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High-Performance Liquid Chromatography with Amperometric Detection of Medroxyprogesterone Acetate in Human Plasma with 2,4-Dinitrophenylhydrazine as Derivatizing Agent and Solid-Phase Extraction for Sample Clean-up

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ABSTRACT: A specific and sensitive method is presented for the analysis of medroxyprogesterone acetate (MPA) in human plasma using reversed phase high-performance liquid chromatography (HPLC) with amperometric detection. The blood plasma spiked with trace amount of MPA was cleaned up to remove natural interfering matrices by solid-phase extraction (SPE). The MPA extract was then derivatized with 2,4-dinitrophenylhydrazine (DNPH) as an electroactive agent. The MPA-DNPH derivative was re-extracted using SPE prior to analysis by reversed phase HPLC. Quantitative analysis of the MPA-DNPH using prednisolone-DNPH as an internal standard were optimized on a Hypersil ODS column using acetonitrile: methanol:30 mM phosphate buffer, pH 3 (39:39:22; v:v:v) as mobile phase at a flow-rate of 1.0 ml min^{-1}. It was found that the method was selective and gave linear calibration curve for a concentration range of 1.0 – 10.0 ng ml^{-1} for 2 ml spiked plasma samples. The relative standard deviation (RSD) of inter-day precision for a period of three validation days was 11.5 $\pm$ 3.4 % for all concentrations used. The RSD of intra-day precision (n = 5) was 5.05 $\pm$ 2.7 % with accuracy (n = 5) of 102.3 $\pm$ 7.4 %. The average recovery was found to be 102.9 $\pm$ 4.4 %. The correlation coefficient of the calibration curve was 0.9985. The limits of detection and quantitation were found to be 0.2 and 1.0 ng ml^{-1}, respectively. Using DNPH as a derivatizing agent can enhance both selectivity and sensitivity of MPA in plasma and is suitable for routine analysis.

KEYWORDS: medroxyprogesterone acetate, 2,4-dinitrophenylhydrazine, human plasma, HPLC, amperometry, solid-phase extraction.

INTRODUCTION

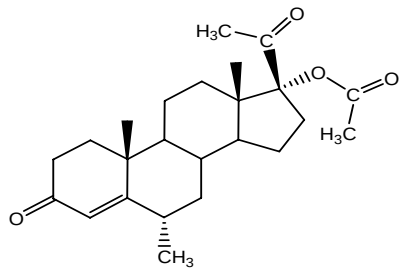
Depot-medroxyprogesterone acetate (Depot-MPA) is commercially available and commonly known as a hormonal contraception used by women. It is a synthetic steroid hormone, which mimics natural progesterone, but its contraceptive activity has been shown to be about 30 times higher. Depot-MPA is widely used by intramuscular administration for long-term contraception[1-3]. Research concerning its pharmacokinetics has been performed in order to improve the hormonal activity of the drug formulation[2,4]. This will help to standardize the dosage of the drug.

Analysis of MPA in plasma or serum has been performed to study its pharmacokinetics and to monitor its residual levels in cancer patients after oral or intramuscular administration[4,5]. Normally, the levels found in plasma range from 1.75 to 9.00 ng ml^{-1} [5]. Formerly, measurement of MPA in a plasma sample was mostly carried out by radioimmunoassay (RIA)[6]. Trace analysis of MPA in dog plasma was achieved using cyclohexane extraction, followed by heptafluorobutyric anhydride derivatization and gas chromatography with electron capture detector (GC-ECD). However, this procedure could not be applied for MPA analysis in human plasma due to its high matrix interference. Attempts have been made to optimize the methods for MPA analysis using small amounts of plasma samples[7,8]. Quantitative analysis of MPA in plasma by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) was also carried out, but did not give satisfactory detection limit[9,10]. The high sensitivity of the gas chromatography – mass spectrometry (GC-

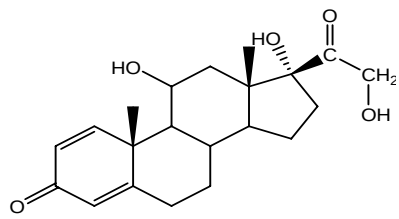
MS) has also been used to monitor the MPA level in human serum[11]. This procedure used trifluoroacetic anhydride derivatization of the extracted portion after SPE and gave the detection limit down to 0.5 ng ml^{-1}.

Although, HPLC-UV analysis of MPA in plasma has been performed, but its sensitivity was about 5 ng ml^{-1} [12]. In order to enhance both selectivity and sensitivity, recent method development and validation of MPA analysis has been focused on HPLC separation with various detection systems. HPLC with chemiluminescence detection was used to trace MPA in serum via 4-(N,N-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole as a fluorogenic agent[13]. Recently, MPA analysis in plasma sample has been conducted by liquid chromatography-electrospray ion trap mass spectrometry (LC-MS/MS) after liquid phase extraction and this gave 10 times higher sensitivity than GC-MS[11,14].

From these reviews, both GC-ECD and HPLC-UV did not give sensitivity high enough for MPA analysis in pharmacokinetic studies. In clinical aspects, the RIA method has a rather high sensitivity but sometimes gives positive result due to metabolite interference. Both GC-MS and LC-MS/MS are methods of choice with high selectivity and sensitivity, but may be considerably inconvenient for routine analysis due to the high instrument cost. The HPLC with amperometric detector (AD) would be a highly sensitive tool. From the molecular structure, MPA is a keto steroid in which the ketone group can undergo reduction. Since the reduction is normally easily interfered by oxygen molecule dissolved in mobile phase, this may be a problem[15]. Hydrazines including 2,4-dinitrophenylhydrazine (DNPH) have been introduced as an electroactive labelling reagent for carbonyl compounds[16]. Until now there is no report of the derivatization of MPA with DNPH (Fig. 1). From the chemical structures condensation reaction can occur between the keto group of MPA and DNPH to give the MPA-hydrazone which can be detected by HPLC-AD. The optimum conditions of DNPH derivatization with MPA were studied. Therefore, development and validation of the method for MPA analysis in spiked blood plasma using prednisolone (P) (Fig. 1) as internal standard (I.S.) by HPLC-AD after derivatization with DNPH and clean-up by solid-phase extraction have been carried out. The method sensitivity obtained is suitable for pharmacokinetic study of MPA.



medroxyprogesterone acetate



prednisolone

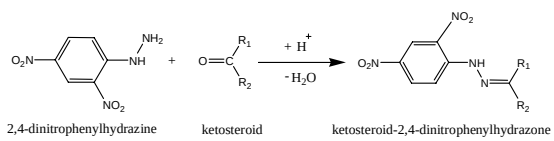


Fig 1. Chemical structure of medroxyprogesterone acetate and prednisolone, and their 2,4-dinitrophenylhydrazine derivatized products.

Materials and Methods

Reagents, Standard Solutions and Plasma Samples

Medroxyprogesterone acetate, 99.27%, used as working standard was obtained from A.N.B. Laboratory Co. Ltd. (Thailand). Prednisolone was an analytical reagent (A.R.) grade from Sigma (Germany). 2,4-Dinitrophenylhydrazine (Fluka, Switzerland) was also of A.R. grade. Methanol (MeOH, Merck, Germany), acetonitrile (ACN, Merck, Germany), di-potassium hydrogen phosphate-3-hydrate (BDH, England) and potassium dihydrogen phosphate (BDH, England) were of HPLC grade. Other chemicals used were also A.R. grade including hydrochloric acid (Merck, Germany), phosphoric acid (Merck, Germany), and sodium dihydrogen phosphate monohydrate (BDH, England).

Standard stock solution (500 mg ml^{-1}) of MPA was prepared in methanol. Dilute standard solutions of MPA were also prepared in methanol. The 2,4-dinitrophenyl hydrazine stock solution used as a labelling reagent (1,000 mg ml^{-1}) was prepared by dissolving the hydrazine in 1.2 ml concentrated HCl prior to adjusting the final volume to 25 ml with methanol.

Stock phosphate solution (2 M) was prepared from $NaH_2PO_4.H_2O$ in deionized water. A buffer solution of 30 mM KH_2PO_4, pH 3.0 was made by dissolving KH_2PO_4 in deionized water and adjusting to pH 3.0 with 6 M

H_3PO_4. Mobile phase was prepared and used to optimize the separation of the mixture of MPA-DNPH and P-DNPH derivatives by amperometric detection (HPLC-AD). The suitable solvent system was composed of ACN : MeOH : 30 mM KH_2PO_4 (pH 3.0) in the ratio of 39:39:22 by volume according to Snyder et al.[17]. The mobile phase was filtered through nylon membrane (0.45 μm, 47 μm) and degassed for 15 min in ultrasonic bath before use. Sep-pak C_{18} cartridge, 100 mg (Waters Associates, U.S.A.) was used.

Human blood plasma samples were kindly obtained from the Blood Bank, Srinagarin Hospital, Khon Kaen University, Khon Kaen, Thailand.

Apparatus

HPLC system (Perkin Elmer, U.S.A.) used in this study included LC200 HPLC pump with electrochemical detector (Coulochem II, ESA, U.S.A.). The standard analytical cell (ESA 5011, U.S.A.) consisted of porous graphite working electrode, palladium reference electrode and platinum counter electrode. The analytical column used was stainless steel tube packed with Hypersil ODS, 5 μm particle size (125 x 4.0 mm i.d., Agilent, U.S.A.). Integrator model 1022 (Perkin Elmer, U.S.A.) was used. UV-Visible spectrophotometer (Cecil 3000, England) was also used. Analytical balance (AE 200) and pH meter (Delta 350) were from Mettler, U.S.A. SPE manifold -12 place vacuum manifold (Lida, Germany) was used. Autopipette (Eppendorf, Germany) with volume adjustments was used throughout the experiment.

Method Development

Derivatization of MPA with DNPH

Study on the hydrazine derivatization of MPA was carried out using 2,4-dinitrophenylhydrazine (DNPH). The standard solution of MPA (10 mg ml^{-1}) was reacted with an excess amount of DNPH for an hour. The MPA-DNPH derivative was then injected into HPLC with detection at 375 nm[18]. The MPA-DNPH derivatives obtained from reacting 0.1 mg MPA with various concentrations (12.5, 25.0, 75.0 and 100.0 mg ml^{-1}) of DNPH solution in a 2.5 ml capped vial were used to study the effect of DNPH concentration and reaction time.

Prednisolone (P) was also tested for derivatization with DNPH and was used as I.S. There should be a single peak and no overlap with any interfering peaks in the sample. The P-DNPH derivative was obtained from the reaction of 0.10 g DNPH in 10 ml MeOH and 0.48 ml conc. HCl with 0.05 g prednisolone[19]. The reaction mixture was placed on a heated water bath at 60 °C for 30 min, allowed to cool down to room temperature and deionized water (60 ml) added until the derivative product had completely precipitated. The derivative product was filtered through a glass filter and washed with 5 ml 2 M HCl twice, followed by 50 ml deionized water. It was then recrystallized in a mixture of chloroform and methanol prior to use.

Solid-Phase Extraction of MPA-DNPH Derivative

The clean up method using solid-phase extraction (SPE) was found to be a crucial step for plasma sample both before and after derivatization with DNPH. Spiked standard plasma (MPA 6 ng ml^{-1}) was extracted by Sep-pak C_{18} cartridge using a mixture of MeOH : H_2O as extracting solvent. The C_{18} cartridge was first washed with 2 ml MeOH and followed by 2 ml water. The spiked plasma (2 ml) was added into the cartridge and then washed with 2 ml water, followed by 250 ml 50 %(v/v) MeOH (3x), and the final elution was made with 1 ml MeOH. The MPA extract portion was kept for derivatizing with DNPH. The solution of DNPH containing 0.1 mg was added into the MPA extract portion and allowed to stand for 30 min, followed by 0.9 ml water and 0.2 ml solution of P-DNPH (10 ng ml^{-1}). The sample of the derivative mixture was then introduced into the cartridge followed by 3 times rinsing with 1 ml 50 %(v/v) MeOH. The cartridge containing MPA- and P-DNPH products was washed by 250 ml 50 %(v/v) MeOH (3x) and again allowed to stand for 15 min before elution by 1 ml MeOH. The eluate was then dried over a stream of N_2, re-dissolved in 60 ml MeOH, then 40 ml MeOH added and 60 ml was injected into the HPLC system.

Analysis of MPA-DNPH by HPLC

The separation of both MPA- and P-DNPH derivatives was carried out on Hypersil ODS column by HPLC-AD (0.85 V) using an electrolyte solvent system as mobile phase with a flow-rate of 1 ml min^{-1}. The mobile phase of ACN : MeOH : 30 mM KH_2PO_4 (pH 3.0) (39:39:22; v:v:v) was used in this experiment after optimization of buffer concentration, pH and organic modifier. Both MPA standard solution and MPA spiked plasma sample were prepared at the concentrations of 0.5, 1, 2, 4, 6 and 8 ng ml^{-1}.

Method Validation

Various concentrations of MPA spiked in a plasma sample with 10 ng ml^{-1} P-DNPH as I.S. were extracted by Sep-pak C_{18} cartridge, and run by HPLC-AD (0.85 V)[20,21]. Limit of detection (LOD) is the concentration of MPA giving a peak height 3 times the baseline noise (3SD) and the limit of quantitation (LOQ) is defined as 10SD. The standard curve was obtained from the concentrations of 0.5, 1, 2, 4, 6, 8 and 10 ng ml^{-1}. The accuracy and precision including recovery were determined at three concentration levels (1, 4 and 8 ng ml^{-1}) of the spiked plasma standard.

Results and Discussion

Method Development

Derivatization of MPA and Prednisolone as an Internal Standard with DNPH

Studies on derivatization of some hydrazines with MPA had been studied in acidic solution[15]. It was found that DNPH is a suitable reagent. Many internal standards having similar core structure which is derivatized with DNPH were also investigated by the same manner[19]. Prednisolone was found to be the most suitable keto steroid and its derivatized solid product was prepared. It was eluted along with the MPA-DNPH and was used as I.S. throughout this experiment.

Effects of derivatization time and concentration of DNPH for MPA analysis were studied. The DNPH concentrations used were 12.5, 25, 75 and 100 mg ml^{-1}. MPA (100 ng ml^{-1}) was used to react with various concentrations of DNPH in the ratio of 1:1 at room temperature and their reaction times were also done up to 180 min (Fig. 2). It was found that 75 mg ml^{-1} of DNPH gave the highest peak area within 120 min derivatization time. In this study, the derivatization time of 30 min was enough, since the signal output was not significantly different from that of 120 min derivatization.. The effect of temperature for 30 min derivatization was also investigated using 100 ng ml^{-1} MPA and 75 mg ml^{-1} DNPH in acidic solution. There seemed to be no effect of temperature, except a linear increase of peak area when the reaction was performed at room temperature (Fig. 3). Thus, the reaction was rapidly completed at room temperature.

Optimization Conditions of MPA-DNPH Analysis by HPLC

The optimization conditions of HPLC-AD for MPA-DNPH analysis were then investigated. Effects of pH (3-6) and buffer concentration (10-40 mM) on mobile phase (ACN: phosphate buffer) were carried out. It was found that the optimum buffering system was at pH 3.0 and 30 mM phosphate buffer (data not shown). In addition, the ratios of organic solvents were investigated and a good separation was obtained with the mobile phase of ACN : MeOH : 30 mM phosphate buffer (pH 3.0) at the ratio of 39:39:22 by volume.

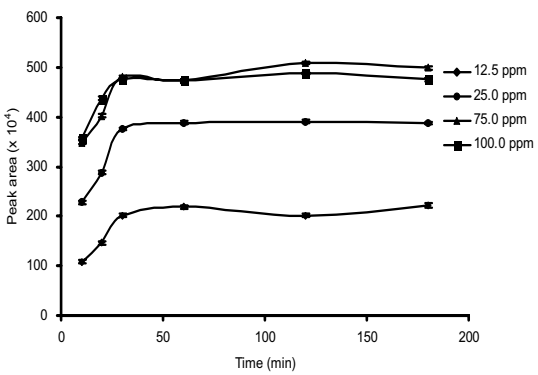


Fig 2. Effects of reaction times and concentrations on the derivatization of DNPH with 100 ng ml^{-1} MPA at room temperature.

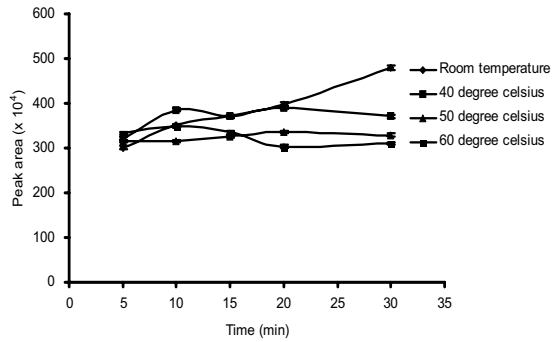


Fig 3. Effect of temperatures on the derivatization reaction of DNPH (75 mg ml^{-1}) and MPA (100 ng ml^{-1}) between 5 min and 30 min.

Fig. 4 shows the hydrodynamic voltammogram (HDV) of MPA- and P-DNPH derivatives. The optimum potential that gave the highest peak area was around 0.8-0.9 V for both derivatized products, and 0.85 V was used in this study.

Method Validation

The method limits for detection and quantitation were found to be 0.2 and 1.0 ng l^{-1} with lower 15% precision and 80-120% accuracy, respectively. In Table 1, the results for intra-day and inter-day precision were 5.05 and 11.5 %RSD, respectively. The intra-day accuracy ranged from 102.1-109.5 %. The average recovery was found to be 102.9 $\pm$ 4.4 %. It was found that this electrochemical detection was considerably selective and gave a working range of 1.0 – 10.0 ng ml^{-1} with linear regression: $y = (3.17 \pm 0.12)\,x - (0.67 \pm 0.03)$, $r^2 = 0.9985$ for $n = 5$ with %RSD = 2.34 – 7.96.

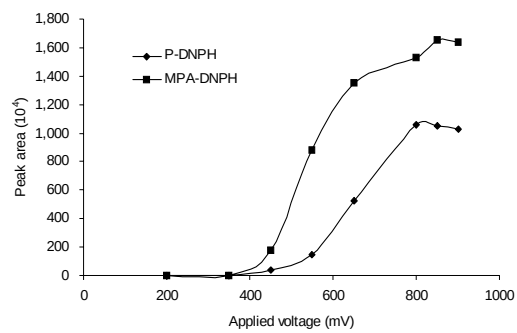


Fig 4. Effect of applied voltammetric potentials on the peak areas of MPA- and P-DNPH derivatives.

Table 1. Intra-day and inter-day precision and method recovery of MPA-DNPH derivative.

Concentration[a] (ng ml^{-1})	Intra-day RSD (%)	Inter-day[b] RSD (%)	Recovery[c] (%)
1.0	7.76	15.4	107.3
4.0	5.06	9.62	98.55
8.0	2.35	9.55	102.9
Mean ± SD	5.05 ± 2.7	11.5 ± 3.4	102.9 ± 4.4

[a] $n = 5$.
[b] within three consecutive days.
[c] Mean of three determinations.
RSD, relative standard deviation.

Method Sensitivity of MPA Analysis in Spiked Plasma Sample

It has been shown that the clean-up step using the SPE method for MPA spiked in human plasma sample using Sep-pak C_{18} cartridge, the clean-up step was necessary probably due to both the reagent used and the sample interference matrices (Fig. 5, a-d). The solution of MPA-DNPH derivative product with excess DNPH was not clean enough, so it must be added through the Sep-pak cartridge before being separated on the HPLC-AD system. Without the clean-up step, there might be trouble for the HPLC column used, which may affect the analytical sensitivity as well. This is the first report of the use of DNPH as an electroactive labeling reagent for MPA analysis. Development and validation of the HPLC-AD of the derivatized product were then carried out. The MPA-DNPH was completely separated from the I.S. within a suitable analysis time (Fig. 5, a-d). The plasma sample size used in this study was still rather high (2 ml) for clinical aspects when compared with other reports[11,13-14], giving LOD and LOQ of 0.2 and 1.0 ng ml^{-1}, respectively. Thus, the developed method with electrochemical detection has sufficiently high sensitivity when compared with other methods, except for the tandem MS, as shown in Table 2. It is, however, satisfactory for pharmacokinetic study of trace MPA in plasma sample, especially in the case of contraceptive administration with very low dose. Since it is rapid procedure and inexpensive, this technique is

Table 2. Some analytical methods and limits of detection of MPA analysis in plasma samples.

Instrument	Extraction method	Sample size (ml)	LOD (ng ml^{-1})	Reference
GC-ECD[a]	liquid phase	4	1-5	[7,8]
GC-MS[b]	solid phase	1	0.5	[11]
LC-MS/MS[c]	liquid phase	1	0.05	[14]
HPLC-UV[d]	solid phase	2	4	[9]
HPLC-PO-Cl[e]	liquid phase	0.1	9	[13]
HPLC-AD[f]	solid phase	2	0.2	This work

[a]: electron capture detection of MPA derivative.
[b]: mass spectrometric detection of MPA derivative.
[c]: tandem mass spectrometry.
[d]: ultraviolet (254 nm) detection of MPA.
[e]: peroxyoxalate chemiluminescence ($\lambda_{ex}/\lambda_{em}$: 480/570 nm) detection of MPA derivative.
[f]: voltammetric (0.85 V) detection of MPA-DNPH derivative.
LOD, limit of detection.

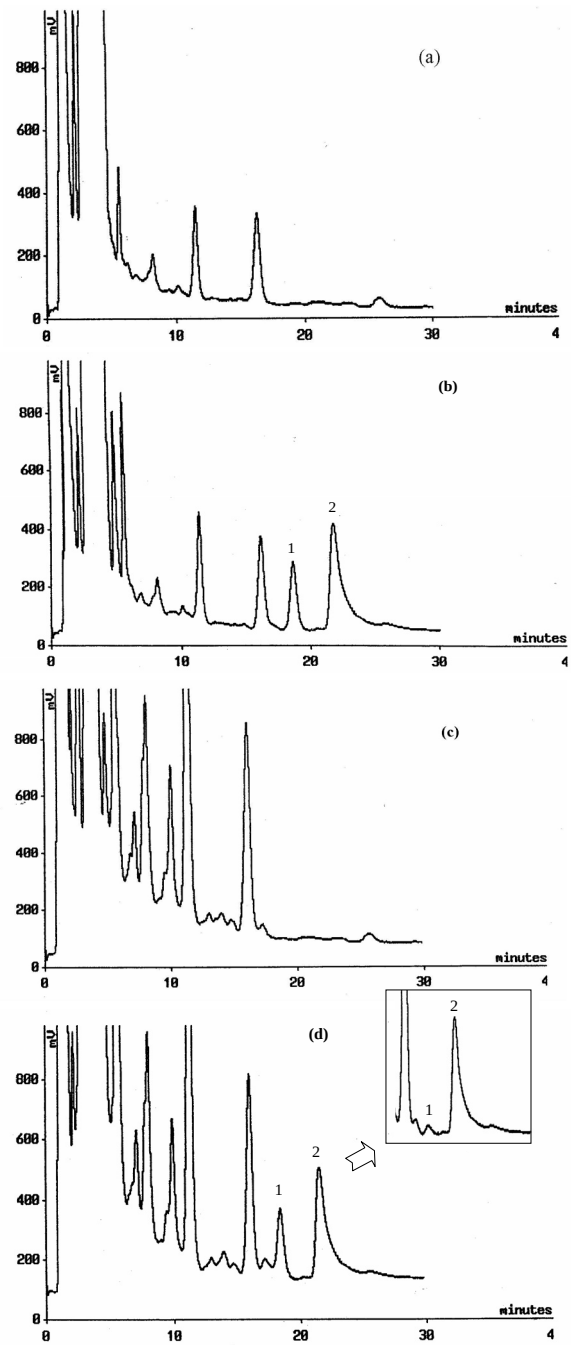


Fig 5. Chromatogram obtained from (a) blank reagent. (b) standard solution of (1) MPA-DNPH (10 ng ml^{-1}) and (2) P-DNPH as I.S. (c) blank of blood plasma sample. (d) blood plasma sample spiked with (1) MPA-DNPH (10 ng ml^{-1}) and (2) P-DNPH as I.S. (inset, (1) MPA-DNPH, 1 ng ml^{-1}).

suitable for routine analysis of the MPA content in most pharmaceutical products and blood samples.

Conclusion

A specific and sensitive method is presented for the trace analysis of MPA in blood plasma using HPLC-AD. For the method development, the plasma sample was spiked with known amounts of MPA standard solution and then extracted on Sep-pak C_{18} cartridge prior to analysis. After solid-phase extraction, the MPA extract was derivatization with DNPH, an electroactive labeling reagent. The MPA-DNPH derivative product was again re-extracted prior to analysis by HPLC-AD (0.85 V). The separation and quantitative analysis of the MPA-DNPH and P-DNPH (I.S.) were optimized on the reversed phase column. Using both SPE and HPLC-AD techniques can enhance both selectivity and sensitivity of trace analysis of MPA and therefore is applicable for routine assay work.

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