Investigative Implications of the Instability and Metabolism of Mebeverine

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Abstract
The anti-spasmodic drug mebeverine is used in the treatment of irritable bowel syndrome. It has been found to be unstable and rapidly metabolized to initially form mebeverine-alcohol and veratric acid. Mebeverine-alcohol is a precursor for a number of amphetamine-like compounds. Consequently, these, in addition to mebeverine and mebeverine-alcohol, can produce false-positive amphetamine immunoassay results. Mebeverine is highly unstable in esterase-containing biological fluid (in particular blood and plasma), but it is largely stable in aqueous solutions and urine. Sodium fluoride did not appear to reduce mebeverine breakdown. Because of its unstable nature, mebeverine analysis should be performed as soon as possible after specimen receipt. Mebeverine, mebeverine-alcohol, and veratric acid concentrations should be measured in the blood/serum to assist interpretation; however, because of rapid metabolism/instability, mebeverine itself is rarely detected. In one fatal case of suspected mebeverine overdosage, mebeverine (1.2 mg/L), mebeverine-alcohol (74 mg/L), and veratric acid (127 mg/L) concentrations were measured in the postmortem blood; a high concentration of citalopram was also detected. In two fatalities involving possible therapeutic use, no mebeverine was detected, but mebeverine-alcohol (6.9 and 5.4 mg/L) and veratric acid (13.7 and 41.8 mg/L) were found by gas chromatography-mass spectrometry and high-performance liquid chromatography—diode-array detection (HPLC-DAD) and measured by HPLC-DAD. Only one case involving mebeverine has previously been published; this paper provides additional data and suggestions of best practice for case investigation.

Introduction
Mebeverine is the veratric acid ester of a substituted ethylamphetamine derivative; 4-{ethyl-[2-(4-methoxyphenyl)-1-methylethyl]amino}butan-lol. It is an anti-spasmodic drug believed to be a direct relaxant of the intestinal smooth muscle, a characteristic that has made it a drug commonly prescribed for the treatment of irritable bowel syndrome. The prescribed maximum daily dose of mebeverine hydrochloride is between 300 and 450 mg (1).

The large mebeverine molecule can be quickly and easily cleaved to produce two different structures; mebeverine-alcohol (MB-OH) and veratric acid. This process can occur both in vivo and in vitro because of esterase-catalyzed hydrolysis (2). As a result of this rapid conversion, in addition to considerable metabolism, mebeverine parent drug is rarely recovered from the blood or urine of patients (2,3). Although, the exact metabolic pathway for mebeverine has not been completely defined, there is a general consensus regarding the reactions and compounds involved (Figure 1). However, the exact proportions of products and the possibility of additional metabolites have been the subject of discussion in the literature (2–9).

Following Hoogewijs and Massart's (4) demonstration of the formation of MB-OH in pigs, Kristinsson et al. (3) studied the metabolism of mebeverine in human volunteers and published a preliminary metabolic pathway showing the production of six metabolites via (and including) the MB-OH and veratric acid intermediates. Subsequent studies by Kraemer et al. (5,6) detected additional MB-OH and veratric acid-derived metabolites and proposed that mebeverine could be metabolized into any 1 of 12 different compounds. Based on rat liver microsome and human urine volunteer analysis, MB-OH was postulated to form eight compounds, including, most notably, the amphetamine derivatives para-methoxyethylamphetamine (PMEA) and para-methoxyamphetamine (PMA), both well-recognized “designer drugs”. Veratric acid was proposed to form another four metabolites, in particular vanillic acid, supporting the initial work by Kristinsson et al. (3).

In a later paper by Stockis et al. (7), the existence of a 13th metabolite, mebeverine acid, was proposed. Although this compound (preferentially extracted under neutral pH conditions) was measured and pharmacokinetic data published, no specific analytical information [e.g., high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS), or UV data] was presented for comparison with other previously identified metabolites. Indeed, mebeverine acid was stated as being present in plasma at concentrations ~1000-fold higher than MB-OH. The production of the acid was proposed...
to have been due to selective oxidation of MB-OH. However, as this was not integrated into the proposed scheme of the metabolic pathway of mebeverine postulated by other researchers (in particular whether this occurs in preference to the production of PMEA, PMA, and other products), the oxidative mechanism and resultant fate of MB-OH remain unclear.

The subsequent problems in detecting intact mebeverine may in part explain the apparent lack of data pertaining to cases of mebeverine ingestion potentially resulting in intoxication, poisoning, and/or death. In life, following a single oral 135-mg mebeverine dose, peak plasma concentrations of 0.67 and 0.138 mg/L were reported for MB-OH and desmethylmebeverine-alcohol, respectively, within 1.5 h (8). Furthermore, no mebeverine was detected, but a peak plasma veratric acid concentration of 13.5 mg/L was found after an oral mebeverine dose of 270 mg (2). Stockis et al. (7) presented maximum plasma mebeverine alcohol and acid concentrations of 0.112 mg/L and 5.0 mg/L, respectively, following a 405-mg oral dose. In the only apparent published case of a fatality attributed to mebeverine ingestion, postmortem mebeverine concentrations of 36 and 24 mg/L were found in the blood and urine, respectively (9). However, no data pertaining to metabolite identification or concentration in this or similar cases have been reported. This paper presents data for mebeverine, mebeverine-alcohol, and veratric acid in three cases involving mebeverine ingestion, including one suspected fatal overdose. Further evidence for the potential for cross-reactivity with amphetamine immunoassay analysis is also investigated.

**Experimental**

**Instrumentation**

HPLC with diode-array UV detection (DAD) analysis was performed using a P580 low pressure pump, STH 585 column oven, ASI-100 autosampler, and a UVD340S DAD all from Dionex (Camberley, U.K.). A Waters Spherisorb SSOD/CN 4.6-mm x 150-mm cartridge column (Eistree, U.K.), protected by a 4-mm x 10-mm guard column of Spherisorb SSODS2 was used for the analysis. Data acquisition was handled by a Dionex Chromelon software package with the DAD recording spectral data between 200 and 595 nm. A wavelength of 220 nm was used for quantitative analysis.

GC–MS analysis was performed using an Agilent HP 6890 GC system fitted with a HP 5973 series mass selective detector (West Lothian, U.K.). A J&W Scientifics DB5 MS capillary column (30 m x 0.25-mm i.d., 0.25-μm film thickness) was used (J&W Scientifics, Bracknell, U.K.). Electron impact data was obtained in full-scan mode.

Amphetamine immunoassay analysis was performed using the Microgenics CEDIA amphetamine assay (Microgenics, St. Albans, U.K.) on an Olympus AU600 autoanalyzer (Olympus, Southall, U.K.). The assay was calibrated such that a “positive” rate of 100 would be equivalent to a 1000 pg/L amphetamine calibration standard.

**Reagents and standards**

Triethylammonium phosphate buffer (TEAP, 1.0M, pH 3.0)
was supplied by Fluka (Dorset, U.K.), and the HPLC-grade acetonitrile was supplied by Rathburns Chemicals (Walkerburn, U.K.). The HPLC-grade 1-chlorobutane was obtained from Fisher Scientific International (Loughborough, U.K.). Sulfuric acid and pyridine were supplied by BDH Chemicals (Poole, U.K.). Acetic anhydride and sodium fluoride was purchased from Sigma-Aldrich (Dorset, U.K.). Desipramine and chloramphenicol (internal standards) were kindly donated by Servier Laboratories (Wexham, U.K.). Mebeverine hydrochloride was supplied by Solvay Healthcare (Southampton, U.K.); veratric acid and vanillic acid were purchased from Sigma-Aldrich (Dorset, U.K.). These were used to prepare fresh reference and calibration standards for the formal identification and quantitation of these drugs in the specimens analyzed. Mebeverine was also used to produce MB-OH standards. A calibration range of 1.25 to 10 mg/L was produced for mebeverine, MB-OH, and veratric acid using blank (pre-screened) equine plasma. Internal quality control standards of 2 mg/L (veratric acid) and 4 mg/L (mebeverine and MB-OH) were also produced in equine plasma.

Preparation of mebeverine, MB-OH, and veratric acid standards

Mebeverine calibration and quality control standards were freshly prepared from an aqueous 1000 mg/L stock mebeverine solution diluted in equine plasma (pre-checked to be MB-OH-free). The highest calibration standard (10 mg/L) was serially diluted to 5, 2.5, and 1.25 mg/L and analyzed immediately after preparation.

MB-OH calibration and quality control standards were produced by maintaining a 10 mg/L mebeverine plasma standard at room temperature overnight to facilitate natural conversion to MB-OH (and veratric acid). The standard was then analyzed by HPLC–DAD and GC–MS to confirm complete conversion, thus producing a 10 mg/L MB-OH standard available for serial dilution for calibration. However, because of the unavailability of pure reference material, such calibration concentrations are approximate.

Veratric acid calibration and quality control standards were freshly prepared in plasma using pure reference material.

Extraction method for biological specimens

For HPLC–DAD analysis of mebeverine and MB-OH, 0.5 mL of internal standard (2 mg/L desipramine in 0.2M Na₂CO₃ solution, pH 10) was added to 0.5 mL of sample/standard followed by 5 mL of hexane/ethyl acetate (7:3) extraction solvent in a polypyrrole 12-mL tube. After 3 min mechanical shaking and centrifugation at 3500 rpm for 3 min, the upper aqueous layer was aspirated and the lower solvent layer was transferred to a second tube via a filter funnel through chloroform-saturated filter paper. The solvent was evaporated to dryness at 45°C under air, reconstituted with 100 μL of methanol, and transferred to a vial for injection. The injection volume was 30 μL.

For GC–MS analysis, 100 μL of 5M NaOH and 150 μL butyl acetate were added to 0.7 mL of sample/standard in an Eppendorf tube. This was vortex mixed for 30 s and centrifuged at 13,500 rpm for 2 min. For acetylated derivatization, 100 μL of the upper solvent layer was transferred into a 15-mL glass container, evaporated to dryness at 45°C under air, reconstituted with 75 μL of acetic anhydride/pyridine mixture (3:2), and then heated at 90°C for 5 min in a heating block. The derivatized extract was transferred to an insert, placed in a GC vial, and capped, ready for analysis. The injection volume was 1 μL.

Chromatographic conditions

Liquid chromatographic elution was based on 30% acetonitrile (70% 25mM TEAP buffer, pH 3) isocratic elution conditions at a flow rate of 2 mL/min for both mebeverine and veratric acid analysis. The column temperature was maintained at 25°C. For mebeverine analysis following basic pH extraction, mebeverine eluted at 10.55 min, mebeverine-alcohol eluted at 1.85 min, and desipramine (internal standard) eluted at 6.30 min. For veratric acid analysis following acidic pH extraction, veratric acid eluted at 1.92 min and chloramphenicol (internal standard) eluted at 2.37 min.

Gas chromatographic elution was based on a temperature gradient starting at 110°C (for 1 min), ramping to 300°C at 25°C/min, then holding at 300°C for 3.4 min. Total run time was 12 min with MS electron impact (EI) data acquired in the full scan mode between 4.5 and 11.5 min. Acetyl-derivatized veratric acid eluted at 6.22 min, and acetyl-derivatized MB-OH eluted at 8.17 min.

Mebeverine instability

From a 1000 mg/L aqueous stock solution of mebeverine, 2 mg/L solutions were prepared in water, plasma, and blood with a 10 mg/L solution prepared in urine. Sodium fluoride (1%) and >3% sodium fluoride were added (w/v) to additional 2 mg/L plasma and blood solutions. These were all stored under various conditions overnight (e.g., −20°C, room temperature, and 37°C). The samples were extracted and analyzed using the outlined HPLC methods for the extraction and detection of mebeverine, MB-OH, veratric acid, and/or other related breakdown products.

Cross-reactivity of mebeverine or breakdown products with CEDIA amphetamine immunoassay

Previous studies had shown a potential cross-reactivity of the amphetamine-based mebeverine products (e.g., PMEA) with an Abbott TDx fluorescence polarization immunoassay (FPJ) amphetamine/methamphetamine II assay (6). However, an aim of this paper was to determine the cross-reactivity of mebeverine breakdown products for a monoclonal antibody amphetamine CEDIA assay. Urine MB-OH standards of 20, 10, 5, 2.5, 1.25, and 0.625 mg/L were produced from a converted mebeverine standard (checked by HPLC–DAD and GC–MS)
and analyzed. The rate of reaction (based on optical density) was recorded. As the converted standard would also contain veratric acid at the same concentrations, potential veratric acid cross-reactivity was determined using up to 100 mg/L aqueous standards produced from pure veratric acid reference material. A veratric acid metabolite, vanillic acid, was also analyzed in the same way. Pure PMA and PMEA reference material was not available for evaluation.

Case histories

Mebeverine is not commonly mentioned as a prescribed drug in the majority of clinical and postmortem cases investigated by our laboratory. Indeed, mebeverine was noted as being prescribed to the deceased in only 10 fatalities between 2001 and 2004. Of these, following routine screening by HPLC-DAD, mebeverine or metabolites was only detected in two of the cases but was also detected in one previous case in 1999 (where mebeverine had not been prescribed). Mebeverine, MB-OH, and veratric acid concentrations were measured in the blood and serum samples submitted for analysis; however, there was insufficient urine available for additional quantitation. All samples had been stored at -20°C prior to analysis.

Results

Identification of mebeverine and metabolites/breakdown products

The published literature presents mainly acetylated MS data for mebeverine and its metabolites (3,5,6). Although other analytical data (e.g., nuclear magnetic resonance, HPLC, infrared) is referred to in some publications, none is shown (3,7). Therefore, during this investigation the identification of mebeverine, MB-OH, and veratric acid was confirmed based on acetylated derivatives analyzed by GC-MS in the full scan mode (5,6). However, additional UV spectral data for mebeverine, MB-OH, and veratric acid were also obtained during this study (Figure 2). As the compounds exhibit very different spectra, UV-DAD would be a useful technique for rapid identification in the absence of MS data. There was no apparent confirmation of the presence or existence of mebeverine acid [as proposed by Stockis et al. (7)] despite pH neutral extraction, but identification of any candidate analyte would have been hampered by the lack of any published analytical data. However, as Stockis et al. (7) indicated, this was the predominant species of mebeverine metabolism (additional work is needed to confirm the authors' findings) in relation to the metabolic pathway as suggested by other researchers in particular (Figure 1).

Mebeverine instability

HPLC–DAD analysis of the 2 mg/L plasma and blood mebeverine standards stored at room temperature and at 37°C showed complete conversion of mebeverine to MB-OH and veratric acid, even in the presence of 1% and > 3% sodium fluoride. However, 2 mg/L water and 10 mg/L urine standards showed no conversion at these conditions. In particular, even at 37°C, a 10 mg/L urine mebeverine standard was stable with no breakdown products detected. However, a 2 mg/L plasma standard stored at -20°C overnight exhibited 72% conversion. To further study the rapidity of reaction, 81% of a 100 mg/L mebeverine plasma standard was found to be converted within 2.5 h when stored at 37°C.

Cross-reactivity of mebeverine or breakdown products with CEDIA amphetamine immunoassay

As shown in Figure 3, the amphetamine CEDIA assay (based on a “positive” cut-off of 1000 mg/L) showed a positive result occurs at MB-OH concentrations of greater than 4.5 mg/L and at mebeverine concentrations greater than 6 mg/L. Veratric acid and vanillic acid did not show a measurable rate, even at higher concentrations (100 mg/L), and therefore are not likely to pro-

Figure 2. UV spectra of mebeverine, mebeverine-alcohol (MB-OH), and veratric acid.
duce "false positive" results using this technique. Kraemer et al. (6) suggested that the intact hydroxybutyl side chain of mebeverine and MB-OH prevented cross-reactivity with the FPIA assay and that the amphetamine-positive immunoassay results could be due to N-dehydroxybutylated metabolites of mebeverine, such as PMA or PMEA. Their studies showed only a negligible cross-reactivity with mebeverine and MB-OH (< 0.1%). However, Figure 3 indicates that this is not the case for CEDIA immunoassay.

Case histories
Case 1. A 44-year-old female was found dead at home. She had been prescribed mebeverine. Citalopram (3.72 mg/L) and ethanol (209 mg/dL) were also found in the femoral postmortem blood. An overdose of prescribed medication was suspected.

Case 2. A 32-year-old female was found dead at home. She had been prescribed mebeverine. Therapeutic amounts of fluoxetine, chlordiazepoxide, paracetamol, valproate, and ethanol (27 mg/dL) were also found in the postmortem blood.

Case 3. A 20-year-old female was found collapsed at home and later died in hospital. She was not thought to have been prescribed mebeverine. Therapeutic amounts of nortriptyline, carbamazepine, and ethanol (51 mg/dL blood) were also found in the antemortem serum and postmortem blood.

The concentrations of mebeverine, MB-OH, and veratric acid in the three cases are shown in Table I. It should also be noted that a presumptive positive result for "amphetamines" was obtained for each case following immunoassay analysis of the urine. The presence of any other amphetamines (including PMA, PMEA, amphetamine, MDMA, and phenyl-2-ethylamine) could not be detected/confirmed using GC–MS, GC–nitrogen-phosphorus detection, and HPLC–DAD.

Discussion
Mebeverine instability
The results indicate mebeverine is more stable in urine or aqueous media compared to blood/plasma and support the findings of other researchers. This has implications for the storage of both case samples and calibration standards. Dickinson et al. (2) demonstrated that the use of the esterase inhibitor, physostigmine, reduced mebeverine hydrolysis. However, in this study, another esterase inhibitor, sodium fluoride, did not appear to reduce mebeverine hydrolysis. Alternatively, it is recommended that, as detailed in this paper, an aqueous stock standard of mebeverine is produced and stored at -20°C prior to being freshly diluted in the required matrix (e.g., plasma) for analysis of case samples. This would also apply to the production and storage of any internal quality standards. For case material, it is recommended that samples be analyzed as soon as possible following receipt. Otherwise, storage at -20°C would reduce the degree of mebeverine breakdown for a short time prior to analysis. However, significant breakdown may occur during long-term storage (particularly in blood/plasma) and during the thawing process prior to analysis (use of an increased temperature to aid thawing is not recommended). Nonetheless, as also detailed in this paper, measurement of the metabolites and breakdown products (MB-OH and veratric acid) is possible and is necessary following a period of degradation. However, it is not possible to accurately determine what proportions of the concentration are present as a product of mebeverine breakdown or as a product of mebeverine metabolism.

Cross-reactivity of mebeverine or breakdown products with CEDIA amphetamine immunoassay
Based on evaluation of mebeverine, MB-OH, veratric acid, and vanillic acid standards, a "positive" result for amphetamines using CEDIA-based immunoassay was obtained for mebeverine and MB-OH at concentrations greater than 6 and 4.5 mg/L, respectively. This was in contrast to other studies using FPIA.
amphetamine analysis (6) and suggests the CEDIA assay is not affected by the presence of an intact hydroxybutyl side chain. The lack of cross-reactivity with veratric and vanillic acids suggests immuno-binding occurs on the methoxylbenzene portion of the mebeverine molecule, which has greater structural similarity to amphetamine than the veratric acid portion (Figure 1). Because of the rapid metabolism and in vitro degradation of mebeverine, it is likely, therefore, that MB-OH would be responsible for any “positive” amphetamine result obtained using CEDIA amphetamine analysis. Consequently, investigators should consider the involvement/presence of mebeverine in cases where apparent unconfirmed amphetamine immunoassay findings have been found.

Case histories

Mebeverine parent compound was only detected in Case 1, albeit at a relatively low concentration (1.2 mg/L). However, much higher concentrations of MB-OH (74.0 mg/L) and veratric acid (127.0 mg/L) were measured in the postmortem blood. These higher concentrations and the presence of intact mebeverine (possibly due to enzymic saturation) strongly indicated a large dose of mebeverine followed by some degradation and/or metabolism. Comparison to a previously published fatality (mebeverine 36 mg/L in the blood) is possible but limited as no methodological or metabolite data are available for that case (9). Nevertheless, the significance of mebeverine to the cause of death in Case 1 was unclear because of the additional presence of a high concentration of citalopram (3.27 mg/L), but an overdose of both drugs was likely.

The much lower concentrations in Case 2 (no mebeverine, 6.9 mg/L MB-OH, and 13.7 mg/L veratric acid in postmortem blood) and Case 3 (no mebeverine, 5.4 mg/L MB-OH, and 41.8 mg/L veratric acid in antemortem serum) are more difficult to interpret. In particular, it is not possible to accurately determine what proportion of the MB-OH or veratric acid concentration is present as a breakdown product or metabolite. However, mebeverine ingestion would result in the initial production of equimolar amounts of MB-OH and veratric acid. Studies have shown higher concentrations of veratric acid exist in biological fluid compared to MB-OH (3). This is likely to be due to the increased number of metabolic options for the conversion of MB-OH (potentially 9 metabolites) compared to veratric acid (3 metabolites). Therefore, following ingestion, although MB-OH and veratric acid concentrations may be initially similar, with time, higher concentrations of veratric acid will remain (2). If mebeverine has not been previously ingested, the concentration of veratric acid would be more representative than MB-OH of the original mebeverine concentration because of continued MB-OH metabolism post dose. However, if mebeverine has been used previously (in particular chronic therapy), the veratric acid concentration may be elevated and less representative of the last mebeverine dose. The resulting MB-OH to veratric acid concentration ratio would be weighted toward veratric acid, which could explain the results in Case 3. Furthermore, if it is assumed that the concentrations are solely derived from in vitro hydrolytic breakdown, the original mebeverine concentration cannot be greater than the highest MB-OH or veratric acid concentration measured, and the concentration ratios should be similar. These observations may be useful in ruling out possible overdose.

Despite these suggestions, it is, as previously stated, difficult to determine a simple interpretative “cut-off” for either MB-OH or veratric acid concentrations because of the paucity of both therapeutic and toxic data. The published concentrations of veratric acid (13.5 mg/L) and MB-OH (0.57 mg/L) found in two separate therapeutic situations (acute single dose) provide some comparative data for the cases analyzed (2,8). In particular, the results of Case 2, where overdosage of mebeverine was not suspected, could be due to chronic therapeutic use given the low MB-OH (6.9 mg/L) and veratric acid results (13.7 mg/L). A more appropriate comparison for the cases would be with Case 1, where excessive mebeverine ingestion was likely. Consequently, although the veratric acid concentration measured in antemortem serum in Case 3 was higher (41.8 mg/L), the MB-OH concentration was still comparatively low (5.4 mg/L) and therefore did also not necessarily indicate mebeverine overdosage prior to hospital admission. The subsequent low postmortem blood concentrations (4.8 mg/L veratric acid and 0.8 mg/L MB-OH) and absence of mebeverine did not suggest continued absorption post-admission.

Conclusions

The instability and metabolism of mebeverine presents both an analytical and interpretative challenge to toxicologists. This paper has attempted to evaluate these aspects in order to provide some suggestions for investigating such cases. Firstly, regardless of the storage conditions, it is advised to perform mebeverine analysis and quantitation as soon as possible following sample receipt. In the absence of a detailed drug history, initial suspicion of mebeverine involvement may arise from an amphetamine “positive” finding by immunoassay. For interpretative purposes, mebeverine, mebeverine-alcohol, and veratric acid concentrations should be measured. The presence of mebeverine itself may be an indication of overdosage (especially if there has been an extended postmortem or analytical interval), and a concentration ratio biased toward veratric acid may indicate previous use. In one suspected case of mebeverine overdosage, a mebeverine concentration of 1.2 mg/L was measured in the postmortem blood with corresponding MB-OH and veratric acid concentrations of 74 and 127 mg/L, respectively.

References

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