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Determination of menthol and menthol glucuronide in human urine by gas chromatography using an enzyme-sensitive internal standard and flame ionization detection

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SUMMARY

The rate of peppermint oil absorption and excretion, following peroral administration, was determined by measuring urinary levels of menthol glucuronide. Menthol, a major component of peppermint oil, was liberated from its glucuronide metabolite by treating raw urine with β-D-glucuronidase (*Patella vulgata*). Phenyl glucuronide was employed as an enzyme-sensitive internal standard. Menthol and phenol were recovered by ethyl acetate extraction and quantitated by capillary gas chromatography using flame ionization detection. Standard curves were linear between 25 and 250 μg/ml with a detection limit (signal-to-noise ratio = 2) of 0.25 μg/ml. Assay precision was shown to be ±1.2% relative standard deviation.

INTRODUCTION

Irritable bowel syndrome (IBS) is characterized by recurrent attacks of colicky abdominal pain, a feeling of distention and altered bowel habit [1]. Although the cause of this syndrome is not fully understood, treatment may include peroral administration of peppermint oil (PO) in the form of an enteric-coated capsule [2]. PO, a major constituent of which is menthol, is a naturally occurring carminative which relaxes gastrointestinal smooth muscle both in vitro and in vivo. Menthol is metabolized in vivo to menthol glucuronide (MG) and is excreted in the urine. The appearance of MG in urine can serve as an index of PO absorption and the systemic availability of menthol [2].

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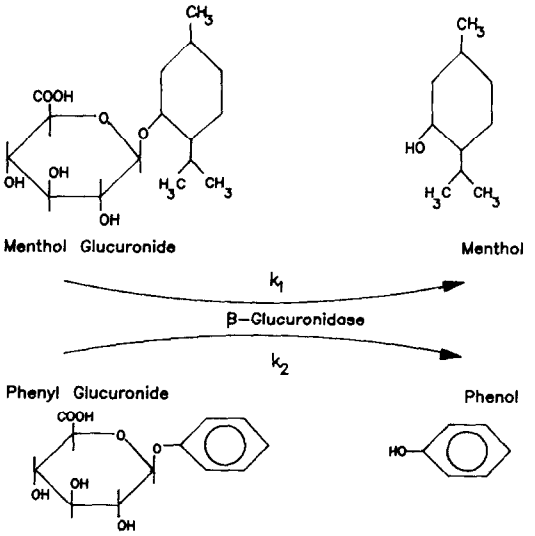

Fig. 1. Use of an enzyme-sensitive internal standard (PG) for the determination of menthol.

Several reports have appeared in the literature describing approaches for the determination of MG in biological matrices. The first reported determination of urinary menthol was that of Gleispach and Schandara in 1970 [3]. Their work involved enzyme hydrolysis of MG, ethereal extraction and gas chromatography with flame ionization detection (GC–FID). Later, Bell et al. [4] reported a more facile method using a chemical (cineole) internal standard for quantification. In addition, Leroy et al. [5] have developed a general method for glucuronides which involved derivatization of the free glucuronic acid prior to reversed-phase high-performance liquid chromatographic analysis.

To more accurately assess the pharmacokinetics of orally dosed PO, we modified the method of Bell et al. [4] to include an internal standard, phenyl glucuronide (PG), which also undergoes enzymatic hydrolysis. The use of an 'enzyme-sensitive' (Fig. 1) rather than 'chemical' internal standard is advantageous in that changes in enzyme activity, due to either endogenous or exogenous factors, should not adversely effect the analytical determination. The glucuronidase-based method described herein has been validated for the measurement of menthol in human urine and should be a useful tool when monitoring PO (menthol) pharmacokinetics.

EXPERIMENTAL

Chemicals and reagents

Aldehyde-free, glacial acetic acid was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.) and anhydrous ethyl acetate was purchased from E.M. In-

dustries (Cherry Hill, NJ, U.S.A.). Both materials were HPLC-grade reagents. Menthol, mentholglucuronic acid, phenol, phenyl-β-D-glucuronide and β-D-glucuronidase (*Patella vulgata*, Type L-11) were all of the highest available purity and purchased from Sigma (St. Louis, MO, U.S.A.). The water used was Milli-Q reagent water (Millipore, Bedford, MA, U.S.A.).

Preparation of reagents

A 20 mg/ml solution of β-glucuronidase was prepared in water, protected from light and stored at 4°C until use. Stock calibration standard solutions containing 25, 50, 75, 100, 150 and 200 μg/ml menthol as well as 50 μg/ml phenol were prepared in ethyl acetate. MG (5 mg/ml) and PG (6.25 mg/ml) aqueous stock solutions were also prepared.

Preparation of samples, controls and calibration standards

All samples, control samples and calibration standards were prepared in screw-cap (125 mm×16 mm) glass centrifuge tubes (Fisher Scientific, Cincinnati, OH, U.S.A.). Typically 5-ml aliquots of urine were taken for analyses. Blank urine, collected from volunteers prior to PO administration, was used as individual blank and control samples. Control samples were prepared by adding 0.2 ml of the MG stock solution (1 mg) to 5 ml of blank urine. The pH of all samples and analytical controls was adjusted to 3.8 with acetic acid using an Accumet 610 pH meter (Fisher). To each sample and control, 0.115 ml PG stock solution (0.72 mg) was added as the internal standard. Exactly 0.2 ml β-D-glucuronidase solution (5000 U) was immediately added to each tube. The tubes were incubated for 24 h in a 37°C water bath (Magni Whirl, Fisher Scientific) to effectively hydrolyze the glucuronides. After cooling to room temperature, 5 ml ethyl acetate were added to each tube (1:1, v/v) and the tube contents were rotomixed (Bioquest Rotator, Cockeysville, MD, U.S.A.) for 30 min and centrifuged (2000 *g* for 10 min). The organic phase was removed for analysis.

Calibration standards were prepared by adding 5-ml aliquots from each of the six menthol/phenol standard stock solutions described above into separate 5-ml portions of blank urine. The 10-ml mixture containing urine and calibration standards in ethyl acetate were rotomixed for 30 min and centrifuged, and the organic phase was separated.

Chromatographic conditions

Samples were analyzed using a Hewlett Packard (Avondale, PA, U.S.A.) Model 5890 gas chromatograph equipped with a flame ionization detector and a Model 7673A automatic sampler. Sample injections (35:1 split) were programmed to be 1 μl in volume. Helium (linear velocity=20 ml/s) was employed as the carrier gas. The injection port temperature was held at 220°C and the flame ionization detector maintained at 250°C. A 30 m×0.25 mm DB-

5 (J&W Scientific, Folsom, CA, U.S.A.) capillary column (0.1-μm film) was used to separate menthol and phenol from urine matrix components. The oven was programmed from 80 to 200°C at a 10°C/min rate, followed by a 3-min hold at 200°C (total run time, 15 min). Data were collected using a Hewlett Packard Model 3393A reporting integrator and Model 9114B disk storage device.

Pharmacokinetics

Four male volunteers were administered 180 mg of PO in an enteric-coated capsule following a 16-h fast. There were no dietary restrictions post-dosing except a conscious effort to increase water intake throughout the day. Total urine output was collected every 2 h for up to 14 h after PO ingestion and the volume accurately measured prior to sample preparation.

RESULTS AND DISCUSSION

Chromatographic characterization

A typical capillary GC–FID profile for the determination of menthol (retention time, $t_R = 10.4$ min) and phenol ($t_R = 8.1$ min) in human urine is shown in Fig. 2. Each analyte is well resolved ($R_s > 2.0$) and both are free of interference from the urine matrix.

Enzyme optimization

β-D-Glucuronidase from *P. vulgata* was evaluated for its usefulness in catalyzing the hydrolysis of MG. It has recently been reported that *P. vulgata*-derived enzyme was superior to other β-D-glucuronidase isolates for hydrolysis of drug conjugates [6]. Factors affecting the hydrolysis rate (enzyme concen-

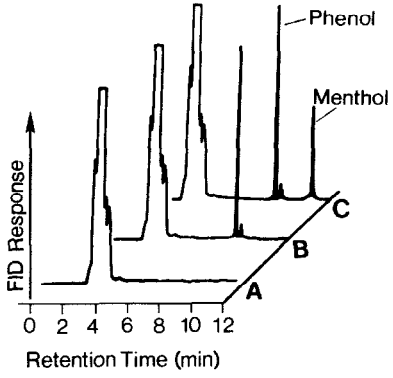

Fig. 2. Typical capillary GC–FID profiles (after enzyme treatment) of (A) blank human urine sample, (B) human urine spiked with 0.72 mg PG and (C) human urine at 4 h following peroral dosing of PO (180 mg) spiked with 0.72 mg PG.

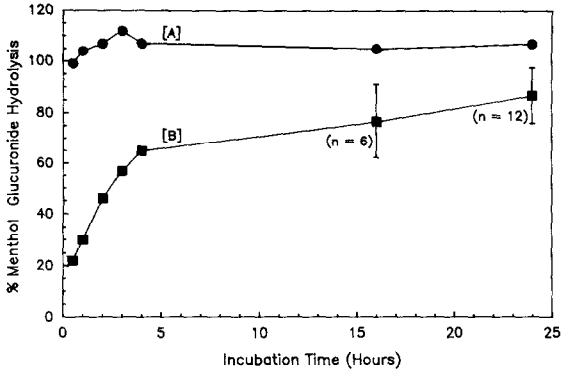

Fig. 3. Comparison of the extent of MG (1 mg) hydrolysis in (A) acetate buffer and (B) human urine as a function of incubation time. Incubations were performed using 5000 U of enzyme at pH 3.8 and a temperature of 37°C.

tration, substrate concentration, temperature and pH) were optimized during these studies. It was determined that a 24-h incubation at 37°C with ~5000 U of β-glucuronidase per 5 ml of urine resulted in maximal (>80% recovery) release of menthol from 1 mg of MG (Fig. 3). Enzyme optimization was carried out using this level of MG since it gives rise to 445 μg of menthol in a 5-ml urine sample (89 μg/ml). This level of menthol approximates the maximum concentration reported for humans following a single peroral dose (360 mg) of PO [1,2]. Nearly 70% of MG is hydrolyzed after a 5-h incubation while hydrolytic incubation times of less than 5 h resulted in significantly reduced recovery of menthol from urine relative to hydrolyses carried out in an acetate buffer (pH 3.8). Assay precision was much improved at 24 h as compared to a 16-h hydrolysis. For convenience sake, a 24-h enzyme incubation period (overnight) was chosen for all subsequent analyses.

Assay evaluation

The procedure described by Bell et al. [4] employed cineole as an internal standard which was added just prior to the extraction step. Though this approach adequately accounted for losses of analyte during liquid–liquid extraction and chromatographic sequences, it would not account for deviations in enzyme activity which may occur as a result of changes in urine composition (e.g. protein content) or the presence of inhibitors (e.g. drug–enzyme interaction). The utilization of an internal standard which also undergoes enzymatic hydrolysis (in a reproducible manner) would minimize the impact of these factors on the analytical determination.

Menthol standards ($n=3$) were spiked into 5-ml blank urine samples at the 50 and 100 μg/ml level and extracted with an equal volume of ethyl acetate, as described in the Experimental section. Recovery of menthol was 90 and 96%,

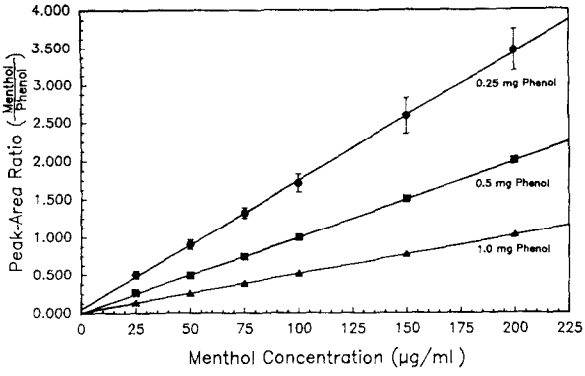

Fig. 4. Calibration curves for the determination of menthol in urine with various amounts (0.25, 0.5 and 1.00 mg) of phenol, as PG (internal standard).

respectively, which demonstrates that a single ethyl acetate (5 ml) extraction of urine efficiently recovers the majority of the available menthol.

PG was chosen as an internal standard because it was commercially available in high purity at low cost. Also, phenol, the enzymatic product, is soluble in ethyl acetate and is easily separated from menthol chromatographically (Fig. 2). A significant amount of data were generated to verify that hydrolysis of PG did not adversely affect the recovery of menthol over a wide range of concentrations. As shown in Fig. 4, the addition of 0.72 mg PG (internal standard) (0.25 mg of liberated phenol) yields the most sensitive (slope = 0.017) menthol calibration curve. All three calibration curves were linear ($r^2 > 0.999$) throughout the menthol concentration range (25–200 µg/ml) examined. The limit of detection (signal-to-noise ratio = 2) for menthol was determined to be 0.25 µg/ml. As little as 8 pg of menthol can be detected using this methodology.

As seen in Table I, phenol recovery at the 0.72 mg PG (50 µg phenol per ml) level is 94.8 ± 1.2% calculated against a phenol calibration curve. Menthol recovery, calculated against a menthol standard curve, averaged 100 ± 10% regardless of the level of phenol present in the urine. As such, the addition of 0.72 mg PG as an internal standard results in maximum recovery of liberated phenol and does not interfere with the quantification or reproducibility of the menthol determination in urine. As the amount of PG added to urine increases, the recovery of phenol reproducibly decreases. This is likely a consequence of differences in the rate of PG and MG hydrolysis as well as phenol/menthol extraction efficiency. The use of a glucuronide conjugate with greater structural homology to MG (e.g. cineole glucuronide) may alleviate this phenomenon.

Two separate blank urine samples (50 ml) were spiked with 0.56 and 1.68 mg MG. Five 5-ml aliquots were taken from each solution and assayed using the PG internal standard in order to generate precision and accuracy data.

TABLE I

EFFECT OF THE ADDITION OF PG TO URINE ON THE RECOVERY OF MENTHOL

Theoretical concentration (μg/ml)		Recovery			
		Menthol		Phenol	
Menthol[a]	Phenol[b]	μg/ml	%	μg/ml	%
25	50	33.0	132.1	47.7	95.4
50	50	54.8	109.5	46.8	93.5
75	50	78.0	103.9	48.2	96.4
100	50	97.8	97.8	47.1	94.2
150	50	151.0	100.7	48.0	96.0
200	50	191.2	95.6	46.7	93.4
Mean			106.6		94.8
S.D.			12.3		1.2
25	100	33.4	133.7	85.4	85.4
50	100	57.5	114.9	87.5	87.5
75	100	81.3	108.5	86.2	86.2
100	100	105.1	105.1	84.9	84.9
150	100	154.6	103.1	84.7	84.7
200	100	204.2	102.1	85.1	85.1
Mean			111.2		85.6
S.D.			10.9		2.4
25	200	33.4	133.6	159.5	79.8
50	200	55.6	111.1	154.3	77.2
75	200	80.7	107.6	156.6	78.3
100	200	105.2	105.2	156.1	78.0
150	200	153.0	102.0	154.8	77.4
200	200	199.9	99.9	153.3	76.6
Mean			109.9		77.9
S.D.			11.2		1.0

[a]Added as MG to 5 ml of urine.
[b]Added as PG to 5 ml of urine. Corresponds to 0.25, 0.50 and 1.0 mg phenol, respectively.

Using molecular mass conversions (MG, 349.4; menthol, 156.26), the theoretical amount of available menthol was 50 and 150 μg/ml in each 5-ml ethyl acetate extract, assuming quantitative recovery. The level of menthol determined was 108 and 102% of theoretical, with a relative standard deviation of 1.0 and 1.3%, respectively.

It should be noted that endogenous phenolic species (e.g. flavonoids) may be present in the urine of patients who have consumed alcohol or tea several

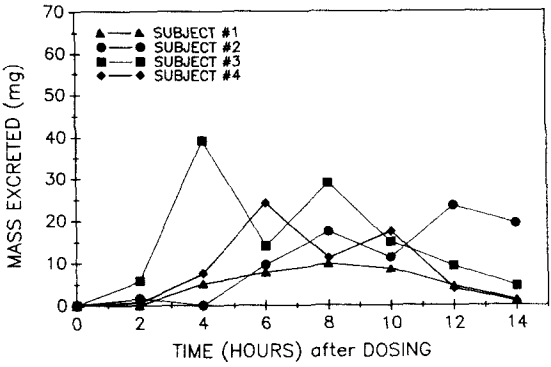

Fig. 5. Pharmacokinetic curves showing the urinary elimination of menthol from four volunteers following a single dose (180 mg) of PO.

hours prior to supplying a urine sample. This should pose no problem with our determination of menthol since most of the phenolic compounds are well resolved chromatographically from the peaks of interest. The presence of endogenous phenolic substances is also readily determined by analyzing the requisite patient blank and control urine samples.

Pharmacokinetic analyses

The utility of this analytical methodology was demonstrated for the determination of urinary menthol output in four male volunteers following a single peroral dose of PO (180 mg). MG urinary excretion profiles appear in Fig. 5. There was a significant amount of variation among each individual which was likely a consequence of differences in PO absorption and dietary habits. Area under the curve analysis indicated that between 37 and 116 mg of menthol were excreted by each subject within 14 h of PO ingestion. That level of menthol corresponded to an average 40% recovery of the original menthol content for a 180 mg PO dose. These results are in good agreement with those (35% recovered in 14 h) reported by Bell and co-workers [2,7] for similar studies.

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REFERENCES

1 W.D.W. Rees, B.K. Evans and J. Rhodes, Br. Med. J., 2 (1979) 835.
2 K.W. Somerville, C.R. Richmond and G.D. Bell, Br. J. Clin. Pharmacol., 18 (1984) 638.
3 H. Gleispach and E. Schandara, Z. Anal. Chem., 252 (1970) 140.
4 G.D. Bell, D.A. Henry and C.R. Richmond, Br. J. Clin. Pharmacol., 12 (1981) 281.
5 P. Leroy, S. Chakir and A. Nicolas, J. Chromatogr., 351 (1986) 267.
6 J. Combie, J.W. Blake, T.E. Nugent and T. Tobin, Clin. Chem., 28 (1982) 83.
7 D.A. White, S.P. Thompson, C.G. Wilson and G.D. Bell, Int. J. Pharm., 40 (1987) 151.