Journal of Chromatography, 343 (1985) 329—338
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2683

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ANALYSIS OF METHYLPHENIDATE AND *p*-HYDROXYMETHYLPHENIDATE USING DEUTERATED INTERNAL STANDARDS

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(Received February 28th, 1985)

SUMMARY

A gas chromatography—mass spectrometry assay is described for the simultaneous determination of *threo-dl*-methylphenidate and *threo-dl-p*-hydroxymethylphenidate in plasma and urine using selected ion monitoring of electron impact generated fragments of their pentafluoropropionyl derivatives. The use of recently available deuterated analogues as internal standards improves overall performance relative to previous methods. The practical limit of quantifiable detection of the assay is 0.5 ng/ml for both methylphenidate and *p*-hydroxymethylphenidate. *p*-Hydroxymethylphenidate appears to be a significant urinary metabolite of methylphenidate in rats but not in humans.

INTRODUCTION

The diagnosis and treatment of the hyperkinetic syndrome represents a

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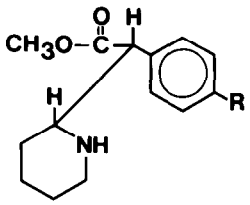

Fig. 1. Structures of MPH (R=H) and HOMPH (R=OH).

major challenge to paediatric psychiatrists [1]. The central stimulant methylphenidate (MPH, methyl *threo-dl*-2-phenyl-2-(2′-piperidyl)acetate, Ritalin®, Fig. 1) is the drug of choice for the treatment of the syndrome with attention deficit disorder with hyperactivity (ADD/H) being the targeted symptoms. Approximately 60—70% of behaviorally defined hyperkinetic children respond favorably to MPH [2]. The considerable inter-individual differences in the pharmacokinetics of MPH [3], the apparent curvilinear relationship between dose of MPH and improvement in learning deficits [4] and impulsivity [5] and the biotransformation of MPH to potentially pharmacologically active metabolites [6—8] further suggest that the monitoring of blood concentrations of MPH and one or more of its metabolites may be of value in the improvement (individualization) of the pharmacotherapy of ADD/H with MPH. However, the practical application of such techniques is limited by the analytical requirements necessitated by the low concentrations of MPH in blood (ca. 2—20 ng/ml) observed following the oral administration of therapeutic doses of MPH [3, 9, 10].

Attempts to quantify circulation concentrations of MPH have predominantly involved gas chromatography (GC) with flame ionization [11, 12], electron-capture [13], thermionic (nitrogen–phosphorus) selective [14, 15] or mass spectrometric (MS) [16—19] detection. A quantitative assay for MPH based upon high-performance liquid chromatographic separation with ultraviolet absorbance detection [20] has been reported, but lacks the requisite sensitivity to quantitate circulating MPH concentrations following therapeutic doses. Of the GC methods, problems such as insufficient sensitivity, undefined method accuracy and precision, elaborate and time-consuming sample preparation, the thermal degradation of underivatized MPH and incomplete chromatographic resolution of MPH and a structurally homologous internal standard limit their routine application. The assay that is the subject of this paper involves GC—MS of MPH and the internal standard, deuterium labelled MPH [21], as pentafluoropropionyl (PFP) derivatives with fragmentation by electron impact (EI) and detection by selected ion monitoring (SIM). The simultaneous determination of *p*-hydroxymethylphenidate (HOMPH, Fig. 1), a pharmacologically active [7] metabolite of MPH that has been suggested to contribute to the stimulant actions of the parent drug [9], is also described. The details of this method and its advantages relative to previously published assay procedures for MPH are discussed. Applying this method, concentrations of MPH and HOMPH were determined in the 24-h urine of man and rat after administration of MPH.

EXPERIMENTAL

Chemicals and reagents

Diethyl ether, hexane, ethyl acetate and acetonitrile were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Sodium carbonate and sodium bicarbonate were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Pentafluoropropionic anhydride (PFPA) was obtained from Pierce (Rockville, IL, U.S.A.). MPH · HCl was a generous gift from Ciba Geigy (Summit, NJ, U.S.A.). The HOMPH · HCl was synthesized as described in detail in a previous publication [7] from this laboratory as was the deuterium enriched MPH · HCl and HOMPH · HCl [21].

Sample preparation procedure: clinical plasma samples

Screw-top centrifuge tubes (15 ml) were treated with a 10% solution of dimethyldichlorosilane in toluene, then rinsed with methanol and oven-dried. The caps were fitted with PTFE-liners. A 1-ml aliquot of plasma was added to each tube, which contained 100 ng of [^2H_n]MPH ([^2H_n]HOMPH was omitted since HOMPH was never detected in human plasma). The pH was adjusted to 2.5—3.5 by adding 1 ml of 0.1 *M* hydrochloric acid. The mixture was extracted with 3 ml of diethyl ether—hexane (3:1) by vortexing for approximately 10 sec followed by brief centrifugation (500 *g*). The organic layer, often gelatinous, was aspirated and discarded and the remaining aqueous phase was adjusted to pH 9 by addition of 0.75 ml of 0.75 *M* carbonate buffer (pH 9.5) and extracted with 3 ml of diethyl ether—hexane (3:1) by vortexing for 10 sec and further mixing for 15 min on a mechanical shaker followed by centrifugation (500 *g*). The organic phases were then transferred to disposable 4-ml screw cap vials likewise fitted with PTFE-cap liners and evaporated to dryness under nitrogen with gentle heating using a heat gun.

The residue obtained upon evaporation was dissolved in 25 μl of ethyl acetate and 50 μl of PFPA, and reacted for 45 min at 60°C. The samples were then removed from the oven and allowed to cool to room temperature. Volatiles were then removed in a fume hood by evaporation under nitrogen at room temperature, and the samples were placed on ice for same-day analysis. Just prior to GC—MS analysis, the samples were individually reconstituted in 10 μl of ethyl acetate and 2—3 μl were injected.

Urine samples

Two male Sprague—Dawley rats (450 and 510 g) were administered 20 mg of MPH · HCl per kg body weight by the intraperitoneal (i.p.) route, and placed in metabolic cages. Total urinary output was collected for 24 h in flasks containing 2 ml of 0.1 *M* acetic acid to stabilize the analytes against hydrolysis [22]. Aliquots (0.2 ml) of urine from each rat were adjusted to 2 ml total volume with distilled water and 20 μg each of deuterated MPH and HOMPH were added, then the samples were extracted by the previous procedure.

Three male volunteers, ages 22–30 years, were orally administered 0.3 mg/kg MPH · HCl. Their urines were collected and frozen as voided over a 24-h period. Aliquots (2 ml) of urine were combined with 1 μg of [^2H_n]MPH and 100 ng of [^2H_n]HOMPH and extracted as before.

Instrumentation

All GC—MS analyses utilized a Finnigan 3300 gas chromatograph–mass spectrometer interfaced to a Finnigan 6000 data system. Fragmentation was accomplished by electron impact at 70 eV ionizing voltage and 500 μA ionizing current. The data system acquired two channels of selected ion current: that at *m/e* 230.2 representing the derivatized piperidyl fragment of MPH and HOMPH, and that at *m/e* 235.2 representing the corresponding pentadeuterated piperidyl fragments of the internal standards [$^2H_5$]MPH and [$^2H_5$]HOMPH. The mass spectrometer was tuned using perfluorotributylamine introduced into the analyzer via the variable leak valve. The injector port, separator and transfer line were operated at 230°C.

Chromatographic separation was accomplished using a 2 m × 2 mm I.D. silanized glass column packed with 3% SP-2100 on 80–100 mesh Supelcoport (Supelco) operated isothermally at 185°C. The carrier gas (helium) flow-rate was 15 ml/min. Under these conditions, the retention times for MPH and HOMPH were 2.2 and 3.3 min, respectively.

Calculations

Blank-corrected standard curves (7–8 points) were prepared by analyzing a series of 1-ml aliquots of drug-free plasma or urine to which the stated quantities of [^2H_n]MPH and [^2H_n]HOMPH and varying amounts of MPH (0–20 ng for clinical serum samples, 0–20 μg/ml for the urine samples in rat and 0—1 μg/ml for human urine) and HOMPH (none for human plasma samples, 0—20 μg/ml for rat urine samples, 0—100 ng/ml for human urine) had been added. These standards were then subjected to the same preparative steps used to extract unknown samples. The ion current ratios generated by monitoring the ions characteristic for MPH/[$^2H_5$]MPH and HOMPH/[$^2H_5$]-HOMPH (i.e., 230.2/235.2) were determined from their peak areas at the appropriate GC retention time for each unknown sample and standard. The concentrations of MPH and HOMPH in an unkown sample were calculated from their ratios using the slope and intercept of the appropriate standard curve.

RESULTS

Solvent extraction

The partitioning of MPH into nonpolar organic solvents from alkalinized plasma samples has been reliably used to solvent extract MPH with a high absolute recovery. An examination of the effect of extraction pH on the recovery of MPH using 1-ml vols. of buffered aqueous solutions (0.75 *M* phosphate buffer, pH 6.5—8.0; 0.75 *M* carbonate buffer, pH 8.5—11) indicated an increasing absolute recovery of MPH with increasing extraction pH up to pH 7.5, above which no further increase was obtained (Fig. 2). Conversely, similar manipulations involving HOMPH indicated a range of optimal extraction pH (9—10) below and above which the yield decreases (Fig. 2). The recovery of this amphoteric phenolic metabolite of MPH from increasingly alkaline solutions is evidently limited above a pH of 10 by the formation of the phenoxide ion. Therefore, samples were routinely buffered using a pH 9.5 carbonate buffer to obtain an efficient extraction of both MPH and HOMPH.

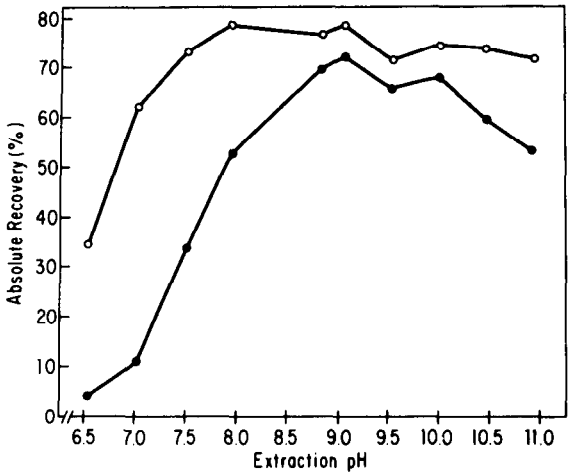

Fig. 2. Solvent extraction recovery of MPH (○) and HOMPH (●) from aqueous solutions as a function of pH.

Derivatization and chromatography

Reaction of MPH and HOMPH with PFPA for 45 min at 60°C produced the relatively volatile N-PFP and N,O-diPFP derivatives, respectively. Such derivatives are stable for at least one week when stored at −10°C if the unreacted PFPA and ethyl acetate are not removed by evaporation after derivatization. Though controlled for by the internal standard, after evaporation of the derivatizing reagent, appreciable amounts of the HOMPH appear to O-deacylate after 24 h. In this event, re-derivatization restores 100% of the peak size. The formation of PFP-MPH derivatives and their EI and chemical ionization (CI) mass spectra have previously been described [18]. The described reaction conditions were found to be optimal for the simultaneous formation of PFP derivatives of MPH and HOMPH, e.g., higher temperatures or longer reaction times decreased the yield of PFP—MPH without appreciably increasing the yield of $(PFP)_2$—HOMPH. The GC separation of PFP—MPH/PFP—$[^2H_5]$MPH from $(PFP)_2$—HOMPH/$(PFP)_2$—$[^2H_5]$HOMPH on 3% SP-2100 was very efficient as illustrated in the selected ion chromatogram (Fig. 3). These samples were extracted from spiked urine. The lack of background interference is a reflection of the molecular specificity of SIM detection.

The choice of PFP derivatives rather than the typically utilized trifluoroacetyl [13, 17, 19, 20] derivative was based upon the higher mass imparted to the SIM-derivatized piperidyl fragment. Monitoring this heavier fragment resulted in a decrease in interfering ion currents when biological samples were analyzed.

Assay linearity, precision, accuracy and sensitivity

Examination of typical standard curves by computing a regression line of peak area ratios $[^2H_0]/[^2H_5]$MPH on concentration ($[^2H_0]$MPH) using a least-squares fit demonstrated a linear relationship with correlation coefficients being consistently greater than 0.99 for both MPH and HOMPH. The within-

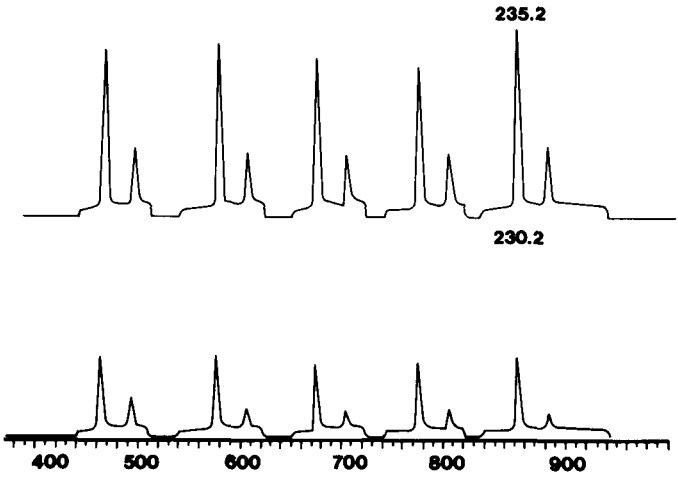

Fig. 3. Lower peaks (*m/e* 230.2) represent computer-plotted mass fragmentograms of MPH and HOMPH (smaller peaks) extracted from five spiked urine samples. The upper peaks represent the corresponding ion current of the pentadeuterated standards. Abscissa time scale 2 sec per scan number.

TABLE I

WITHIN-RUN PRECISION AND ACCURACY

Compound	Concentration (ng/ml)			
	n	Added	Found*	C.V. (%)
MPH	10	1	0.88	5.2
	10	2	2.07	7.3
	8	9	8.72	6.0
HOMPH	9	2	2.14	5.4
	9	9	8.90	2.3

*Values represent mean ± 1 standard deviation.

run precision and accuracy of the method were determined by assaying 1-ml aliquots of plasma to which either 1, 2 or 9 ng of MPH and 2 or 9 ng of HOMPH were added (Table I). The deviation of the amount of MPH found from the known amount added was greater at 1 ng/ml than at 2 or 9 ng/ml, with the coefficient of variation (C.V.) being independent of concentration. Both the accuracy and precision of replicate determinations of 2 or 9 ng/ml concentrations of HOMPH were greater at the higher concentration. The high sensitivity of the method is a consequence of the use of highly-enriched deuterated internal standards, efficient solvent extraction, the presence of an abundant EI-generated fragment for SIM and an essentially complete formation of stable, volatile derivatives. The practical limit of detection was 0.5 ng/ml for both MPH and HOMPH based upon an assayed plasma volume of 1 ml. These limits were defined by a minimal signal-to-noise ratio of 4 and a C.V. for replicate determinations of 15% or less. The present method permits the accurate quanti-

tation of plasma concentrations of MPH following clinical administration [22] in samples obtained for pharmacokinetic analysis [3] or other dispositional studies [8].

DISCUSSION

Using the present method, MPH and HOMPH were resolved from their respective internal standards by differences in their mass rather than their GC properties as has been previously used for the resolution of MPH from an alternative internal standard, ethylphenidate (ritalinic acid ethyl ester, ETPH) [13, 17—19]. This latter practice of SIM of a fragment (derivatized piperidine ring) common to both MPH and ETPH dictates a complete and consistent GC separation of these two compounds if usable assay accuracy and practical limits of detection are to be achieved. In practice, these criteria were difficult to attain in this laboratory and in others [18, 19]. Such chromatographic difficulties have led to the use of SIM of the dissimilar quasimolecular ions of MPH and ETPH generated by CI as an alternative means of resolving MPH and ETPH [18].

Iden and Hungund [18] first proposed the use of MPH with deuterium incorporated in the piperidine ring as an internal standard for EI—GC—MS analysis of MPH. However, the appropriate deuterium-labelled derivative was not a known compound at the time due to its rather involved synthesis [21]. With the recent availability of the deuterated compound as well as its *p*-hydroxylated derivative, use of these stable isotopic compounds as internal standards has improved overall assay performance relative to other literature

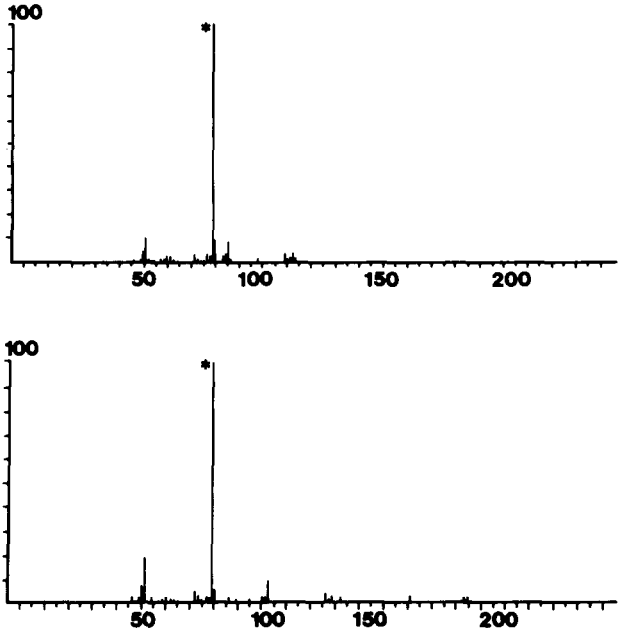

Fig. 4. EI mass spectra of MPH · HCl (upper) and HOMPH · HCl (lower). Indicated (*) ion represents the piperidyl fragment (*m/e* 84) of each.

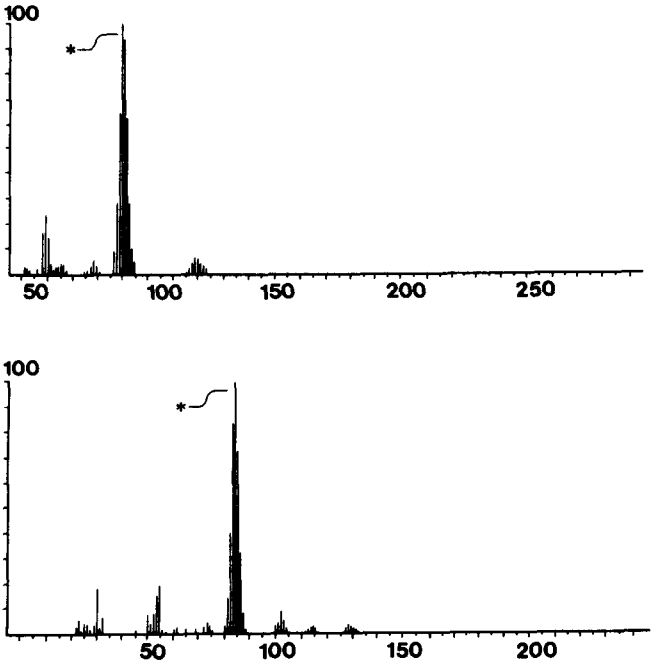

Fig. 5. EI mass spectra of [^2H_n]MPH · HCl (upper) and [^2H_n]HOMPH · HCl (lower). Indicated (*) ion represents pentadeuterated fragment (*m/e* 89) of each.

methods. The described procedure has been routinely used with consistently good results in this laboratory for the past three years, being applied to the determination of MPH in clinical plasma samples [3, 23] as well as analysis of MPH and HOMPH in other tissues from pre-clinical studies [3, 8].

The EI mass spectra of MPH and its *p*-hydroxy (see Fig. 4 and ref. 7), carboxy [7], N-acyl [17, 18] and deuterated derivatives (see Fig. 5 and ref. 21) are all dominated by the piperidine ring fragment which constitutes the base peak of each spectrum. Therefore, the incorporation of deuterium into the piperidine ring is essential to the use of deuterated variants as internal standards for a high sensitivity EI—GC—MS assay of MPH and HOMPH. The mass spectra of the deuterated compounds (Fig. 5) demonstrates the extensive incorporation of deuterium atoms into the piperidine ring, with all combinations of deuterons between [$^2H_3$] and [$^2H_9$] appearing in appreciable abundance as a consequence of exchange reactions concurrent with the deuterogenation during synthesis [21]. The contribution of trace amounts of [$^2H_0$]MPH from the internal standard to the ion current of the protium form (MPH) was exceedingly small, with the total isotopic enrichment (atom percent [^2H]) being 99.95% for [$^2H_{n\neq 0,1}$]MPH and 99.8% for [$^2H_{n\neq 0,1}$]HOMPH. However, in urine samples, where microgram quantities of internal standards were added, low nanogram blank values for MPH and HOMPH were necessary to subtract from unknowns. The most abundant isotopic variants were the pentadeutero forms of MPH and HOMPH, which represented approximately 25% of the deuterated species in each case. Accordingly, the corresponding PFP-derivatized piperidyl fragment ions (*m/e* 235) were chosen for SIM of the internal standards.

It is well-established that a stable isotopic derivative of an analyte is

expected to out-perform structurally-related compounds as GC—MS internal standards [24]. Besides analytical improvements in accuracy and precision, the sensitivity can be improved when the stable isotopic standard acts as a carrier compound, competing for losses of analyte occurring during sample work-up and chromatography. The deuterated standards used in the present method are particularly appropriate as carriers since only 25% of deuterated MPH and HOMPH exists in the pentadeutero form. The remaining 75% of the deuterated variants in the standards does not interfere with the assay while being available to function in a carrier capacity. Consequently, larger amounts of internal standard can be added to unknowns to improve work-up and chromatographic yield of the analytes without greatly imbalancing the relative ratios of the protium-form SIM fragments in favor of the pentadeuterated fragment. Large imbalances were observed to decrease assay sensitivity and contribute to peak integration difficulty in our current study.

Urinary excretion of MPH and HOMPH

The extent of *p*-hydroxylation in the biotransformation of MPH, as with amphetamine [25], assumes significant species-related differences. HOMPH appears to be a major metabolite in rats, but exists as only a reportedly minor metabolite in humans [6, 8]. As HOMPH is more efficacious than MPH in enhancing the functional activity of the putative neurotransmitter dopamine in the central nervous system [7], an action thought to be associated with the therapeutic effects of MPH, HOMPH has been suggested to represent a pharmacologically or clinically significant metabolite of MPH [6, 9].

In the present study, the 24-h urinary excretion of HOMPH in the two rats given 20 mg/kg MPH · HCl (i.p.) represented 11.5% and 10.5% of the administered dose. Conversely, less than 0.1% of an oral dose (0.3 mg/kg) of MPH · HCl administered to humans was excreted in the urine as HOMPH within 24 h of receiving the dose (Table II). The rat would therefore not appear to be a viable metabolic model of MPH biotransformation in humans, at least as involves the bioformation of phenolic metabolites. Further, it appears unlikely that HOMPH will contribute to the clinical response of the parent drug since HOMPH has not been detected in clinical plasma samples and is present in only very low concentrations in human urine.

TABLE II

CONCENTRATION OF MPH AND HOMPH IN 24-h URINE

Rats were administered MPH · HCl, 20 mg/kg i.p.; the human subjects received MPH · HCl, 0.3 mg/kg orally.

Compound	Rats (μg/ml)		Human subjects (ng/ml)		
	1	2	A	B	C
MPH	15.4	17.9	274.2	51.0	139.7
HOMPH	47.4	54.4	5.0	2.0	5.5
Urine volume (ml)	35.5	21.0	721	1379	821

ACKNOWLEDGEMENTS

This work was supported by PHS Grant MH-36294 and HD-03110. The authors wish to thank Josephene Hill and Patricia Childs for their help in preparing the manuscript.

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