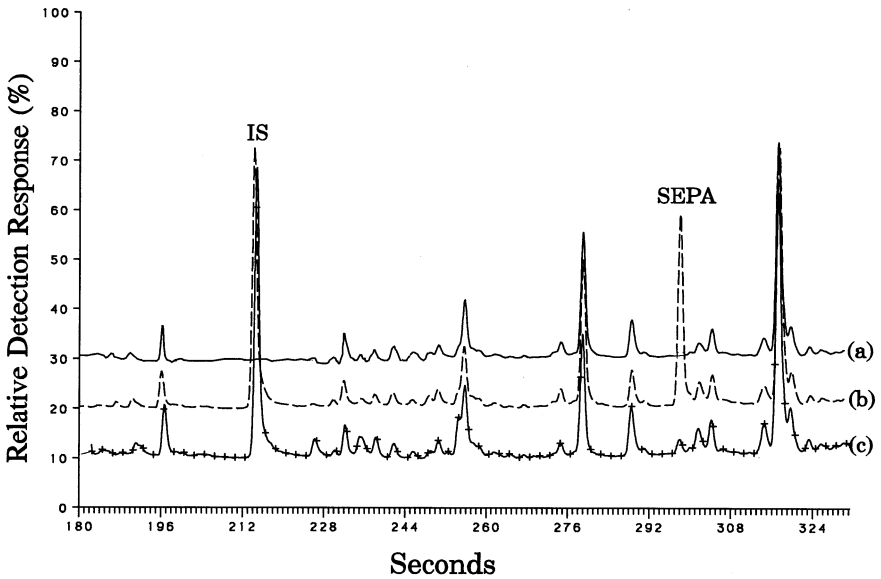

Fig. 5. Typical chromatograms of SEPA in human serum. Extract of (a) a human serum blank, (b) a fortified human serum standard (100 ng ml^{-1}), and (c) a 2-h post-dose serum sample from a clinical bioavailability study.

2.5. Quantification

Quantification was accomplished by peak–height ratio (PHR) analysis using a structurally related compound, 2-*n*-heptyl-1,3-dioxolane, as the assay internal standard (IS). Chromatographic peak heights were integrated using an UPACS chromatographic system software on an Harris Nighthawk Computer System. Standard curves derived from analysis of the serum standards were constructed by linear regression analysis of the PHR versus the spiked SEPA concentration. The intercept was found to be not significantly different from zero ($p > 0.05$) for calibration curves analyzed during the method validation and thus, the equation, $Y = aX$ (forced through the origin) can be used to calculate the unknown and QC sample concentrations by inverse prediction against the calibration curve.

2.6. Validation

To determine the linear range of the method as well as the assay accuracy and precision, freshly prepared human, rat or rabbit serum standard curves were analyzed on four different days along with the low, medium, and high QC samples assayed in triplicate [7]. The intra-assay precision was determined from the relative standard deviation (RSD) of QC sample concentrations analyzed on the same day and the inter-assay precision was obtained from the RSD of QC sample concentrations analyzed on four days. The assay accuracy was evaluated by comparing the given amounts of the substances with those determined for QC samples (bias). The limit of quantitation (LOQ) was estimated from the low concentration standards in the calibration curves, at which the precision and accuracy are acceptable (RSD or bias $\leq 20\%$). The absolute extraction recoveries were determined based on the comparison of the areas under the peaks of the extracted samples with those of unextracted reference standard solutions containing the corresponding concentrations. In all cases, the means, standard deviations (SD), and RSD were calculated. Analysis of variance was performed using a SAS program to evaluate the stability of SEPA in serum after storage and freeze/thaw cycles. A p value of < 0.05 was considered as significant in statistical analysis.

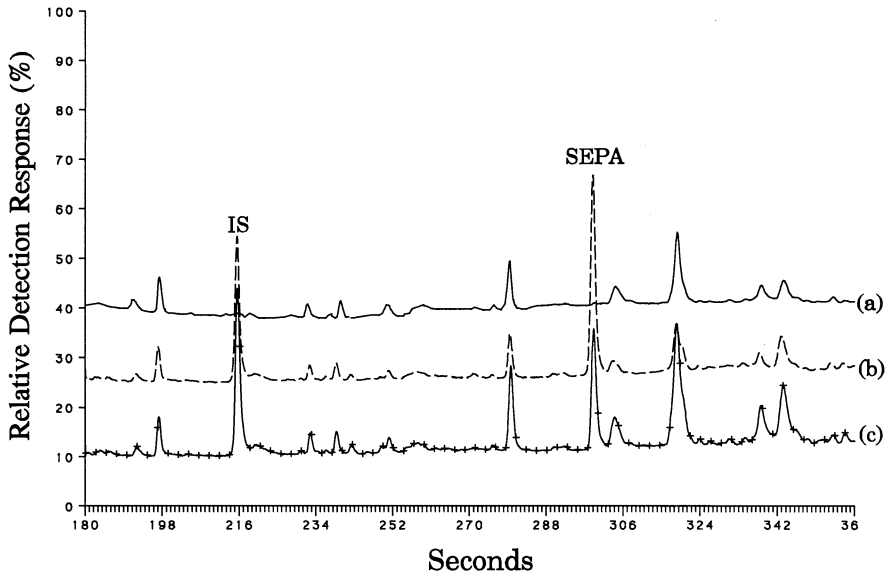

Fig. 6. Typical chromatograms of SEPA in rat serum. Extract of (a) a rat serum blank, (b) a fortified rat serum standard (1000 ng ml^{-1}), and (c) a 1-h post-dose serum sample from a toxicokinetic study.

3. Results and discussion

3.1. Gas chromatography

SEPA and the IS have no chromophore for either ultraviolet or fluorescence detection using liquid chromatography. The gas chromatography with flame ionization or mass spectrometry detection was more suitable for quantitation of SEPA. The GC–FID was the first choice for the method development since it was commonly available for use. A capillary GC column with cross-linked methyl silicone phase was selected based on the SEPA structure. A 0.2 mm diameter and 12 m length capillary column with cross-linked methyl silicone gun phase (Ultra-1, 12 m × 0.2 mm × 0.33 μm, Hewlett Packard, Avondale, PA) gave sharpest peak of SEPA. To achieve the requested sensitivity, a splitless inlet was used for sample injection. The splitless inlet purge delay time was set at 1 min, by which, the majority of the injected sample was introduced into the column and the reproducibility of peak heights for both SEPA and IS were maximized.

Detector temperatures ranging from 250 to 350°C had no effect on the SEPA peak height and therefore, a temperature of 250°C for FID was selected. The peak heights of SEPA and IS increased when the injection inlet temperature increased from 100 to 200°C, but had no further increase from 200 to 300°C (Fig. 3). There was no evidence showing thermal decomposition of SEPA and IS with the inlet temperature up to 300°C. Therefore, an inlet temperature of 250°C was selected to achieve a better assay sensitivity and reproducibility. The oven temperature, which was programmed from 80°C to 185°C at a rate of 20°C min^{-1} and held at 185°C for 1 min, and thereafter to 220°C at a rate of 35°C min^{-1} and held at 220°C for 2 min, provided the best resolution of SEPA or IS from endogenous components, and cleaned the column after each injection as well. Under these chromatographic conditions, the system precision, determined by injecting prepared sample five times, was found to be no greater than 0.5% in most cases.

3.2. Application of solid-phase extraction in sample preparation

The detection limit of this GC–FID method for SEPA, based on a signal-to-noise ratio of 3:1, was

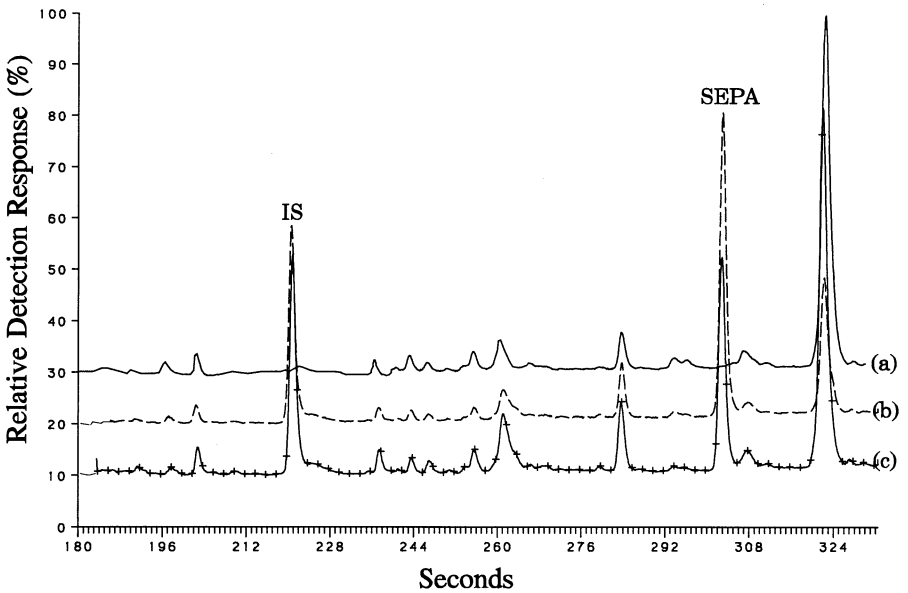

Fig. 7. Typical chromatograms of SEPA in rabbit serum. Extract of (a) a rabbit serum blank, (b) a fortified rabbit serum standard (1000 ng ml^{-1}), and (c) a 1-h post-dose serum sample from a toxicokinetic study.

approximately 25 ng ml^{-1}. However, the maximum SEPA serum concentrations for subjects received the topical treatment of the minoxidil-SEPA formulation were expected to be approximately 20 ng ml^{-1} in humans. Obviously, the instrument sensitivity was far behind from what requested. Since SEPA has no functional group for derivatization, the assay sensitivity can only be improved by introducing a more selective and concentrated sample through sample preparation. Nevertheless, SEPA and IS are very volatile compounds. Any extraction procedures involving evaporation will result in low recovery of SEPA and IS. Both protein parcipitation or liquid–liquid extraction methods need large volume of organic solvents to achive high extraction recovery, as well as the evaporation procedure to generat a more concentrated extract for GC analysis. Therefore, the effort was focused on a solid-phase extraction method for the serum sample preparation since it has been well known that a smaller volume of solvent can be used to elute the compounds from SPE columns to obtain relatively selective and concentrated sample without the need of evaporation. After the investigation of several SPE columns, a 50 mg 1.0 ml^{-1} phenyl (PH) SPE column was found to be sufficient to retain the analytes in 1 ml serum and also provided the possibility of using a minimum volume of elution solvent (100 μl). Consequently, hexane, toluene, methanol, and chloroform as an elution solvent and the elution volume of 100, 200, and 300 μl were evaluated for the absolute extraction recovery. As indicated in Fig. 4, chloroform was the best elution solvent among these solvents as far as the SEPA extraction recovery was concerned. However, as an injection solvent, hexane was the best when the density and expansion ratio were considered. Therefore, a 100 μl of the combination of the two solvents (hexane–chloroform, 1:1, v:v) was finally chosen as the best compromise giving a extraction recovery of 80–95% for both SEPA and IS. By loading 1 ml serum sample to the SPE column and using 100 μl solvent for elution, a 10-fold concentrated extract was obtained for GC analysis without involving any evaporation procedure. 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

Table 1
Intra- and inter-assay accuracy and precision for determination of SEPA in human, rat and rabbit serum

Matrix	Added (ng ml^{-1})	Intra-assay ($n=3$)			Inter-assay ($n=12$)		
		Mean (ng ml^{-1})	RSD (%)	Bias (%)	Mean (ng ml^{-1})	RSD (%)	Bias (%)
Human serum	4.95	5.2	2.4	+4.0	5.2	5.0	+4.0
	49.5	50.0	9.0	0	51.0	8.0	+3.0
	198	213	4.0	+7.6	199	10	+0.9
Rat serum	49.5	48.5	4.0	−2.0	52.0	7.0	+5.0
	495	510	6.0	+3.0	510.0	8.0	+3.0
	1980	2030	7.0	+2.5	1960.0	8.0	−10
Rabbit serum	49.5	49.0	6.0	−2.0	48.6	5.0	−1.8
	495	468	2.4	−5.4	512	4.0	+3.0
	1980	1770	4.0	−11	1890	8.0	−4.6

chromatograms. With the previously described extraction procedures, the limit of quantitation reached 2.5 ng ml^{-1}. The extraction recoveries were comparable when various amounts of serum ranging from 0.1 to 1 ml were used for extraction as long as an additional amount of water was added to make an approximately total volume of 1 ml prior to the extraction. Representative chromatograms after extraction of serum blank, fortified serum standards, and post-dose serum samples for human, rat, and rabbit are shown in Figs. 5–7, respectively.

3.3. Linearity and sensitivity

Linear calibration graphs were obtained over the concentration range of 2.5–250 ng ml^{-1} (using 1 ml human serum) or 25–2500 ng ml^{-1} (using 0.1 ml rat or rabbit serum) with correlation coefficients greater than 0.999 and intercepts not significantly ($p > 0.05$) different from zero. Based on the low concentration data of these calibration curves, the limit of quantitation was 2.5 or 25 ng ml^{-1} when 1 ml or 0.1 ml serum sample was used for extraction.

3.4. Specificity

Specificity of this method has been demonstrated by the representative chromatograms for human, rat, and rabbit serum shown in Figs. 5–7. SEPA and IS were well resolved from the human, rat, or rabbit serum endogenous material peaks. Additional blank human serum from several individuals and blank rat and rabbit serum from different pools have also been tested and showed no significant interference at the retention times of the compounds of interest.

3.5. Precision and accuracy

The relative standard deviation (RSD) of the slopes from the calibration curves was 0.8, 4.0 and 3.8% for human, rat and rabbit serum, respectively, showing excellent system precision. The intra- and inter-assay precision and accuracy data expressed by the QC data from each analytical run for the human, rat, and rabbit serum are summarized in Table 1. Both RSD and Bias for intra and inter assays were $\leq 10\%$, showing that the measured concentrations of QC samples are in good agreement with the actual concentrations, and the assay reproducibility is excellent.

3.6. Stability

As shown in Table 2, SEPA concentrations of low, medium, and high QC samples during the assay validation were not significantly different ($p > 0.05$) when analyzed freshly after preparation, after 8 h of storage at room temperature, or after two-week, four-week, and 4 months of storage at $-15°$ to $-25°$C, indicating that this compound was stable at least for 8 h in human serum

Table 2
Stability of SEPA in human and rat serum after storage

Matrix	Added (ng ml^{-1})	Measured SEPA concentration (ng ml^{-1})					p[a]
		Fresh	8 h at 22°C	2 weeks at −20°C	4 weeks at −20°C	4 months at −20°C	
Human serum	5.0	5.2 ± 0.1	5.0 ± 0.2	5.2 ± 0.2	5.0 ± 0.1	5.2 ± 0.2	0.575
	49.5	50.0 ± 4.5	45.6 ± 1.3	48.0 ± 3.4	51.3 ± 2.0	46.8 ± 2.8	0.410
	198.0	213.0 ± 8.5	188.0 ± 8.8	187 ± 20.6	188.0 ± 24.2	182.4 ± 6.3	0.241
	Overall						0.994
Rat serum	49.5	54.6 ± 2.2	—[b]	48.5 ± 1.9	53.0 ± 5.3	51.8 ± 2.1	0.220
	495.0	508 ± 10.7	—	510.0 ± 30.6	469.0 ± 18.8	503.0 ± 25.2	0.164
	1980.0	1760.0 ± 52.8	—	2030.0 ± 149.7	2020.0 ± 161.6	2040 ± 102.0	0.071
	Overall						0.996

[a] p-value was derived by analysis of variance.
[b] Not determined.

when stored at room temperature and for 4 months in human and rat serum when stored at −15° to −25°C. The mean recoveries for each concentration of the QC samples after one, two, three, or four freeze/thaw cycles ranged from 90–110% when quantitated against a freshly prepared serum standard curve (Table 3), indicating that SEPA was stable in human serum after four normal freeze/thaw cycles. The stability of SEPA in rabbit serum was similar to rat and human serum. The extracts of prepared samples (in hexane:chloroform, 1:1, v:v) were stable at least for 24 h when placed in an autosampler tray (0–4°C) and the vials were tightly caped.

3.7. Application

The GC–FID method was successfully applied to assay serum samples from preclinical toxicokinetic studies and clinical pharmacokinetic studies with the topical treatment of the minoxidil-SEPA formulation. As an example, SEPA serum concentration–time data on day 21 for subjects who received topical treatment twice a day for 20 days and a single dose on day 21 are presented in Fig. 8. Serum concentrations could be detected over 12 h for subjects who received treatment A (50 mg SEPA) and 48 h for subjects who received treatment C (150 mg SEPA) by using this method.

4. Conclusion

The described GC–FID method for the quantitative determination of SEPA in serum has sufficient sensitivity to make it possible for evaluating toxicokinetics and pharmacokinetics of SEPA in animals and in man for the newly developed minoxidil-SEPA formulation. The assay is rapid

Table 3
Stability of SEPA in human serum after freeze/thaw cycles

Added (ng ml^{-1})	Measured SEPA concentration (ng ml^{-1})					p[a]
	Fresh	1 cycle	2 cycles	3 cycles	4 cycles	
5.0	5.2 ± 0.1	5.2 ± 0.2	5.2 ± 0.1	5.0 ± 0.4	5.0 ± 0.1	0.407
49.5	50.0 ± 4.5	46.8 ± 2.8	46.8 ± 1.5	53.6 ± 4.3	48.9 ± 3.7	0.552
198.0	213.0 ± 8.5	182.4 ± 6.3	200.0 ± 4.8	202.7 ± 6.5	186.0 ± 7.2	0.057
Overall						0.998

[a] p-value was derived by analysis of variance.

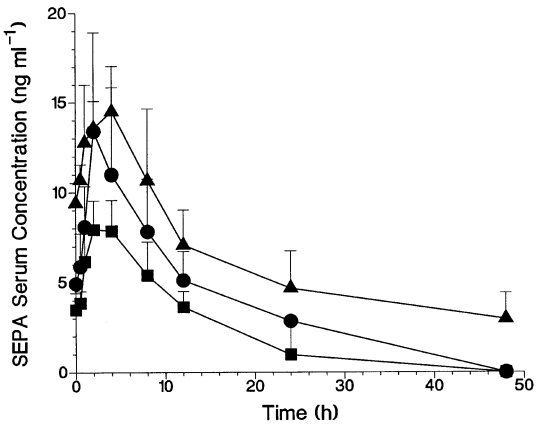

Fig. 8. Mean SEPA serum concentration–time data on day 21 for health volunteers who received the minoxidil-SEPA formulation twice a day for 20 days and once a day for day 21. (■), (●), and (▲) represent treatment A (50 mg SEPA), treatment B (100 mg SEPA), and treatment C (150 mg SEPA), respectively.

and simple, and requires only commonly available instruments. A large number of samples can be processed daily (about 100). This work also shows the success of solid-phase extraction for producing a selective and concentrated sample with high extraction efficiency to achieve a great improvement in the assay sensitivity for a volatile compound like SEPA, particularly.

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