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Automated Gas Chromatography/Tandem Mass Spectrometry with On-line Chemical Derivatization for the Determination of Tebufelone and Two Metabolites in Human Plasma

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An automated capillary gas chromatography/tandem mass spectrometry (GC/MS/MS) assay for the simultaneous quantitation of tebufelone (TE) and its two major metabolites (PGE-1802413 and PGE-6285825) in plasma was developed. Using a 1:1 BSTFA:pyridine derivatization cocktail to solubilize plasma extracts, trimethylsilylation (TMS) of labile alcohol and carboxylic acid functional groups occurred instantly upon introducing each sample into a 300 °C GC injection port. This on-line chemical derivatization process rendered these three diverse analytes equally amenable to GC analysis and circumvented laborious off-line derivatization procedures.

The selectivity of MS/MS conducted on a triple-quadrupole instrument allowed minimal sample preparation and rapid analysis. Electron ionization produced molecular ions ($M^{\cdot+}$) for TMS-TE, TMS_2-PGE-1802413, TMS_2-PGE-6285825 and their respective stable-isotope-labeled internal standards, which were selected in Q1 to undergo collisionally activated dissociation in Q2. Quantitation was achieved through monitoring product ions in Q3 at m/z 320, 445 and 305 for respective analytes, relative to corresponding internal standard ions at m/z 323, 449 and 305. A 2.5–1000 ng per sample (approximately 25 pg to 10 ng injected) quantitation range provided access to an effective 2.5–10 000 ppb plasma concentration range (0.1–1 ml samples) for each analyte.

Based on quality control data accumulated throughout 8 months of method application, the assay showed no bias and composite ($N = 212$) relative standard deviations of 5.6%, 7.0% and 9.5% for the respective analytes (with quality control levels typically covering a range of 10–250 ng per analyte). During this period, more than 2000 plasma study samples were analyzed, attesting to the reliability and ruggedness of this approach for routine application. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Tebufelone (TE), 1-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-5-hexyn-1-one (also referred to as NE-11740), is a member of a class of lipophilic di-*tert*-butyl phenols that have undergone extensive evaluation as potential anti-inflammatory agents. Previous studies have shown TE to have two major active Phase-I metabolites in plasma, an alcohol (4-OH-TE; PGE-1802413) and a carboxylic acid (4-TE-A; PGE-6285825), as shown in Fig. 1(a). As with any new pharmaceutical candidate, studies to determine key pharmacokinetic parameters of the parent drug and major active metabolites must be carried out, under a variety of dosing conditions, across several animal species and in humans. To support these studies, an assay was needed to quantitate these three very different chemical species in plasma, down to low ppb levels. Adding to the method development challenge was the anticipation of several thousand preclinical and clinical samples, where analyte concentrations in plasma might range from low ppb [e.g. 24 h following a single, low peroral (p.o.) dose] up to 10 ppm (e.g. for multiple-p.o.-dose, range-finding pharmacokinetic studies in animals) levels. Thus this assay not only needed to be sensitive and applicable to three compounds of diverse functionality, but a wide linear range and a high throughput (speed) capability were also essential.

Previously reported methodology for TE-only employed gas chromatography with tandem mass spectrometry detection (GC/MS/MS).[1,2] This approach has proven reliable and rugged in application to several thousand preclinical and clinical plasma samples. The electron ionization (EI) selected reaction monitoring (SRM) MS/MS detection scheme uniquely detects TE and its stable-isotope-labeled internal standard. The

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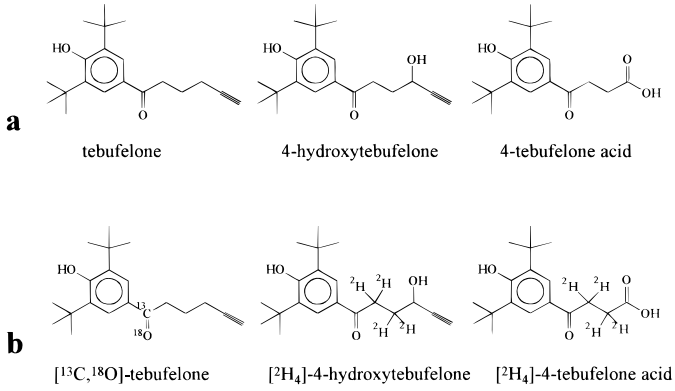

Figure 1. Structures of (a) analytes TE, 4-OH-TE and 4-TE-A and (b) corresponding stable-isotope-labeled internal standards TE-CO, d_4-4-OH-TE and d_4-4-TE-A.

selectivity of this method afforded a very simple sample clean-up (extraction with ethyl ether), followed by run times of about 3 min per sample.

Strategy for development of an assay for the simultaneous quantitation of TE, 4-OH-TE and 4-TE-A sought to build on the successful TE-only method. Not surprisingly, however, difficulties arose in using the GC to introduce the two metabolites into the ion source of the mass spectrometer. Briefly, 4-OH-TE would generally pass through a new GC column without degradation. However, after several injections of crude matrix extracts, increasing proportions of 4-OH-TE underwent dehydration in the injector [evidenced by the growing presence of a molecular weight (MW) 298 degradant in resulting chromatograms]. Even worse, 4-TE-A would not pass through the GC column under any conditions explored.

Recognizing the need for chemical derivatization to stabilize the alcohol and acid functional groups of these metabolites prior to GC/MS/MS analysis, several off-line derivatization approaches were initially explored. These included acetylation, methylation trimethylsilylation (TMS) and combinations thereof. In the end, each off-line derivatization procedure had at least two of the following key shortcomings: (a) lowering pH to catalyze some reactions caused unacceptable loss of isotopic labels from the internal standards; (b) unacceptably low chemical conversion efficiency; (c) instability of some derivatized products (e.g. TMS_2-4-TE-A not observed following off-line procedure); (d) dangerous reagent (e.g. diazomethane) required; (e) laborious multi-step derivatization processes.

To circumvent the above shortcomings, an on-line trimethylsilylation process was discovered and optimized. This technique provides near-quantitative protection of all phenol, hydroxyl and carboxylic acid functional groups, as TMS ethers or esters, upon introduction into the heated injection port of the GC. This approach not only enables simultaneous trace-level quantitation of all three analytes, but it accomplishes this with no more steps than required in the original TE-only GC/MS/MS method.[1] That is, instead of dissolving the dried ether extract in an organic solvent, it is reconstituted in a trimethylsilylation cocktail prior to injection. The final method is described in this paper, along with a discussion of its performance over 8 months of routine application to clinical plasma samples.

EXPERIMENTAL

Chemicals and reagents

The TE reference standard was synthesized at Procter & Gamble Pharmaceuticals, Norwich, NY and determined to be 99.8% pure by HPLC.[3] The 4-OH-TE and 4-TE-A reference standards, as well as all three stable-isotope-labeled internal standards (Fig. 1b), were synthesized at Procter & Gamble's Miami Valley Laboratories, Cincinnati, OH. Chemical purities for the metabolite reference standards were determined to be 94.5% and 99.8% respectively based on HPTLC and elemental analysis. The [^{13}C,^{18}O]-TE (TE-CO) isotope incorporation at the carbonyl position was determined by GC/(EI)MS to be 98.1 and 94.7 at.% excess for ^{13}C and ^{18}O respectively, leaving less than 0.2% devoid of isotopic labels. Full ^{2}H enrichment (i.e. all four deuteriums incorporated) was achieved for 38% of the stable-isotope-labeled 4-OH-TE (d_4-4-OH-TE) material, with less than 0.4% devoid of isotopic labels. Likewise, full ^{2}H enrichment was achieved for 64% of the d_4-4-TE-A, with less than 0.4% devoid of isotopic labels. These levels of isotopic enrichment were adequate to meet the needs of the present assay. The [^{14}C]-TE used in the TE absolute recovery experiment was also synthesized at Procter & Gamble's Miami Valley Laboratories. The level of radioactivity was 14.4 μCi μmol^{-1} TE, corresponding to a ^{14}C incorporation at the carbonyl position of about 23%. Unless otherwise specified, solvents were reagent ACS grade, obtained from standard suppliers, and used without further purification. Silylation grade pyridine was obtained from Alltech Associates, Deerfield, IL (# 275-3050). The bis(trimethylsilyl)trifluoroacetamide (BSTFA) reagent was obtained from Aldrich, Milwaukee, WI (# 15,419-5). Blank human plasma was obtained from Ohio Blood Plasma, Inc., Cincinnati, OH.

Sample preparation

Preparation of working standards. All reference standard and corresponding internal standard stock solutions were individually prepared in absolute ethyl alcohol (USP). Working standards, used to simultaneously generate calibration curves for TE, 4-OH-TE and 4-TE-A, were prepared by combining equal amounts of each

compound along with 40, 80 and 40 ng of TE-CO, d_4-4-OH-TE and d_4-TE-A respectively. Quantitative transfers were made using Wiretrol calibrated micropipets (Drummond Scientific Co., Broomall, PA). These ethanolic solutions were then taken to dryness under nitrogen and subsequently reconstituted in 100 μl of 1:1 BSTFA:pyridine and transferred to autosampler microvials prior to analysis. Working standards were typically prepared at 2.5, 5, 10, 25, 50, 100, 250, 500 and 1000 ng (per analyte) levels.

Preparation of plasma samples. Spiked plasma samples were employed for method validation or for quality control (QC) during method application. Typically, 100 μl of an ethanolic solution containing 40, 80, and 40 ng of TE-CO, d_4-4-OH-TE and d_4-TE-A respectively were applied to the bottom of a 13 mm × 100 mm borosilicate centrifuge tube, followed by addition of an ethanolic solution containing the three reference standards at the desired nanogram per sample level. The ethanol was then removed under nitrogen and an aliquot of blank plasma (volume selected to match study sample volumes used throughout the associated batch run) added to the tube. Tubes were then capped, vortexed and allowed to stand for 10 min at room temperature. Study plasma samples (unknowns) were prepared identically, except only the internal standards were added to the centrifuge tubes. Study plasma sample volumes ranged from 0.1 to 1.0 ml, depending on the anticipated drug and metabolite concentration ranges. For example, 1.0 ml plasma aliquots were employed for single-low-dose studies, while 0.1 ml was more commonly employed for single-high-dose or multiple-dose studies. [*Note.* For reasons not fully understood, addition of ethanolic standards directly to plasma ultimately gave rise to problematic 'over-derivatization'. That is, each analyte tended to form an additional derivative (i.e. addition of one more TMS group than desired) in a difficult-to-reproduce fashion. Qualitative studies indicated that the extra derivative was located at the carbonyl group, which was trapped as a TMS ether, presumably made possible by a keto–enol tautomerism. However, by using the approach of dissolving analyte and internal standard residues in plasma, this phenomenon was avoided.]

Liquid–liquid extraction (LLE) of spiked plasma samples was accomplished using 2 ml of ethyl ether. The aqueous layers were frozen by placing the centrifuge tubes in an acetone/dry-ice bath. Ether layers were then transferred to a clean glass vial, taken to dryness under nitrogen, reconstituted in 100 μl of 1:1 BSTFA:pyridine and then transferred to autosampler microvials prior to analysis.

Absolute analyte recovery from LLE. The absolute recovery of TE from the sample preparation procedure was determined by spiking [^{14}C]-TE into a 1 ml aliquot of blank plasma and carrying it through the usual LLE procedure. Radioactivity present in the ether layer was then measured using scintillation counting and compared with the radioactivity initially spiked in the plasma, as a measure of absolute recovery. The mean TE recovery was 93.9%, with a 6.1% RSD ($N = 6$). However, because radiolabeled metabolite standards were not available, absolute recovery of each metabolite was estimated using ^{14}C-labeled metabolites generated *in vivo* (following administration of [^{14}C]-TE). Glucuronidase-treated human urine and monkey plasma, each selected to contain predominantly (>80%) 4-OH-TE and 4-TE-A respectively (based on HPLC profiling with on-line radiochemical detection), were, in turn, spiked into blank human plasma and processed, as described above. Based on these experiments, the mean absolute recovery for 4-OH-TE was estimated to be 101.6%, with a 6.5% RSD ($N = 6$). The mean absolute recovery for 4-TE-A was estimated to be 77.0%, with an 8.5% RSD ($N = 6$).

GC/MS/MS conditions

A Finnigan CTC-A200S autosampler combined with a Finnigan Model 9611 gas chromatograph was used to derivatize the sample and introduce it into the ion source of a Finnigan TSQ-46C triple-stage quadrupole mass spectrometer equipped with a SuperIncos data system. The GC/MS/MS analysis was automated via user-defined macro programs generated within the data system. This allowed computer control of all assay steps, including data reduction. Total cycle time per GC/MS/MS run was about 6 min. Automated data reduction (i.e. chromatogram deconvolution and peak integration) was typically initiated immediately following the batch run for each day of method application.

A 1.2 μl aliquot of the 1:1 BSTFA:pyridine solution was injected in the splitless mode. The fused silica capillary column was a 10 m × 0.25 mm i.d. Rtx-5 (Restec Corporation, Bellefonte, PA), with a 0.25 μm film thickness. After a 0.3 min hold at 165 °C, oven temperature was ramped to 290 °C at 25 °C min^{-1}, with a final hold of 0.5 min. Other conditions were: 300 °C injector; 300 °C transfer line/interface; 30 cm s^{-1} helium at 165 °C.

EI conditions were as follows: source temperature set at 190 °C; 0.30 mA emission current at 70 eV. The MS/MS detection schemes utilized collisionally activated dissociation (CAD) and SRM, with CAD conditions set at 2.0 mTorr argon and 9.9 eV ion energy for all analytes. A multi-experiment was created within the SuperIncos data system, which divided each analytical run into three parts as follows. *Part 1* was a 'dummy' period where detector and source electronics were 'off' from injection until just prior to elution of TE. This helped method ruggedness (e.g. filament off while solvent and less volatile matrix components eluted) and minimized consumption of data space. *Part 2*, the middle portion of the acquisition, was designed for quantitation of TE. The *m/z* 372-to-320 and *m/z* 375-to-323 SRM transitions were sequentially monitored for detection of TMS-TE and TMS-TE-CO respectively. The dwell time for each ion reaction was 118 ms, resulting in a total cycle time of about 240 ms during this acquisition period. *Part 3*, the last portion of the acquisition, was designed for quantitation of 4-OH-TE and 4-TE-A. The *m/z* 450-to-305, 454-to-305, 460-to-445 and 464-to-449 SRM transitions were sequentially monitored for detection of TMS_2-4-TE-A, TMS_2-d_4-4-TE-A, TMS_2-4-OH-TE and TMS_2-d_4-4-OH-TE respectively.

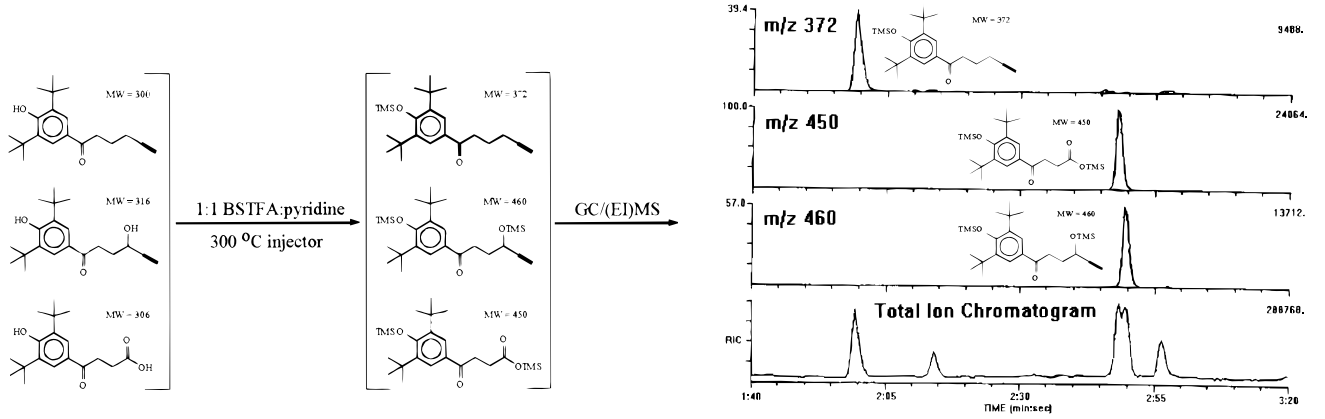

Figure 2. Flow diagram for chemistry (left) and chromatographic separation for on-line trimethylsilylation process, as applied to TE, 4-OH-TE and 4-TE-A. Extracted single-ion chromatograms (right) at *m/z* 372, 450 and 460 represent $M^{\cdot+}$ ions of each major product formed from respective analytes (10 ng of each compound injected).

The dwell time for each ion reaction was 60 ms, resulting in a total cycle time of about 240 ms during this acquisition period. Product ion mass windows were set at 0.250 Da for all detection schemes. The conversion dynode, electron multiplier and preamplifier sensitivities were set at −5 kV, −1800 V (typically) and 10^{-7} A V^{-1}, respectively.

Quantitation procedure

Quantitative determinations were made by first preparing and analyzing working standards covering the range of interest. Response factor (RF) values for TE, 4-TE-A and 4-OH-TE were simultaneously calculated for each working standard, based on peak areas from the *m/z* 372-to-320 (TE), *m/z* 450-to-305 (4-TE-A) and *m/z* 460-to-445 (4-OH-TE) SRM channels, relative to the SRMs of their respective internal standards (IS), according to the general equation

$$RF_a = (A_a/[a])([IS]/A_{IS}) \quad (1)$$

where A_a is the peak area determined for TE, 4-TE-A or 4-OH-TE and A_{IS} is the corresponding internal standard peak area. [*a*] and [IS] are the concentrations (nanograms per sample) of the analyte and internal standard respectively.

Mean response factors (MRFs) were calculated for TE, 4-TE-A and 4-OH-TE from their respective RF values, covering the entire quantitation range. These MRF values were used by the data system to automatically calculate TE, 4-TE-A and 4-OH-TE amounts in each sample subsequently analyzed.

RESULTS AND DISCUSSION

On-line trimethylsilylation

Represented in Fig. 2 is the process by which TE and its two metabolites are efficiently derivatized and chromatographed. All derivatizable functional groups are instantly and efficiently trimethylsilylated upon their introduction into the GC injection port from their 1:1 BSTFA:pyridine solution. Depicted are the results from GC/(EI)MS (full-scan) analysis of an injected mixture containing 10 ng of each analyte. As shown in the extracted single-ion chromatograms, TMS-TE elutes early, while TMS_2-4-TE-A and TMS_2-4-OH-TE essen-

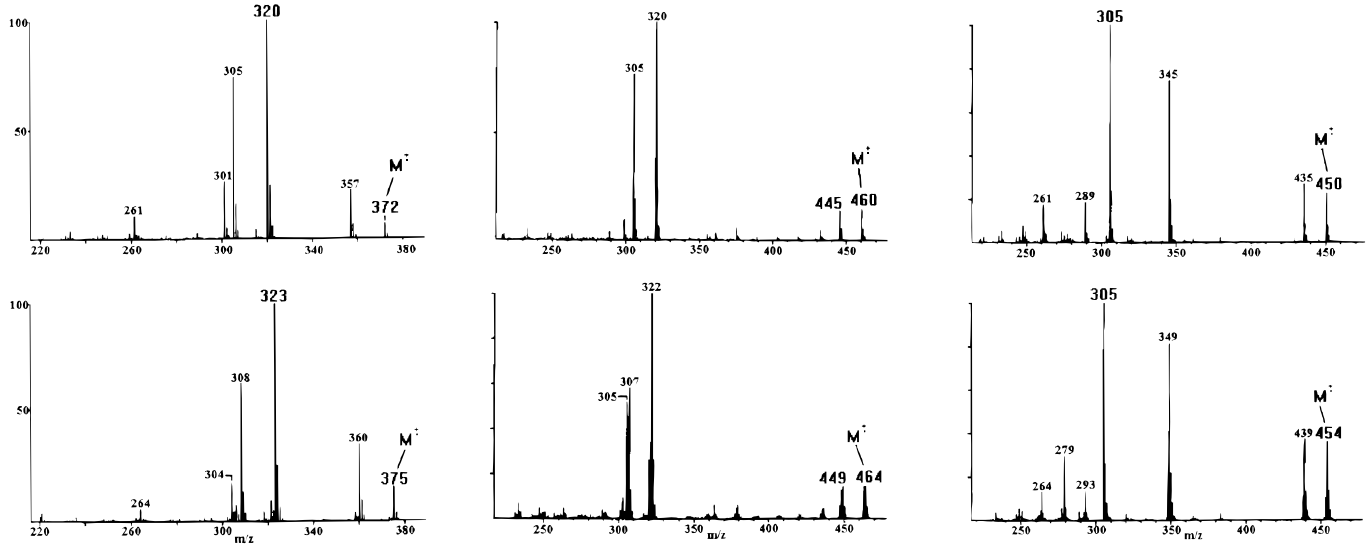

Figure 3. EI mass spectra of TMS-TE (top left), TMS_2-OH-TE (top center) and TMS_2-4-TE-A (top right), along with those of their corresponding stable-isotope-labeled internal standards (bottom).

tially coelute at a later time. This gave rise to the staggered detection scheme utilized (see Experimental). Such full-scan runs were employed in experiments to optimize chromatography (peak shape and analysis time) as well as derivatization efficiency (cocktail composition and injection port temperature). For example, during method development, direct evidence for incomplete derivatization was evident by monitoring underivatized TE and 4-OH-TE (or its degradant) and TMS-4-OH-TE (partially derivatized) *vs.* their fully derivatized counterparts. However, because no degradants or partially derivatized forms of 4-TE-A were detected, optimization for this compound concentrated on maximizing the signal for its fully derivatized form.

In addition to pyridine, hexane and 4-decanone were evaluated as base solvents for the derivatization cocktail. Ratios for each ranging from approximately 1:5 to 5:1 with BSTFA were tested. Optimal performance for all three analytes was obtained with the final 1:1 BSTFA:pyridine composition, from which only the fully derivatized forms of TE and 4-OH-TE were observed and the signal from TMS_2-4-TE-A was maximized (and comparable with that of TMS_2-4-OH-TE in magnitude). Also, even with this optimized cocktail, derivatization efficiency began to diminish when the injection port temperature dropped much below 280 °C. Finally, it was found that trace moisture was detrimental to the on-line derivatization process, prompting extension of the ethyl ether dry-down periods (i.e. beyond the visual dryness point) as a precaution.

SRM detection schemes

For reference, conventional EI mass spectra of fully derivatized TE, 4-OH-TE and 4-TE-A and their corresponding stable-isotope-labeled internal standards are shown in Fig. 3. These were used as starting points for developing optimal SRM (MS/MS) detection schemes for each analyte. In short, molecular ions were used as precursor ions and the CAD processes were optimized for formation of respective product ions that provided the best compromise between sensitivity and selectivity *vs.* matrix components, while minimizing signal overlap between each analyte and its internal standard. Product ion spectra of the derivatized analytes and their corresponding internal standards were obtained under optimized CAD conditions, as displayed in Fig. 4. For TMS-TE and TMS-TE-CO the γ-hydrogen rearrangement product ions at m/z 320 and 323 were selected. For TMS_2-4-TE-A and TMS_2-d_4-4-TE-A the product ion resulting from cleavage α to the side-chain carbonyl group at m/z 305 was selected for both. For TMS_2-4-OH-TE and TMS_2-d_4-4-OH-TE the $(M - CH_3^{\bullet})^+$ product ions at m/z 445 and 449 were selected. Backbone cleavages used in the SRM schemes for TE and 4-TE-A quantitation are highly selective for these analytes, while the somewhat generic 15 Da neutral loss monitored for 4-OH-TE would normally not be preferred. This selection was made because the only major product ion observed from CAD of derivatized 4-OH-TE (under reasonable collision conditions) resulted from loss of a methyl group, unlike CAD product spectra for derivatized TE and 4-TE-A (both of which showed similar fragments to those observed in the EI process). Specifically, the γ-hydrogen rearrangement product and the product resulting from cleavage α to the carbonyl group that were produced strongly for derivatized TE and 4-TE-A respectively under relatively mild CAD conditions and both of which were expected for derivatized 4-OH-TE, based on EI fragmentation

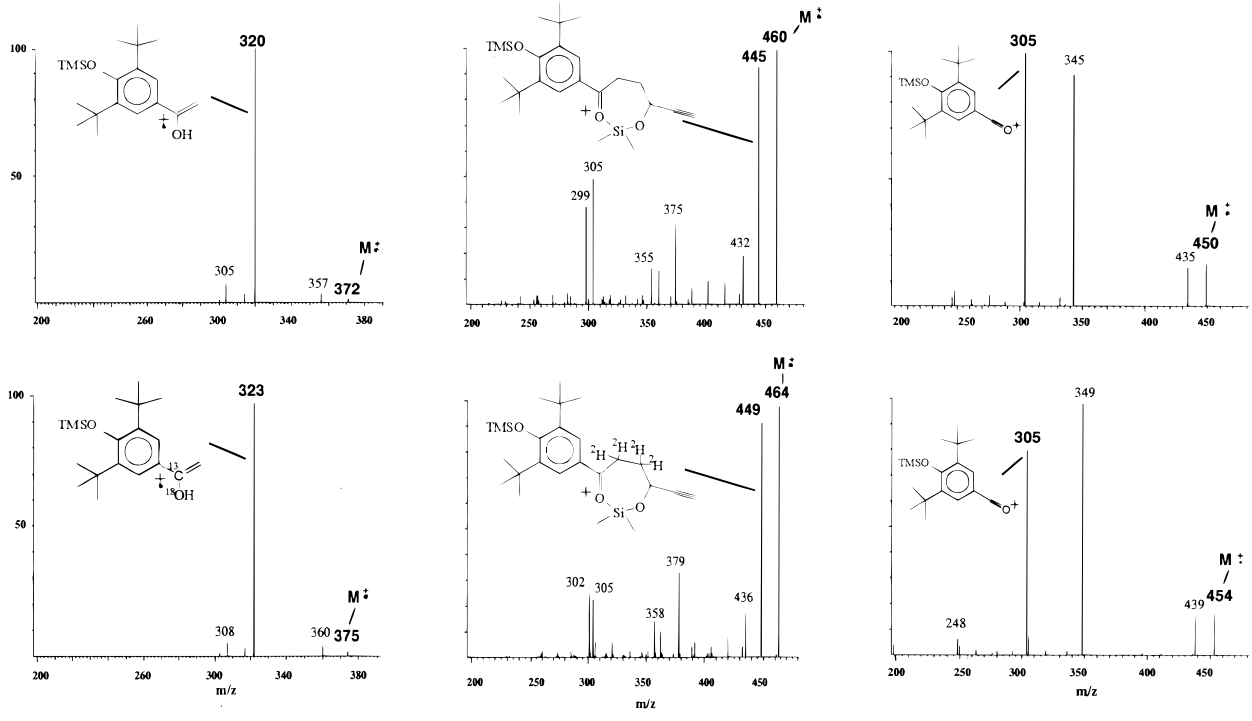

Figure 4. Product ion mass spectra of TMS-TE (top left), TMS_2-4-OH-TE (top center) and TMS_2-4-TE-A (top right) molecular ions, along with those of their corresponding stable-isotope-labeled internal standards (bottom), obtained under optimized CAD conditions. Highlighted, with proposed structures, are product ions selected for the SRM detection schemes.

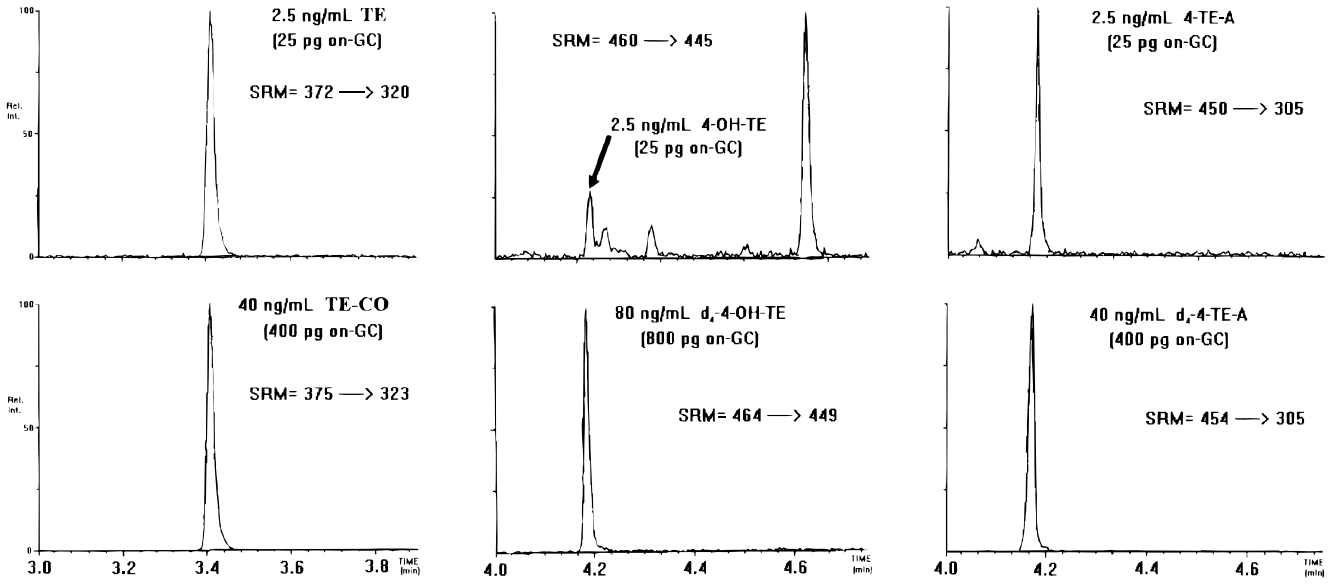

Figure 5. Six SRM chromatograms obtained from a single GC/MS/MS analysis of an extract from 1 ml of human plasma containing 2.5 ng ml^{-1} of each analyte (top) and method-prescribed levels of their respective internal standards (bottom).

data, were nearly absent in the CAD spectra for this compound. One possible explanation for these results stems from the position of the side-chain TMS ether. Although $(M - CH_3^{\cdot})^+$ observed in the EI spectrum of TMS_2-4-OH-TE can result from fragmentation at one of three positions, it is suggested that the structure postulated in Fig. 4 predominates under the less energetic CAD conditions. Once formed, this cyclic product ion would be relatively stable against subsequent fragmentation. This is also consistent with the absence of lower-mass CAD ions that are typically observed for this class of compounds when products-of-$(M - CH_3^{\cdot})^+$ experiments are conducted (data now shown). Nevertheless, all SRM detection schemes that were chosen provided sufficient sensitivity and selectivity to meet the method objectives, as described below.

An indication of the selectivity and sensitivity of the SRM detection schemes used is provided in Fig. 5. Segments of six SRM chromatograms, obtained from the GC/MS/MS analysis of 1 ml of human plasma spiked with 2.5 ng of each analyte plus prescribed levels of their respective internal standards, are shown. This demonstrates adequate signal-to-noise at the 2.5 ng ml^{-1} lower limit of quantitation (LLOQ) required for each compound. Given the signal strength for derivatized TE and 4-TE-A and the fact that their SRM detection schemes uniquely detect them, it is evident that the LLOQ for these analytes could be significantly lowered. However, it is unlikely that the LLOQ could be improved for 4-OH-TE without significant method improvements. This is due to the relatively modest selectivity provided by its SRM detection scheme that also permits detection of some matrix components and must therefore rely more heavily on chromatographic resolution to meet its selectivity requirement.

Method performance

Method performance was monitored each day of method application by evaluating the quality of working standard data. In addition, QC samples were prepared to cover the daily concentration range of interest, typically at two or three analyte levels ($N = 3$, 4 or 5, depending on batch size), and analyzed as though they were unknowns. This permitted a daily assessment of method accuracy and precision. By compiling daily method performance data over an 8 month

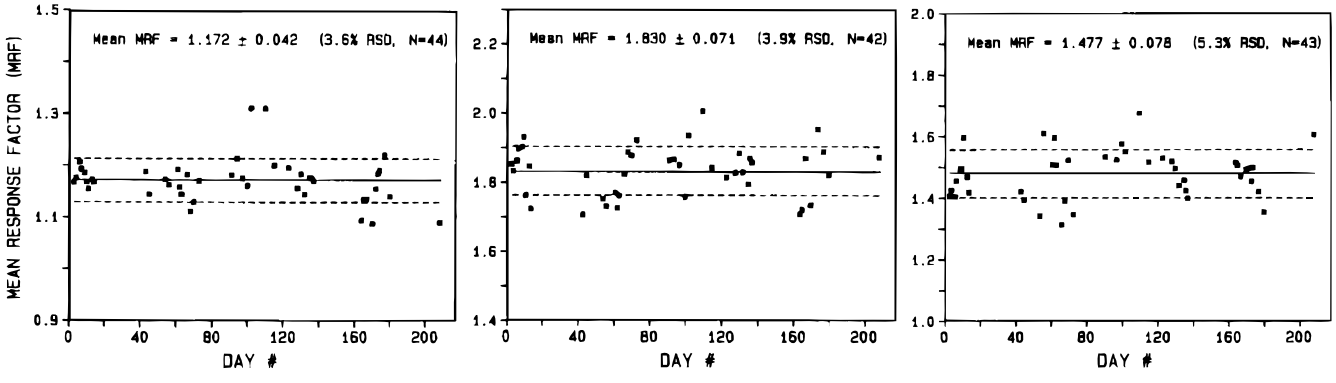

Figure 6. Distribution of mean response factors for TE (left), 4-OH-TE (center) and 4-TE-A (right) from working standard sets analyzed over an 8 month period of method application. The mean MRF is designated with a full line. The broken lines bracket ± one standard deviation from the mean.

Table 1. Quantitative determination of TE, 4-OH-TE and 4-TE-A in spiked plasma (QC) samples[a]

Analyte	Amount spiked (ng)[b]	*N*	% Recovery[c]	% RSD
TE	10	32	110.5	4.6
	25	71	102.9	3.2
	50	29	99.4	2.8
	100	58	100.3	2.5
	250	22	93.4	4.9
Composite TE results		*212*	*101.9*	*5.6*
4-OH-TE	10	32	98.9	9.0
	25	71	98.3	6.5
	50	29	95.3	5.2
	100	58	99.4	6.5
	250	22	92.5	4.1
Composite 4-OH-TE results		*212*	*97.7*	*7.0*
4-TE-A	10	32	106.1	13.0
	25	71	101.0	10.0
	50	29	99.2	5.4
	100	58	99.0	6.5
	250	22	96.5	7.9
Composite 4-TE-A results		*212*	*100.5*	*9.5*

[a] Summary of QC results over 8 months of method application.
[b] All QC samples were prepared through the quantitative addition of known amounts of TE, 4-OH-TE and 4-TE-A to aliquots of blank plasma (0.1–1.0 ml, depending on study).
[c] % Recovery values are the % TE, 4-OH-TE or 4-TE-A measured relative to the amount spiked into each sample.

Table 2. Inter-assay comparison of TE-only results from clinical samples

TE concentration range (ng ml^{-1})	*N*	% Method bias[a]	% MADM[b]
<LLOQ	64	All were consistent[c]	
2.5–9.9	52	−5.2	4.9
10–49.9	74	0.8	5.6
50–199	81	−0.1	3.5
200–650	43	5.3	3.5

[a] The % method bias calculation arbitrarily uses TE results from the present assay as a reference for comparison with TE-only assay results[1] obtained 9 months earlier from the same set of samples.
[b] Percent mean average deviation from the mean (% MADM) is the mean of all individually calculated % ADM values within each concentration range. % ADM is the absolute value of the difference between one of the two independent TE measurements and the mean of the two measurements ($M_{1,2}$), normalized to $M_{1,2}$.
[c] Results from the present assay showing TE levels under 2.5 ng ml^{-1} (i.e. <LLOQ) were all previously determined by the TE-only assay[1] to be either under 1.0 ng ml^{-1} (its LLOQ) or measured in the 1.0–2.4 ng ml^{-1} range.

period of method application, a clear picture of method ruggedness and reproducibility was developed, as described below.

Working standard (calibration) data. The MRF was used, along with analyte and internal standard peak areas, to calculate analyte amounts in unknown plasma samples (see Experimental). However, the % RSD, calculated for a given set of RF values, served as a daily composite indicator of method precision and linearity. Working standard data performance was deemed acceptable for an analyte if its RF% RSD value was less than 15%. Over 8 months of method application, more than 40 sets of working standards were analyzed (2.5–1000 ng per sample working range per analyte), with mean RF% RSDs of 7.1% ($N = 44$), 7.4% ($N = 42$) and 3.6% ($N = 43$) for TE, 4-OH-TE and 4-TE-A respectively. As an added indicator of working standard data quality, linear correlation coefficients (r^2) were consistently greater than 0.999 for all three analytes. Plots of MRF values over this period for each analyte (Fig. 6) demonstrate the remarkable day-to-day consistency of this method.

Overall method accuracy and precision (QC data). Typically, QC plasma samples were spaced evenly throughout each daily GC/MS/MS batch run. Results of the QC assays provided a daily indication of method accuracy, expressed as percent (%) recovery, relative to the known amount spiked for each analyte. Table 1 provides a composite of all QC results obtained over an 8 month period of method application. These data verify acceptable method accuracy (i.e. no significant bias) and precision across all three analytes at the five QC levels employed. Precision values (% RSD) reported here actually represent a worst-case scenario because they were not determined for a 1 day application (as is typically reported). Rather, they represent the superposition of daily and day-to-day variability across more than 40 batch runs.

Another indicator of method consistency was the fact that the mean % recovery (MPR) for any analyte on any given day was never out of the 100% ± 13% range. Plots illustrating the MPR values for each analyte over

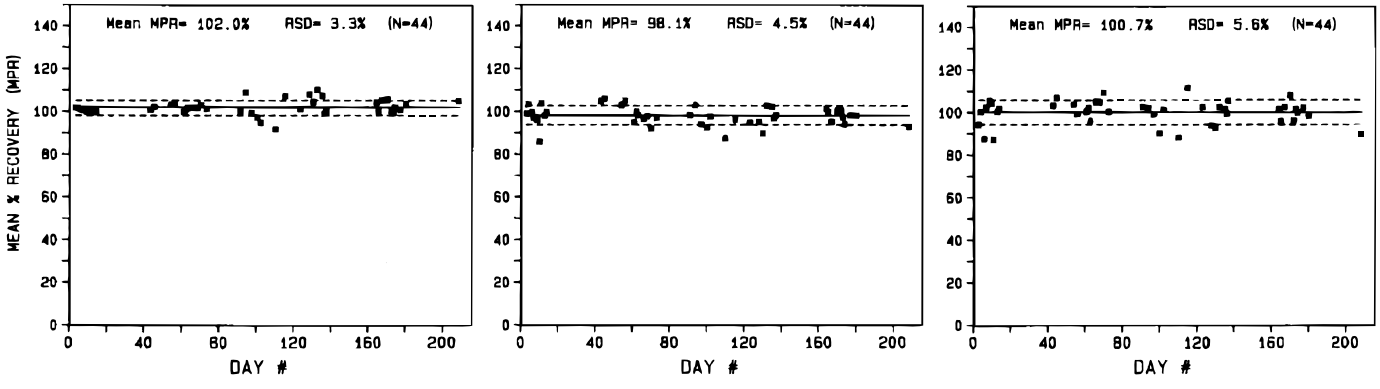

Figure 7. Distribution of mean % recoveries for TE (left), 4-OH-TE (center) and 4-TE-A (right) from daily sets of quality control plasma samples analyzed over an 8 month period of method application. The mean MPR is designated with a full line. The broken lines bracket ± one standard deviation from the mean.

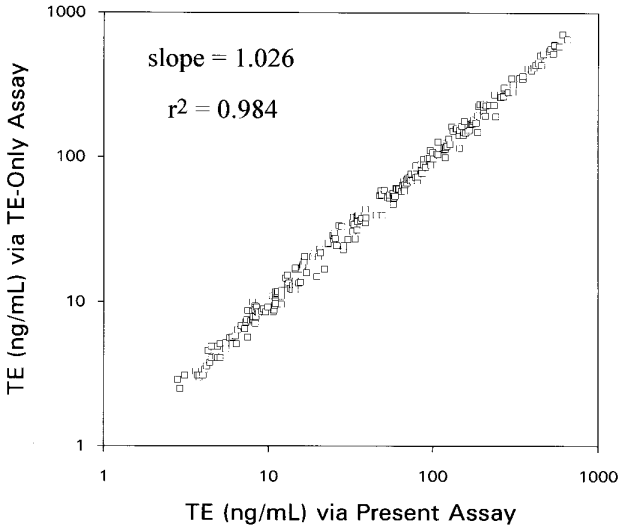

Figure 8. Plot of 250 TE plasma concentrations measured using previous (TE-only) GC/MS/MS method[1] *vs.* TE levels determined 9 months later using present TE plus metabolites assay (same set of samples). Slope (1.026) and r^2 (0.984) values are based on a linear ($1/x^2$-weighted) least-squares fit.

the 8 month evaluation period (Fig. 7) provide a visual indication of this day-to-day consistency.

Inter-assay comparison (TE-only). A set of over 300 clinical plasma samples was originally analyzed for TE using the previously established TE-only GC/MS/MS method.[1] After 9 months of storage at −70 °C, these samples were prepared and reanalyzed for TE, 4-OH-TE and 4-TE-A according to the method described here. Because both assays generated TE plasma level data from the same set of samples, this allowed an evaluation of the potential for inter-assay bias on measured TE levels. Results presented in Table 2 show excellent agreement between the two databases. Note that, for comparison purposes, results of the present assay were arbitrarily selected as the reference values in calculating % method bias for each concentration range. Also, percent 'mean average deviation from the mean' (% MADM) values make no assumption as to which assay results are correct, while providing a measure of variability of TE measurements derived between the two methods. As an additional means of method comparison, all 250 quantifiable TE measurements obtained using the present assay were plotted *vs.* previous results from the TE-only assay. To better visualize the data distribution, a log–log plot is provided (Fig. 8), although the slope and r^2 values were calculated based on linear regression (weighted $1/x^2$). The nearness to unity of both values also attests to the strong agreement between these two, quite different, quantitative TE assays.

Method applications

The GC/MS/MS-based methodology described here was applied to the analysis of more than 2000 real samples in an 8 month period, primarily in support of various clinical pharmacokinetic studies. Daily batch runs typically exceeded 100 samples, with no loss in sensitivity observed (consistent internal standard peak areas from beginning to end of each batch run). This speaks surprisingly well for the ruggedness of an assay where chemical derivatization occurs pre-column and all reagents pass through the GC column and into the mass spectrometer. The 2.5–1000 ng per sample working range, along with applicability to sample sizes ranging from 0.1 to 1 ml, has effectively allowed access to a 2.5–10 000 ng ml^{-1} concentration range for each analyte. This wide range permitted method application in support of studies using many and varied dosing regimes.

As an example, shown in Fig. 9 is a typical three-compound concentration *vs.* time plot obtained from analysis of plasma samples from a subject administered a single p.o. dose of TE. Note that even at this relatively low dose (100 mg) the GC/MS/MS assay provides accu-

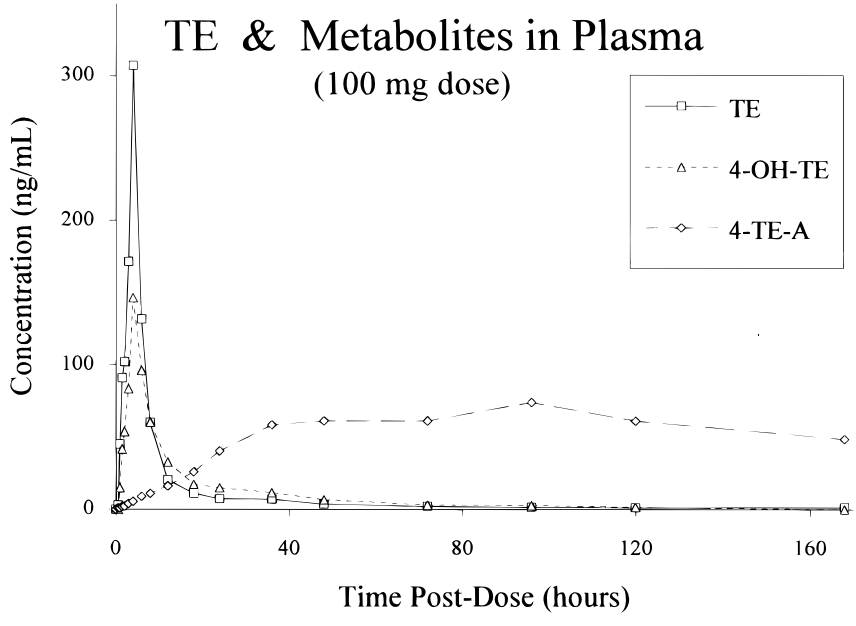

Figure 9. Typical plasma TE, 4-OH-TE and 4-TE-A concentration–time profiles for a subject perorally administered a low (100 mg) dose of TE.

rate TE and metabolite concentration data well into their final elimination phases.

CONCLUSION

Automated GC/MS/MS with on-line chemical derivatization is well suited for the routine, simultaneous determinations of TE, 4-OH-TE and 4-TE-A in plasma, as demonstrated in its application to more than 2000 real samples. The on-line trimethylsilylation procedure greatly simplifies sample preparation requirements, while rendering analytes of diverse functionality equally analyzable using a GC-based approach. The selectivity of MS/MS detection permits short analysis times (typically eight samples per hour) and minimizes the potential for problematic interferences, as applications extend across various animal species. The method has no significant bias and excellent precision for all three analytes, due in large part to the use of stable-isotope-labeled internal standards. The 2.5–10 000 ng ml^{-1} working range of this method permits application to samples from studies using a wide variety of dosing regimes.

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