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Determination of *m*-hydroxymandelic acid, *m*-hydroxyphenylglycol and their conjugates in human plasma using liquid chromatography with electrochemical detection*

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Abstract: An LC method for the analysis of *m*-hydroxymandelic acid (MHMA) and *m*-hydroxyphenylglycol (MHPG) and their conjugates in human plasma was developed and validated. The method for the quantitation involved extraction of acidified plasma (subject to hydrolysis with β-glucuronidase for 120 min with 500 units of enzyme/0.25 ml of plasma at 37°C for the conjugates) with an organic phase (methyl-*tert*-butyl ether). Analysis of MHMA, MHPG and the internal standard (3-hydroxy-4-methoxymandelic acid) was carried out on an ODS stationary phase: 100 × 4.6 mm, 5 μ followed by a 75 × 4.6 mm, 3 μ using 1% acetonitrile in 0.1 M acetic acid as the mobile phase. An electrochemical detector operated at +1.15 V vs Ag/AgCl was employed for the detection. The standard curves were linear in the range of 10.0–250.0 ng ml^{-1} for MHMA and 5.0–125.0 ng ml^{-1} for MHPG. The limit of quantitation was 10.0 ng ml^{-1} for MHMA and MHPG. Acceptable accuracy and precision were obtained during the intra-batch and inter-batch analysis for both the assays.

Keywords: *LC; metabolites; phenylephrine; electrochemical detection;* m-*hydroxymandelic acid;* m-*hydroxyphenylglycol.*

Introduction

Phenylephrine, an alpha-receptor agonist is metabolized to *m*-hydroxyphenylglycol (MHPG) and *m*-hydroxymandelic acid (MHMA) in the human body [1]. The deamination of phenylethylamines is a two-step process [2]: monoamine oxidase converts the amines to aldehydes which are further metabolized to either a glycol (by aldehyde reductase) or to an acid (by aldehyde dehydrogenase). There is a complete lack of information on the plasma concentrations of these metabolites in the literature. The objective was to develop sensitive and specific methods for the analysis of MHMA and MHPG in human plasma. MHMA and MHPG have been quantitated in human urine by gas chromatography–mass spectrometry-selected ion monitoring [3, 4]. The methods involved a derivatization step which was necessary for detection. To date, there are no liquid chromatographic (LC) methods available for the measurement of MHMA and MHPG, especially in human plasma.

Experimental

Chemicals and supplies

m-Hydroxyphenylglycol was synthesized in the Pharmacokinetics Laboratory of the School of Pharmacy at the University of Missouri–Kansas City. The procedure for the synthesis and purification is described elsewhere [5]. *m*-Hydroxymandelic acid, 3-hydroxy-4-methoxymandelic (HMMA) acid and β-glucuronidase from *Helix pomatia* (enzyme activities: 400,000 units g^{-1} of β-glucuronide glucuronosohydrolase; 22,000 units g^{-1} of sulphatase) were obtained from Sigma (St Louis, MO). Methyl-*tert*-butyl ether and acetonitrile were of HPLC grade and obtained from Burdick and Jackson (Muskegon, MI). Glacial acetic acid (GAA) was obtained from J.T. Baker (Phillipsburg, NJ). Concentrated hydrochloric acid (HCl) (36%) and sodium acetate were obtained from

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Fisher (Springfield, MO). Drug-free human plasma was obtained from Biological Specialty Corporation (Lansdale, PA).

Standard solutions

The internal standard (IS) solution (3-hydroxy-4-methoxymandelic acid, 1000 ng ml^{-1}) was prepared in distilled water (DW). 1.0 N and 0.1 N HCl were prepared by diluting 42.5 and 0.85 ml of concentrated HCl to 500 and 100 ml with DW. Sodium acetate buffer (100 mM; pH 5.0) was prepared by adjusting the pH of 0.82 g of sodium acetate dissolved in about 90 ml DW with GAA, and making up the volume to 100 ml with DW. β-Glucuronidase solution (5000 units ml^{-1}) was prepared by dissolving 12.5 mg of enzyme in 1 ml of sodium acetate buffer (100 mM; pH 5.0). Six ml of GAA was diluted to 1 l with DW and filtered through 0.45 μ filter to obtain 0.1 M acetic acid. The mobile phase for LC (1% acetonitrile in 0.1 M acetic acid) was prepared by making up a volume of 10 ml of acetonitrile to 1 l with 0.1 M acetic acid. The mobile phase was mixed thoroughly and degassed.

Separate stock solutions of MHPG and MHMA were prepared by dissolving a precisely weighed quantity in DW to give a concentration of 1 mg ml^{-1}. A primary plasma stock solution (PPS) was prepared by mixing 50 μl stock solution of MHPG with 100 μl stock solution of MHMA and diluting volumetrically to 10 ml with blank plasma (conc. 10/5 μg of MHMA/MHPG per ml of plasma). The calibration standards were prepared by further serial dilutions of the PPS. Blank plasma was used for the 0.0 ng ml^{-1} standard. A 250 μl quantity of the calibration standards was aliquoted into the tubes and frozen at −20°C.

For the preparation of validation pools, separate stock solutions (conc. 1 mg ml^{-1} of DW) and primary plasma stock solutions were prepared for each analyte. Further dilutions with plasma were carried out for each analyte separately until a solution with double the desired concentration was obtained. Equal volumes of the concentrated validation pools for each analyte were mixed to get the appropriate validation pools.

The calibration standards and the validation pools for the assay of the conjugates of MHMA and MHPG were prepared in a similar fashion as mentioned above. For the sake of distinction, these calibration standards and validation pools will be referred to as being hydrolysed with β-glucuronidase, or MHMA-GLU and MHPG-GLU, for short.

LC system and chromatographic conditions

The LC system consisted of a Model 110A pump (Altex, Berkeley, CA), Shimadzu SIL-6B injector operated by Shimadzu SCL-6B system controller (Shimadzu, Kyoto, Japan), Model LC-4B amperometric detector with LC-22A temperature controller and glassy carbon working electrode (Bioanalytical System, West Lafayette, IN) and Shimadzu C-R1B integrator. An Omniscribe Series B-5000 strip chart recorder (Houston Instruments, Austin, TX) with dual voltage pens was simultaneously used for the signal measurement. MHMA, MHPG and the internal standard (HMMA) were separated on Altex Ultrasphere octadecylsilane (ODS), 5 μg, 100 × 4.6 mm column followed by a 3 μ, 75 × 4.6 mm column (supplier P.J. Cobert, St Louis, MO) at room temperature using an isocratic mobile phase consisting of 1% acetonitrile in 0.1 M acetic acid. The flow rate was maintained at 1.5 ml min^{-1} and the detection was by oxidation at +1.15 V vs Ag/AgCl at 30°C. The response generated by the detector was measured as peak height at a detection range of 100 nA V^{-1}. After the elution of MHMA, MHPG and HMMA from the column, 9.5 min were allowed before the next injection to remove the rest of the late eluting peaks.

A mobile phase consisting of acetonitrile–acetic acid (0.1 M) was employed at a flow rate of 1.5 ml min^{-1} for the analysis of MHMA and MHPG. This provided about 2933, 5158 and 3873 theoretical plates on the columns for MHMA, MHPG and the IS. With a capacity factor of 2.5, 3.7 and 4.6 for MHMA, MHPG and the IS, good resolution from each other (MHMA–MHPG resolution = 2.4; MHPG–IS resolution = 3.3) and the co-extracted compounds were observed. MHMA was found to have a retention volume of 7.2 ml and MHPG had a retention volume of 9.5 ml, while the retention volume for the IS was 11.3 ml.

Extraction procedure for LC analysis

MHMA and MHPG. The stored samples were equilibrated to room temperature and vortexed briefly. A 250 μl aliquot of stored validation pools was pipetted into conical tubes and the calibration standards were used as

such. Internal standard (50 μl of 1000 ng ml^{-1} of 3-hydroxy-4-methoxymandelic acid) was added to each sample. After delivering 200 μl of 1.0 N hydrochloric acid to each sample, the samples were vortexed again for a brief period and 5 ml of methyl-*tert*-butyl ether was added to the samples. The samples were vortexed for 2 min (1 min on each side of the vortexer) on the SMI Multitube vortexer (Scientific Manufacturing Industries, Emeryville, CA). The organic layer was separated into clean borosilicate culture tubes after centrifugation for 10 min at 1200*g* in the Dynac centrifuge (Clay Adams, Parispany, NJ). The organic layer was evaporated to dryness under a gentle stream of nitrogen using Pierce Reacti-Vap III (Pierce, Rockford, IL). The residue was reconstituted in 150 μl of distilled water and a 50 μl portion of each sample was injected into the LC system.

MHMA-GLU and MHPG-GLU. After appropriately preparing the calibration standards and the validation pools (250 μl plasma), 75 μl of 0.1 N HCl was delivered to each sample. The samples were equilibrated to 37°C in a shaker bath (Precision Scientific, Chicago, IL) and 100 μl of β-glucuronidase solution was added to the plasma samples. The tubes were capped and incubated at 37°C for 120 min at 60 Osc min^{-1}. After the incubation, internal standard (50 μl of 1000 ng ml^{-1} of HMMA), 200 μl of 1.0 N HCl and 5 ml of methyl-*tert*-butyl ether were delivered to each sample. Extraction was effected by shaking horizontally at 1 Osc s^{-1}. The rest of the separation procedure and evaporation of the organic layer was as described above.

Validation protocol

MHMA and MHPG. Seven validation batches were analysed. Each batch contained duplicate calibration standards at the following concentrations for MHMA/MHPG: 0.0/0.0, 10.0/5.0, 20.0/10.0, 50.0/25.0, 100.0/50.0, 166.7/83.3 and 250.0/125.0 ng ml^{-1} plasma. There were a total of four validation pools at the following concentrations for MHMA/MHPG: 10.0/5.0, 20.0/100.0, 100.0/50.0 and 200.0/10.0 ng ml^{-1} plasma. Each validation pool was assayed five times in a batch. These validation pools functioned to evaluate sensitivity of the assay, inter-batch as well as intra-batch accuracy and precision. The standards and validation pools were assayed in a random order.

MHMA-GLU and MHPG-GLU. A total of five validation batches were run. Each batch contained calibration standards and validation pools as described above. In addition, a validation pool containing an unknown amount of the MHMA/MHPG conjugates and obtained in an actual pharmacokinetic study from a subject dosed with phenylephrine was used. The unknown validation pool functioned to evaluate reproducibility of the hydrolysis procedure.

Data evaluation and calculations

Calibration curves were generated by linear regression of all the calibration standards (excluding 0.0 ng ml^{-1} standard). Regression statistics, i.e. intercept, slope, correlation coefficient and standard error of estimate were calculated for each curve. The per cent relative standard deviation (RSD) was used as a measure of precision. It is the standard deviation (SD) expressed as a percentage of the average computed concentration (RSD = (SD × 100)/average conc.). The percentage analytical recovery (% AR) was used to assess accuracy and is defined as average computed concentration expressed as a percentage of the amount of analyte added (% AR = (Average conc. × 100)/amount of analyte added). Limit of quantitation (LOQ) was defined as the smallest detectable concentration which can be estimated with an acceptable degree of precision and accuracy. Limit of detection was set at the lowest concentration level that can be determined to be statistically different from an analytical blank (0.0 standard). It was found by taking twice the standard deviation of the 0.0 standard in units of concentration, i.e. ng ml^{-1}. The acceptance criteria for the calibration standards and the validation pools was as described by Shah *et al.* [6]. The accuracy and precision of the calibration standards and validation pools were to be within 15%. A RSD of 20% was considered acceptable for the lowest validation pool.

Results

Chromatography

Typical chromatograms following a 50 μl injection of extracted standards prepared to contain 20.0/10.0 and 250.0/125.0 ng ml^{-1} of

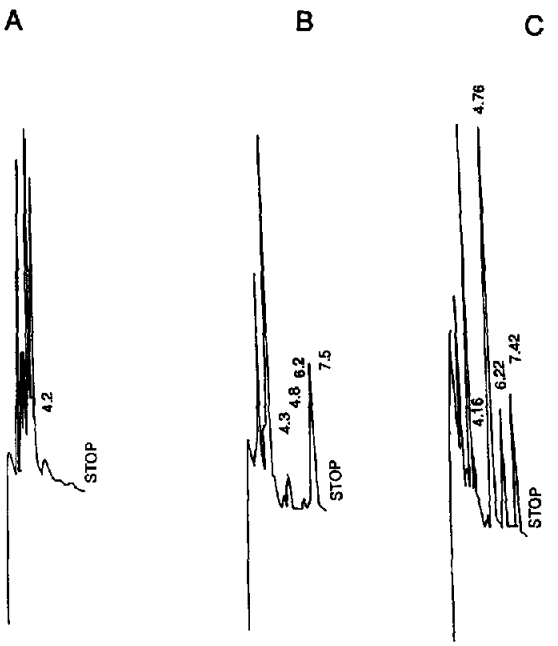

Figure 1
Typical chromatograms of (A) blank plasma sample from pharmacokinetic study and *m*-hydroxymandelic acid (MHMA) and *m*-hydroxyphenylglycol (MHPG) standards, (B) 20.0/10.0 ng ml^{-1} and (C) 250.0/125.0 ng ml^{-1} of MHMA/MHPG with 50 ng ml^{-1} of internal standard (IS). MHMA, 4.8 min; MHPG, 6.2 min; IS, 7.4 min.

MHMA/MHPG and 200 ng ml^{-1} of internal standard and a blank plasma from a pharmacokinetic study are presented in Fig. 1.

Detection

Detection was done electrochemically in the oxidation mode on a glassy working electrode at an applied potential of +1.15 (reference electrode Ag/AgCl). To select the proper detection potential, a hydrodynamic voltammogram was constructed (Fig. 2). A sensitivity of 100 nA V^{-1} was found to be appropriate for all the samples.

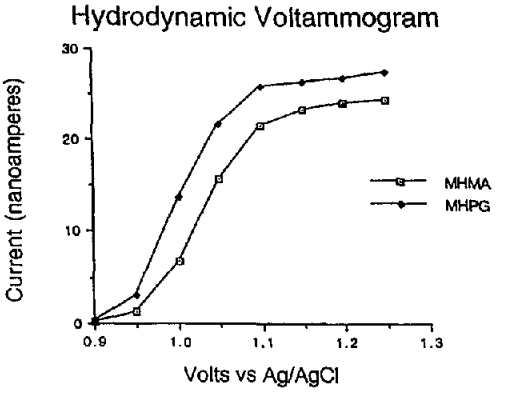

Figure 2
Hydrodynamic voltammogram of *m*-hydroxymandelic acid (MHMA) and *m*-hydroxyphenylglycol (MHPG).

Linearity and reproducibility

The calibration curves were reproducibly linear in the range of 10.0–250.0 ng ml^{-1} for MHMA and in the range of 5.0–1250.0 ng ml^{-1} for MHPG. Linearity over the same ranges was also reproducibly demonstrated for the β-glucuronidase hydrolysed calibration curve of these two analytes. Regression statistics from the calibration standard curves for MHMA and MHPG and their hydrolysed counterparts, MHMA-GLU and MPHG-GLU during the validation study are presented in Table 1.

The linearity of the standard curves was further extended by the validation of a dilution procedure of 1 to 5 (using a validation pool of 500.0/250.0 /ng ml^{-1} for MHMA/MHPG).

Accuracy and precision

Table 2 presents the parameters used to verify the accuracy and precision of the methods for the analysis of MHMA and MHPG, MHMA-GLU and MHPG-GLU in human plasma. The average precision for

Table 1
Regression statistics for *m*-hydroxymandelic acid (MHMA), *m*-hydroxyphenylglycol (MHPG) and enzymatically hydrolysed *m*-hydroxymandelic acid (MHMA-GLU) and *m*-hydroxyphenylglycol (MHPG-GLU)

Analyte/Range	*n*	Slope	Intercept	Corr.	SEE
MHMA	6	83.60	−0.219	0.997	6.32
10.0–250.0 ng ml^{-1}		(±)7.51	(±)1.57	(±)0.003	(±)3.73
MHPG	7	149.97	−1.67	0.997	3.37
5.0–125.0 ng ml^{-1}		(±)11.8	(±)1.27	(±)0.003	(±)1.83
MHMA-GLU	5	70.82	0.336	0.998	5.71
10.0–250.0 ng ml^{-1}		(±)2.87	(±)1.57	(±)0.001	(±)1.71
MHPG-GLU	5	149.9	−0.534	0.997	3.40
5.0–125.0 ng ml^{-1}		(±)10.7	(±)2.24	(±)0.002	(±)0.82

Corr.: correlation coefficient.
SEE: standard error of estimate.

Table 2
Accuracy and precision for validation pools of *m*-hydroxymandelic acid (MHMA), *m*-hydroxyphenylglycol (MHPG) and enzymatically hydrolysed *m*-hydroxymandelic acid (MHMA-GLU) and *m*-hydroxyphenylglycol (MHPG-GLU)

Analyte	*n*	Mean	SD	RSD	%AR
MHMA (ng ml^{-1})					
10.0	30	9.88	1.24	12.6	98.8
20.0	30	19.93	1.62	8.1	99.7
100.0	30	105.1	7.35	7.0	105.1
200.0	30	207.2	12.9	6.2	103.6
MHPG (ng ml^{-1})					
10.0	34	9.50	1.56	16.4	95.0
50.0	35	50.77	5.34	10.5	101.5
100.0	35	103.4	10.0	9.7	103.4
MHMA-GLU (ng ml^{-1})					
10.0	26	11.07	1.37	12.4	110.7
20.0	29	22.49	3.17	14.1	112.5
100.0	29	108.8	9.59	8.8	108.8
200.0	30	219.3	24.3	11.1	109.7
MHPG-GLU (ng ml^{-1})					
10.0	27	10.08	1.63	16.2	100.8
50.0	29	53.52	5.73	10.7	107.0
100.0	29	105.6	9.20	8.7	105.6
Total MHMA (unknown conc.)	30	185.4	20.4	11.0	
Total MHPG (unknown conc.)	35	38.41	4.77	12.4	

SD: standard deviation.
RSD: per cent relative standard deviation.
%AR: per cent analytical recovery.

MHMA was 8.5%, and the same for MHMA-GLU was 11.6%. The average precision for MHPG and MHPG-GLU was 12.2 and 11.9%, respectively. The average accuracy for *m*-hydroxymandelic acid was 101.8%, and the same for MHMA-GLU was 110.4%. The average accuracy for *m*-hydroxyphenylglycol and MHPG-GLU was 100.0 and 104.4%, respectively. The accuracy and precision for 5.0 ng ml^{-1} validation pool for *m*-hydroxyphenylglycol and its enzymatically hydrolysed part are not reported because they did not meet the acceptance criteria.

Limits of quantitation and detection

The limit of quantitation for the assay of MHMA and MHMA-GLU was 10.0 ng ml^{-1} (Table 2), and the same for the analysis of MHPG and MHPG-GLU was also 10.0 ng ml^{-1}. The validation pool at the concentration of 5.0 ng ml^{-1} for both MHPG and MHPG-GLU failed to meet the acceptance criteria required for the limit of quantitation. The limits of detection for the analysis of MHMA and MHMA-GLU in human plasma were 3.9 ng ml^{-1} and 3.4 ng ml^{-1}, respectively, whereas, the same for MPHG and MHPG-GLU were 8.8 ng ml^{-1} and 3.3 ng ml^{-1}, respectively.

Reproducibility and optimization of hydrolysis of the conjugates

The amount of enzyme (β-glucuronidase) required, and the length of incubation for the complete hydrolysis was optimized using varying amounts of the enzyme and different lengths of time. A sample with an unknown amount of conjugates of MHMA and MHPG was obtained from a human pharmacokinetic study and used for the optimization procedure. After incubation for an appropriate length of time with an appropriate concentration of enzyme the samples were extracted as described above and then subject to the LC procedure. A ratio of peak height of analyte to internal standard was used as a measure of the extent of hydrolysis; an increase in the ratio indicating an increase in the analyte liberated from the conjugates. β-Glucuronidase enzyme (500, 2750 or 5000 units) dissolved in 0.1 ml of sodium acetate buffer (100 mM, pH 5.0) was incubated with 250 μl of plasma for 30, 75 or 120 min at 37°C in a water bath. The results from the enzymatic optimization procedure indicated that 500 units of enzyme incubated with 250 μl of plasma for 30 min were sufficient for complete hydrolysis of the MHMA conjugates, however, there was an increase in the concentration of MHPG with increasing

time and enzyme amount. Therefore, another study was carried out with 500 units and 5000 units of enzyme over a period of 24 h (0, 2, 6, 12, 24 h). Based on the results it was concluded that 120 min of incubation at 37°C with 500 units of β-glucuronidase enzyme added to 0.25 ml of plasma was sufficient for the complete hydrolysis of the conjugates of MHMA and MHPG.

Reproducibility of the hydrolysis was estimated as the RSD (precision) obtained during the validation procedure and the results are presented in Table 2. Since the enzymatic hydrolysis with β-glucuronidase gave the total MHMA and total MHPG, it was assumed that unconjugated forms of the analytes were either constant or negligible. Therefore, RSD of 11.0% for total MHMA and 12.4% for total MHPG conjugates indicated that the hydrolysis of the conjugates in plasma was being carried out reproducibly.

Specificity

Specificity was determined by evaluating a minimum of 10 plasma samples devoid of drug to verify the absence of interfering substances present at the retention times of MHMA, MHPG and the IS.

Stability of analytes on freeze–thaw

A validation pool with concentration of 100.0/50.0 ng ml^{-1} for MHMA/MHPG was prepared and frozen at −20°C. The pool was thawed (0 cycle) and analysed repeatedly (one and two cycles) for two freeze–thaw cycles to evaluate the effects on the stability thereof. The results presented in Table 3 indicate that MHMA and MHPG were stable during the two freeze–thaw cycles.

Table 3
Stability of *m*-hydroxymandelic acid (MHMA) and *m*-hydroxyphenylglycol (MHPG) frozen at −20°C during two freeze–thaw cycles

Freeze–thaw cycle	Mean	SD	RSD	%AR
MHMA (100.0 ng ml^{-1})				
0	103.3	5.74	5.6	103.3
1	103.3	1.69	1.6	103.3
2	101.6	4.22	4.2	101.6
MHPG (50.0 ng ml^{-1})				
0	47.23	3.12	6.6	94.5
1	54.10	4.74	8.8	108.2
2	48.79	3.15	6.5	97.6

SD: standard deviation.
RSD: per cent relative standard deviation.
%AR: per cent analytical recovery.

Discussion

Since the nature of the functional groups on MHPG and MHMA was very similar, the objective was to isolate both the analytes into an organic solvent in a single extraction step. Extraction of acidified (pH 1–2) aqueous solution of MHMA and MHPG (100 ng ml^{-1} each) with ethyl acetate and methyl-*tert*-butyl ether gave fairly good recovery (60 and 76% with methyl-*tert*-butyl ether and 75 and 76% with ethyl acetate for MHPG and MHMA, respectively). However, with the plasma samples, presence of interfering peaks with MHPG in blank plasma along with higher sensitivity requirements (objective was 5 ng ml^{-1}) clearly showed that further 'clean up' steps would be required.

One of the initial techniques used to clean up the plasma samples was a double-extraction procedure. After extraction of the analytes into an organic solvent (ethyl acetate), back extraction with 0.1 N NaOH followed by re-extraction of the acidified basic layer was carried out. This procedure did not extract either of the two analytes. An attempt was made to facilitate separation of the analytes from the potential interferences on solid-phase extraction (SPE) columns. Various SPE columns covering a wide range of polarity were evaluated; amino, cyano, silica, diol, phenyl and ODS. Extraction of the interferences and the analytes from the plasma onto the SPE columns and elution from SPE columns occurred more or less in a parallel pattern.

Since the sensitivity requirements of the two analytes were different and the interferences were present only with MHPG, an attempt was made to separate the two analytes based on the manipulation of the ionization of their functional groups. This procedure of first extraction at pH of about 7 followed by a second extraction at pH of about 2–3 was not met with success because of partial extraction of the MHPG at both the pH ranges. Ion-pair extraction procedure was also evaluated taking advantage of the phenolic and carboxyl groups on MHPG and MHMA. These functional groups would be ionized under basic conditions and therefore, be available for ion-pair formation which would be extracted into an organic solvent. Tetramethylammonium hydroxide and tetrabutylammonium hydroxide were used as ion-pairing agents. There was no significant difference in the extraction ability of

the two ion-pairing agents used. Of the various extraction solvents evaluated: hexane, *n*-butyl chloride, methylene chloride, chloroform, ethyl acetate, diethyl ether and methyl-*tert*-butyl ether, only ethyl acetate was able to extract MHPG (70%) with no co-extraction of MHMA. None of the other solvents could extract either of the two analytes. Following the ion-pair extraction the plasma residue was acidified and re-extracted with ethyl acetate. This second step extracted 79% of MHMA but also 25% of the remaining MHPG. Construction of a calibration standard curve for MHPG in the range of 10–200 ng ml^{-1} gave a lot variation in the internal standard recovery during extraction and high scatter in the standard curve. This probably was due to high variability associated with ion-pairing extraction procedure.

A variety of mobile phases were also used in an effort to construct a mobile phase optimization chart and to separate the interference from the analytes of interest. Some of the mobile phases evaluated included varying concentrations and combinations of acetonitrile, methanol and tetrahydrofuran. However, when the mobile phase optimization failed to provide the desired results, a longer column length was provided which seemed to solve the problem of resolution from interferences at the required levels of sensitivity for MHPG and MHMA.

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