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Sensitive and selective gas chromatographic methods for the quantitation of camphor, menthol and methyl salicylate from human plasma

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Abstract

Analytical methods using gas chromatography–flame ionization detection (GC–FID) for the quantitation of camphor and menthol and GC–MS for the quantitation of methyl salicylate have been developed for measurement of low concentrations from human plasma. Anethole serves as the internal standard for camphor and menthol and ethyl salicylate serves as the internal standard for methyl salicylate. Plasma samples undergo multiple, sequential extractions with hexane in order to provide optimal recovery. For menthol and camphor, the extracting solvent is reduced in volume and directly injected onto a capillary column (Simplicity-WAX). Extracted methyl salicylate is derivatized with BSTFA prior to injection onto a capillary column (Simplicity-5). Between-day variation (% RSD) at 5 ng/ml varies from 6.2% for methyl salicylate to 13.5% for camphor. The limit of detection for each analyte is 1 ng/ml and the limit of quantitation is 5 ng/ml. These analytical methods have been used in a clinical study to assess exposure from dermally applied patches containing the three compounds.

Keywords: Camphor; Menthol; Methyl salicylate

1. Introduction

Camphor, menthol and methyl salicylate are common ingredients found in topically applied analgesics and rubifacients. These products find use in the treatment of minor muscle aches and pains. A recent trip to a community pharmacy revealed 35 different topical preparations containing one or more of these ingredients. Of those products, 16 contained only menthol in amounts ranging from 1.27% to 16%. One spray product listed menthol as the only active ingredient but no content was given. Two products contained camphor and menthol only, but neither listed a content for camphor and only one product listed menthol at 2.5%. Fourteen products contained menthol and methyl salicylate in amounts ranging from 1% menthol and 15% methyl salicylate to 10% menthol and 30% methyl salicylate. One of the store brand products contained menthol and methyl salicylate as active ingredients in unlisted amounts. Three products contained all three ingredients, with

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the most concentrated product containing 30% methyl salicylate, 10% menthol and 4% camphor. These products are manufactured and sold in a variety of forms including: creams, gels, rubs, patches, balms, sprays, rub-on stick, and as a pain relieving ''mousse''. This long list of products is most likely a reflection of increased physical activity in the population, especially among the so-called ''weekend warriors'', whose exertions are rewarded with muscle strain, aches and pains.

These products, whose site of action is believed to be local, are generally assumed to be safe, since dermal absorption is thought to be negligible. This may not be the case, however, for methyl salicylate and its metabolite, salicylic acid. Several studies have suggested, based primarily upon measurement of salicylic acid in blood, skin and urine, that methyl salicylate, if not absorbed per se, provides a source for systemic absorption of salicylic acid [1–4]. Distinct toxic symptoms are associated with each of these compounds if sufficient systemic exposure is achieved. Ingestion of camphor can result in hallucinations, tremors, fainting, cyanosis, arrhythmia and convulsions [5–9], abortion [10], and chronic ingestion has hepatotoxic effects [11]. Methyl salicylate ingestion may result in the salicylate poisoning syndrome resulting in nausea, vomiting, tinnitus and metabolic acidosis which can lead to death [12,13]. Direct contact with a decongestant containing camphor, menthol and eucalyptol caused corneal lesions and encephalopathy in an infant [14]. Excessive use of topical methyl salicylate formulas can cause local necrosis, nephritis [15], and interfere with warfarin therapy resulting in abnormal bleeding reactions [4]. In addition to their individual potential toxic effects, each of these products is believed to be a dermal penetration enhancer [16,17], thus, suggesting the possibility of increased toxicity when used in combination. There is also some evidence to suggest that methyl salicylate may promote its own dermal absorption by enhancing cutaneous blood flow [18]. Absorption through the skin may also be enhanced by heating the skin, such as with a heating pad or exercise [15,19].

With this wide array of products on the market having the potential for producing toxic effects, a sensitive analytical method would be useful for quantitating these compounds in plasma so that appropriate pharmacokinetic and topical bioavailability studies may be performed. While high-performance liquid chromatography (HPLC) methods for quantitation of intact methyl salicylate from biological fluids have been reported, these lack sufficient sensitively for accurate measurement following low dose exposure. Camphor and menthol lack sufficient absorptivity to permit detection by spectroscopic methods. Methods are available for the detection and quantitation of methyl salicylate in blood or plasma by indirect methods where total salicylates are measured before and after hydrolysis [20,21] and for menthol [22] and camphor [23] in urine. None of these methods could be adapted to quantitate the three intact compounds at low concentrations in plasma. Two major difficulties were encountered in developing analytical methods for the quantitation of these compounds. First, the concentrations expected in plasma after normal dermal application, and certainly the detection limits required to demonstrate that no toxicologically significant amounts are absorbed, are necessarily small. Second, since all the compounds are volatile, concentrating the samples to increase detectability required delicate sample handling. A previous publication from this laboratory [24] noted some of these difficulties during the development of a selective assay for methyl salicylate.

This paper presents a sensitive procedure for the quantitation of methyl salicylate, camphor and menthol in small volumes of human plasma, such as may be expected at sample intervals over the span of a human pharmacokinetic study. Precision and accuracy data are presented as are preliminary findings from a clinical study in which dermal patches containing the compounds were applied and systemic exposure was measured.

2. Experimental

2.1. Chemicals

All chemicals were obtained from manufacturers or suppliers in the USA. (+)-Camphor and menthol were obtained from ICN Chemicals (Costa Mesa,

CA, USA) and Aldrich (Milwaukee, WI, USA). Anethole, which served as the internal standard for camphor and menthol, was manufactured by Magnus, Mabee and Reynard (New York, NY, USA). Methyl salicylate was obtained from Sigma (St. Louis, MO, USA) and Acros (Fairlawn, NJ, USA). Ethyl salicylate, which served as the internal standard for the methyl salicylate assay, was obtained from ICN Chemicals. Hexane was purchased from Burdick and Jackson (Muskegon, MI, USA) and ethanol (100%), was manufactured by Quantum, USI division (Anaheim, CA, USA). Anhydrous sodium sulfate was purchased from Mallinkrodt Baker (Paris, KY, USA). Acetonitrile (ACN), Omnisolv, chromatography grade was purchased from EM Science (Gibbstown, NJ, USA), and *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from Pierce (Rockford, IL, USA).

Blank whole blood was collected from human volunteers into 100×16 mm green top Vacutainer tubes from Becton Dickinson (Franklin Lakes, NJ, USA) containing 143 U sodium heparin per tube. Blood was immediately centrifuged to obtain plasma, and the plasma was stored in polypropylene tubes with screw top caps at −12°C until use.

Stock solutions of individual chemicals were prepared by dissolving in ethanol. Mixed standards of camphor and menthol (ICN Chemicals) were prepared at 1000 and 4000 ng/ml in ethanol and a second mixed standard of camphor and menthol (Aldrich) was prepared at 1000 ng/ml in ethanol for use in spiking samples. The ICN standards were used to prepare the calibration, precision and accuracy replicates and the Aldrich standard was used for quality control (QC) purposes. Anethole was prepared at a concentration of 3 μg/ml in ethanol. Separate calibration and QC standards of methyl salicylate were prepared in ethanol at identical concentrations of 1178 ng/ml. The Acros methyl salicylate standard was used to prepare the calibration, precision and accuracy replicates and the Sigma methyl salicylate standard was used for preparing QC samples. Ethyl salicylate was prepared in ethanol at a concentration of 2250 ng/ml. All QC standards and samples were prepared independently of those used for calibration of standard curves.

2.2. Equipment

2.2.1. Gas chromatography–flame ionization detection (GC–FID) conditions for camphor and menthol

Gas chromatographic analysis was conducted with a HP 5890 Series II GC equipped with a flame ionization detector and a HP 7673 Autosampler. Heated zones were set at 150°C for the injection port, 240°C for the detector, and an initial oven temperature of 90°C. Initial oven temperature was maintained for 7 min and then ramped at 5°C/min to 160°C and held for 2 min. This program allowed sufficient time for all compounds to elute with adequate separation and minimal baseline rise. The temperature program ended with a baking cycle at 230°C for 2 min. The GC column was a Supelco Simplicity-WAX, 30 m×0.25 mm I.D., 0.25 μm film thickness. The column head pressure was set at 15 p.s.i. and the carrier gas was nitrogen with a flow-rate of 10 ml/min (1 p.s.i.=6894.76 Pa). All samples were injected by the autosampler at a volume of 12.5 μl, with a split of 1:10.

2.2.2. Gas chromatography–mass selective detection conditions for methyl salicylate

GC–MS analysis was conducted with a HP 5970 Series II GC with a 5972 Series MSD and a HP 7673A Autosampler. Heated zones were set at 250°C for the injection port, 300°C for the detector, and an initial oven temperature of 110°C. Initial oven temperature was maintained for 3 min and then ramped at 10°C/min to 180°C and then at 20°C/min to 240°C and held for 1 min. The GC column was a Supelco Simplicity-5, 30 m×0.25 mm I.D., 0.25 μm film thickness. The column flow was programmed to be a constant 2 ml/min with helium as the carrier gas. All samples (3 μl) were injected by the autosampler in the splitless mode.

Data were acquired using a selected ion monitoring (SIM) program. The start time (min) and the ions measured included the following compounds: underivatized methyl salicylate, 3.8 min (ions, 92, 120, 152); underivatized ethyl salicylate, 4.4 min (ions, 92, 120, 166); derivatized methyl salicylate, 6.0 min (ions, 209, 210, 179, 193); derivatized ethyl

salicylate and salicylic acid, 7.8 min (ions, 177, 193, 195, 223, 209).

2.3. Standard curve preparation

Standard curves were obtained using non-weighted linear regression analysis of the ratio of the peak areas of analyte to internal standard (I.S.) vs. analyte concentration. Concentrations of analytes used for the precision and accuracy samples were determined from the respective standard curves.

2.3.1. Camphor and menthol assay

Two ml portions of human plasma were pipetted into 75×10 mm borosilicate glass tubes and 25 μl anethole (I.S.) in ethanol was added via a 200-μl Gilson Pipetman. Calibration standards and precision and accuracy replicates were prepared by adding appropriate volumes of the standard solutions (1000 or 4000 ng/ml), via a 20-μl or 200-μl Gilson Pipetman to each plasma sample and adding blank ethanol such that the total volume of solvent added to each sample was 125 μl (including the I.S.). Identical procedures for addition of ethanol were applied to authentic plasma samples. Standards were all added by immersing the pipette tip in the plasma prior to ejecting the solution and mixing immediately afterward to minimize loss of volatiles. Concentrations of calibration standards were 0, 5, 10, 25, 50, 80, 100, 150 and 200 ng/ml and the QC check standard was prepared at a concentration of 40 ng/ml. Concentrations for precision and accuracy replicates were 5, 20, 40 and 100 ng/ml.

Upon spiking, the plasma samples were capped and gently mixed to ensure a homogeneous distribution of the standards in the matrix. Then, 500 μl of hexane was added to each tube and samples were shaken on an Eberbach mechanical shaker for 10 min at a low setting. This procedure allowed for efficient extraction while preventing the formation of an emulsion. Samples were then centrifuged for 10 min at 3000 rpm and the hexane layer was removed via a disposable glass pipette and transferred to a 75×12 mm KIMAX borosilicate glass disposable culture tube containing approximately 0.1 g anhydrous sodium sulfate and the tube was placed in an ice bath. An additional 500 μl of hexane was added to the plasma sample and the extraction was repeated three more times for a total of four extractions. Hexane layers were combined after each extraction in the 75×12 mm tubes and were kept on ice until all extractions were complete. The extracts were then concentrated in an ice bath under a gentle nitrogen stream until a final volume of between 30 and 100 μl was achieved. The extract is not evaporated to less than about 30 μl. Evaporation to smaller volumes results in loss of compound. Concentrates were transferred to glass conical inserts and placed into 2-ml autosampler vials with screw-top caps and PTFE/silcone/PTFE septa. Analysis by GC–FID was carried out by autosampler injection of 12.5 μl of each sample onto the GC column under the conditions described above.

2.3.2. Methyl salicylate assay

Human plasma samples (1.3 ml) were pipetted into 1.7-ml snap-top polypropylene conical tubes and 20 μl of the I.S., ethyl salicylate (in ethanol), was added via a 20-μl Gilson Pipetman. Calibration standards and precision and accuracy replicates were prepared by adding appropriate volumes of a standard solution, 1178 ng/ml, via a 200-μl or 20-μl Gilson Pipetman and blank ethanol to each plasma sample such that the total volume of solvent added to each sample was 86 μl (including the I.S.). The identical total volume of ethanol was added to all authentic plasma samples. Standards were all added by immersing the pipette tip in the plasma prior to spiking and mixing immediately afterward to minimize loss of volatiles. Concentrations of calibration standards were 0, 2.5, 5, 10, 20, 30, 40 and 60 ng/ml and the QC check standard was prepared at a concentration of 40 ng/ml. Concentrations for precision and accuracy replicates were 5, 20 and 40 ng/ml.

Upon spiking, the plasma samples were capped and gently mixed to ensure a homogeneous distribution of the standards in the matrix. A 50-μl aliquot of ACN was added to each tube followed by 100 μl hexane. The ACN improved methyl salicylate recovery and reduced variability, especially for plasma samples with high lipid content. Samples were shaken on an Eberbach mechanical shaker for 10 min at a low setting.

Once this extraction step was completed, the samples were centrifuged for 8 min at 12 000 rpm

and the hexane layer was removed via disposable polypropylene microcapillary pipettes and transferred to 0.7-ml amber crimp-top vials containing 2 μl of ethanol. A second 100 μl portion of hexane was again added to the sample and the extraction was repeated for a total of two extractions. Hexane layers were combined in the amber vials, and 25 μl BSTFA with 1% TMCS was added to each sample, and the caps attached and crimped. Samples were briefly vortexed and heated in a heating block in a sand bath at 55°C for 30 min to derivatize the compounds. Samples were then allowed to cool to room temperature before analysis by GC–MS under the specified conditions.

3. Results

Fig. 1 illustrates gas chromatograms of a human plasma sample (A) spiked with camphor and menthol (20 ng/ml) and a plasma blank (B) brought through the extraction procedure. The retention times for camphor, menthol and anethole were 13.5, 17.0 and 22.1 min, respectively. The blank sample sometimes has a peak which interferes with menthol at 17.0 min. This interference varied between plasma donors, but was consistent between donations from the same donor. The samples were corrected for this interference by subtracting the area ratio found in the blank sample from the area ratio found for the spiked samples.

Fig. 2 illustrates a GC–MS chromatogram of methyl salicylate (A; 20 ng/ml) and a blank sample (B) obtained from human plasma brought through the extraction and derivatization procedures. The retention times for the derivatized methyl salicylate and derivatized ethyl salicylate were 7.2 and 8.2 min, respectively.

Table 1 summarizes the intra-day variation associated with the camphor, menthol and methyl salicylate assays as applied to human plasma and incorporating the procedures outlined. A minimum of three days of validation was conducted to characterize the anticipated variability of the assay. For camphor and menthol this procedure was extended for an additional two days. The range of concentrations employed for camphor and menthol was 5 to 100 ng/ml; whereas, a narrower range was used for

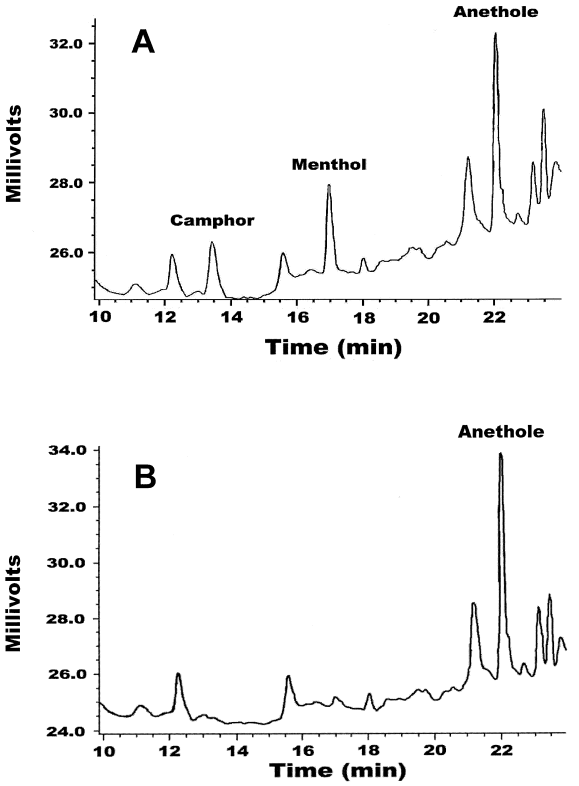

Fig. 1. (A) Gas chromatogram of a human plasma sample spiked with camphor and menthol (20 ng/ml) along with the internal standard, anethole, and brought through the entire extraction procedure. (B) Gas chromatogram of a blank human plasma sample spiked with internal standard and brought through the entire extraction procedure. Note that the chromatograms illustrate times after 10 min.

methyl salicylate, 5 to 40 ng/ml. Any authentic samples having concentrations beyond this range need to be diluted prior to assay or the calibration range needs to be extended.

The within-day percentage relative standard deviation (% RSD) for all three compounds decreased at higher concentrations and, in all cases, the % RSDs were less than about 8% at concentrations equal to or above 20 ng/ml. The only exception to this was seen on day 4 for camphor where the % RSD was 16.4% at 20 ng/ml and was due to one replicate with a low concentration (13 ng/ml). Greater variation was noted at the lowest concentration of 5 ng/ml, but this was less than about 10%. On only two occasions,

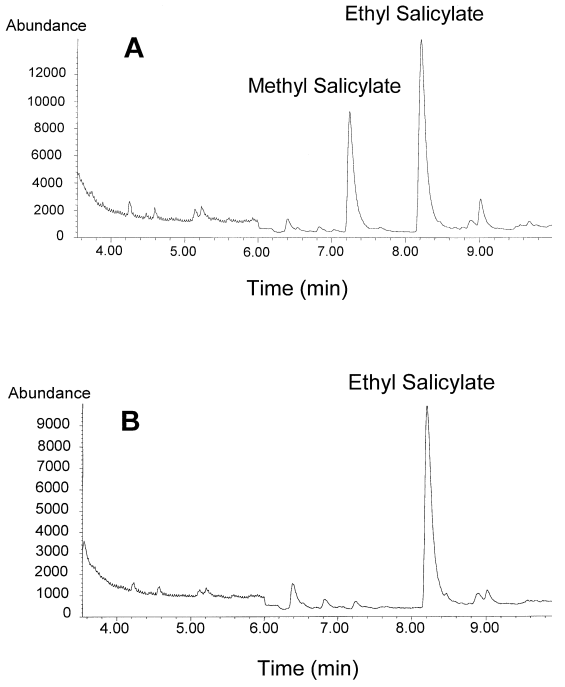

Fig. 2. (A) GC–MS chromatogram of a human plasma sample spiked with methyl salicylate (20 ng/ml) along with the internal standard, ethyl salicylate, and brought through the entire extraction and derivatization procedures. (B) GC–MS chromatogram of a blank human plasma sample spiked with internal standard and brought through the entire extraction and derivatization procedures. Note that the chromatograms begin at 3.5 min.

once for camphor (day 3) and once for menthol (day 1) were the % RSDs greater than 10%.

Table 2 presents the results of the inter-day reproducibility experiments for all three compounds. These values represent the mean of 15 samples; however, the results from 25 samples were used in calculating the mean for camphor and menthol at 20 and 40 ng/ml. The % RSDs were less than or equal to 10% in all cases with the exception of camphor at 5 and 20 ng/ml (13.5 and 11.5%, respectively). The percentage bias at concentrations greater than 20 ng/ml were, with one exception, less than 4%. The only exception was a value of 10.9% for methyl salicylate at 20 ng/ml. For the lowest concentration of 5 ng/ml, the percentage bias was about 12% for all compounds. The values for bias were positive and negative. Although we did not accurately determine the extraction efficiencies for the three compounds, they are estimated to be about 95% on the basis of relative areas achieved with direct injection of the compounds in comparison to those observed from samples taken through the entire procedure.

The analytical methods developed here were applied to a clinical study in which patches containing the three compounds were applied to skin of human subjects. Although these results will be the basis of another report [25], Fig. 3 illustrates the average (±standard deviation) concentration–time profiles for each compound obtained in eight subjects after the application of the dermal patches. Each patch contained the following amounts of camphor, menthol and methyl salicylate: 46.80, 37.44 and 74.88 mg, respectively. The average maximum plasma concentrations of those compounds were approximately 38, 32 and 37 ng/ml for camphor, menthol and methyl salicylate, respectively.

4. Discussion

The work described here was initiated in order to develop sensitive and reproducible analytical methods for the quantitation of camphor, menthol and methyl salicylate from human plasma. Based upon a review of the literature, there appeared to be a need for a reliable assay to quantitate those compounds. This is especially the case in light of the marketing and the apparent increased use of many commercially available over-the-counter products containing various combinations of camphor, menthol and methyl salicylate. The assays developed needed to have sufficient sensitivity to evaluate human exposure (i.e., as assessed by blood concentration measurements) following dermal application of such products.

Initial attempts were made to develop one assay using common extraction procedures for all three compounds that would have the requisite sensitivity, selectivity and reproducibility. For a variety of reasons this proved unsuccessful. One major consideration was the need to concentrate the camphor and menthol in order to improve sensitivity; however, any attempt at concentrating extracts resulted in significant loss of methyl salicylate. Similar losses upon evaporation were also seen for camphor and menthol when using a variety of techniques includ-

Table 1
Intra-day reproducibility of the assays for camphor, menthol and methyl salicylate from human plasma

Concentration added (ng/ml)	Concentration measured (ng/ml) (% RSD)[a]				
	Day 1	Day 2	Day 3	Day 4	Day 5
Camphor					
5	6.1 (8.0)	5.0 (9.7)	5.7 (15.2)	–	–
20	19.6 (2.1)	17.2 (5.6)	20.5 (4.9)	18.1 (16.4)	22.1 (1.6)
40	38.9 (8.7)	36.5 (3.1)	37.3 (6.4)	37.4 (3.1)	43.8 (2.1)
100	–	–	90.7 (6.3)	94.3 (5.8)	105.3 (4.8)
Menthol					
5	5.7 (11.2)	5.9 (6.2)	5.2 (9.8)	–	–
20	19.9 (3.3)	18.1 (8.0)	21.4 (5.8)	18.2 (10.3)	20.4 (3.7)
40	43.2 (3.6)	36.7 (3.8)	40.2 (2.5)	39.7 (0.8)	41.7 (1.5)
100	–	–	96.0 (4.2)	96.9 (3.7)	105.5 (4.5)
Methyl salicylate					
5	4.5 (3.8)	4.2 (4.4)	4.1 (5.3)		
20	22.4 (3.3)	22.0 (2.8)	22.2 (4.2)		
40	40.1 (1.9)	42.7 (1.3)	38.7 (2.1)		

[a] Mean of five samples. % RSD=% relative standard deviation.

ing centrifugal evaporation and dying under nitrogen; recovery was poor and erratic. This problem was solved by not evaporating the sample to dryness and by drying under nitrogen when the tubes were placed on ice. Extracts should not be evaporated to volumes less than about 30 μl to avoid loss of these com-

Table 2
Inter-day reproducibility of the assays for camphor, menthol and methyl salicylate from human plasma

Concentration added (ng/ml)	Inter-day reproducibility	
	Concentration measured (ng/ml) (% RSD)[a]	% Bias[c]
Camphor		
5	5.6 (13.5)	12.0
20	19.5 (11.5)[b]	−2.5
40	38.8 (8.4)[b]	−3.0
100	96.8 (8.4)	−3.3
Menthol		
5	5.6 (10.2)	12.0
20	19.6 (8.9)[b]	−2.0
40	39.6 (6.1)[b]	−1.0
100	99.5 (5.9)	−0.5
Methyl salicylate		
5	4.3 (6.2)	−14.3
20	22.2 (3.3)	10.9
40	40.5 (4.6)	1.3

[a] Mean of 15 samples. % RSD=% relative standard deviation.
[b] Mean of 25 samples.
[c] % Bias=[(Measured−Added)/Added]×100.

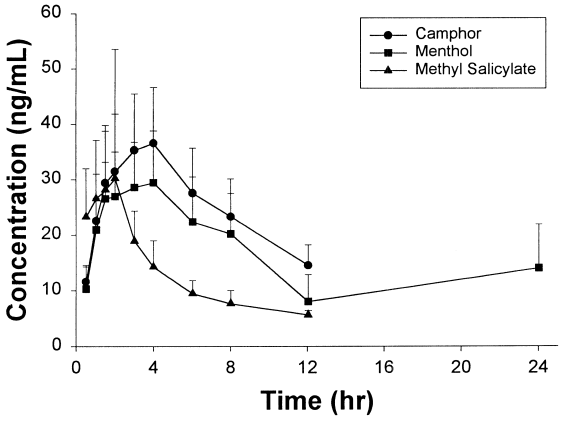

Fig. 3. Average plasma concentration vs. time profiles for camphor (●), menthol (■) and methyl salicylate (▲) following the application of dermal patches. Each value is the mean obtained from eight subjects and the vertical error bars represent the standard deviation of the mean.

pounds by evaporation. Other approaches, such as direct headspace analysis and the use of solid-phase micro-extraction also proved to be unreliable.

It was necessary to exhaustively extract plasma samples for camphor and menthol in order to improve recovery and reduce variability. For that reason, sequential extractions were obtained and combined. This procedure clearly increases the time effort and limits the number of samples that may be processed in one day. The average number of samples extracted per day was 25. If a large number of samples were to be processed, the application of automated extraction techniques might be necessary.

Any attempt to concentrate samples in order to improve sensitivity of methyl salicylate detection was avoided because of the substantial and erratic loss upon evaporation. It was not necessary to do so because of the instrumental sensitivity achieved using the GC–MS in SIM mode. We have reported on a similar procedure used for the quantitation of methyl salicylate and salicylic acid from plasma in which sample concentration was necessarily avoided [24]. Solid-phase extraction also proved to be an unreliable approach to concentration of methyl salicylate. Processing of the methyl salicylate samples was less time consuming than the efforts needed for the assay of camphor and menthol. The average number of samples extracted per day was 50.

The hexane used for the camphor and menthol analysis would sometimes contain contamination peaks which interfered with camphor and menthol. To eliminate this contamination, the hexane was filtered through Alumina N (Act I), 50–200 μ (Scientific Adsorbents, Atlanta, GA, USA) just prior to use. This contamination was not a problem in the methyl salicylate assay.

As a consequence of the sample processing issues noted above, a plasma sample was split into two aliquots; one for the assay of camphor and menthol and the other for the assay of methyl salicylate. In order to achieve the sensitivity noted here, a minimum of a 3–4 ml plasma sample must be obtained and this will provide only one analytical result; the volume is too small for duplicate assays. In order to obtain reliable results for concentrations less than about 5 ng/ml, the assay will need to be scaled-up to handle the necessarily larger plasma volume. An additional concern is the occasional appearance of an extraneous peak at about 17 min in the camphor and menthol chromatograms. Since that peak remains relatively constant within a subject from time-to-time, we recommend obtaining pre-study blank plasma samples. This will permit one to correct concentrations for that additional peak.

One unique aspect of this assay and that of a previous report [24], is the direct measurement of plasma methyl salicylate at low ng/ml concentrations. Direct measurement of plasma methyl salicylate, as opposed to measurement of total salicylate prior to and after sample hydrolysis, provides a more accurate estimation of intact compound. This is especially the case because the concentrations of methyl salicylate, as noted in Fig. 3, are quite small following dermal application. Experimental errors associated with the subtraction approach would likely be unacceptably large at those low methyl salicylate concentrations. It has also been suggested [26], that camphor and menthol may prevent the hydrolysis of methyl salicylate to salicylic acid when those compounds are applied simultaneously.

The analytical methods reported here have a limit of detection of about 1 ng/ml and a limit of quantitation of about 5 ng/ml for camphor, menthol and methyl salicylate. The application of the analytical methods to a clinical study is illustrated in Fig. 3. A total of eight patches were applied to the

skin of eight subjects for 8 h. Plasma concentrations were below the limit of quantitation at about 12 h. The rise in menthol concentration between 12 and 24 h was noted in five of the eight subjects but at this time we can offer no explanation for this observation. The limit of quantitation may not be sufficient for characterizing the dermal absorption (or exposure by other routes) of the three compounds in humans if the product applied has a low content or if a small amount is applied to the skin.

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