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Short communication

Liquid chromatographic method for simultaneous determination of mycophenolic acid and its phenol- and acylglucuronide metabolites in plasma

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Abstract

A simple, sensitive and reproducible HPLC method is presented for the simultaneous determination of mycophenolic acid (MPA) and its metabolites phenolic MPA-glucuronide (MPAG) and acyl glucuronide (AcMPAG) in human plasma. Sample purification requires protein precipitation with 0.1 M phosphoric acid/acetonitrile in the presence of Epilan D as an internal standard (IS). Separation was performed by reversed-phase HPLC, using a Zorbax SB-C18 column, 32% acetonitrile and a 40 mM phosphoric acid buffer at pH 3.0 as mobile phase; column temperature was 50 °C, flow rate 1.4 ml/min, and measurement by UV detection was at 215 nm (run time 12 min). The method requires only 50 μl plasma. Detection limits were 0.1 μg/ml for MPA and AcMPAG, and 2.0 μg/ml for MPAG, respectively. Mean absolute recovery of all three analytes was >95%. This analytical method for the determination of MPA and its metabolites is a reliable and convenient procedure that meets the criteria for application in routine clinical drug monitoring and pharmacokinetic studies.

Keyword: Mycophenolic acid

1. Introduction

Mycophenolate mofetil (MMF), the morpholinoethylester of mycophenolic acid (MPA), is an immunosuppressive agent with proven efficacy in the prevention of rejection in transplant recipients. MMF can be considered a pro-drug, given the fact that immunosuppressive activity is expressed only after hydrolysis to MPA [1].

MPA selectively and reversibly inhibits the enzyme inosine monophosphate dehydrogenase type 2 (IMPDH-2), which is a key enzyme in de novo purine biosynthesis of guanine nucleotides in T- and B-lymphocytes [2]. According to Holt [3], inhibition of this enzyme blocks the proliferation of these cells by impairment of nucleic acid synthesis and leads to a reduction in acute allograft rejection.

After oral or intravenous administration, MMF is rapidly converted to MPA such that no measurable concentrations of MMF in plasma have been found in transplant patients. MPA is then converted to inactive metabolites by glucuronidation mediated by the human uridine diphosphate glucuronosyltransferase (UDP-GT) enzyme family [4]. The glucuronide (MPAG) is eliminated by the kidney and the intestine. MPAG is pharmacologically inactive, but MPAG may be hydrolysed back to MPA during entrohepatic recirculation [5,6]. The enterohepatic recirculation process may change over time. Overall these phenomena may contribute to the inter-patient variability in plasma concentrations observed for MPA [7]. MMF and MPAG are absorbed quickly. High concentrations of MPAG are found in some kidney transplant patients with severe renal impairment [8]. In addition, MPA is further metabolised to 7-*O*-Glucoside (M1), acyl glucuronide (AcMPAG, M2), and finally via microsomal cytochrome P450 to the metabolite (M3) [9,10] (Fig. 1).

At present, the activity or toxicity in vivo of those metabolites cross-reacting in the immunological measurement (enzyme-multiplied immunoassay technique, EMIT) is unknown. AcMPAG has been observed regularly in the plasma of liver, kidney, and heart transplant recipients undergoing treatment with MMF[11]. The glucuronide MPAG has a half-life of 80 h and is eliminated at decreased rates as

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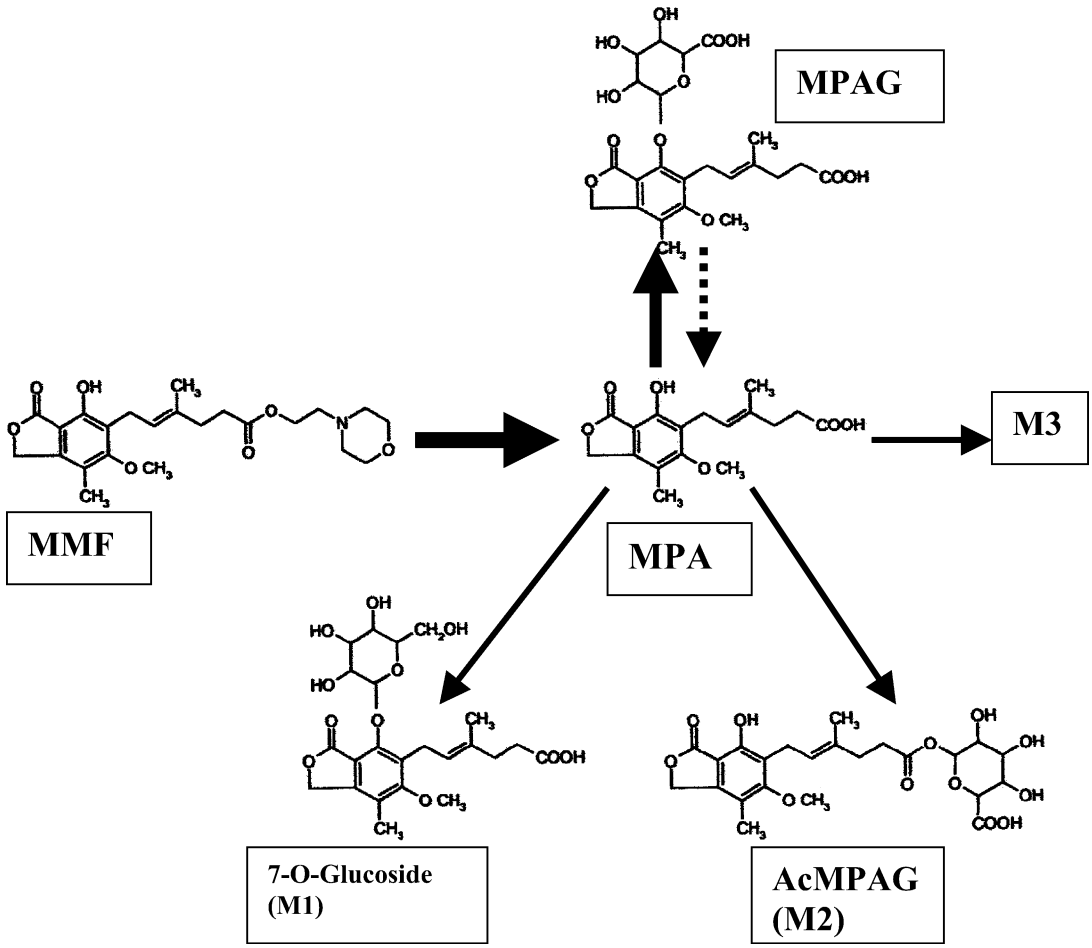

Fig. 1. Chemical structures of mycophenolate mofetil and its metabolites: mycophenolic acid, phenolic MPA-glucuronide, acyl glucuronide and M3.

kidney function decreases [12]. As kidney failure becomes more pronounced, the half-life of MPA increases [13]. In the presence of elevated creatinine values, MPAG and thus mycophenolate as well can accumulate [14], and AcMPAG plasma concentrations are dependent on renal function [15].

A number of HPLC methods have been used to determine MPA in plasma. Most of the HPLC methods described in the literature are used to monitor a single drug or required hydrolysis of sample with β-glucuronidase of MPAG to MPA [16]. Investigators have approached MPA or MPAG without AcMPAG analysis in many different ways; liquid–liquid [17,18], atmospheric pressure chemical ionization tandem mass spectrometry with HPLC [19] and solid-phase extractions [20,21], or complicated post-column derivatization [22] have been used to pre-treat serum or plasma samples, and different chromatographic approaches using normal-phase or reversed-phase columns and different wavelengths have been applied. AcMPAG measurement is the more interesting with respect to monitoring of MPA; not all HPLC assays are validated or calibrated to measure MPAG, and few centres have an assay for the measurement of AcMPAG [3].

We describe a very simple, fast and efficient optimized HPLC method for simultaneous one-cycle determination of MPA, MPAG, and AcMPAG in human serum or plasma. Our modified HPLC method is based on published method [23]. An additional advantage of this method is that it does not require uncommon or sophisticated HPLC instruments and is thus suitable for analysis of drugs in hospital laboratories with high numbers of samples.

2. Experimental

2.1. Chemicals

All chemicals were reagent or HPLC grade. Orthophosphoric acid (H_3PO_4) and acetonitrile were purchased from Merck (Stuttgart, Germany), mycophenolic acid was purchased from Sigma (Vienna, Austria), and MPAG from Syntex (Palo Alto, CA, USA). Acyl glucuronide is not available commercially; it was kindly supplied to us by Shipkova et al. [9]. Epilan D (internal standard, IS) was provided by Gerot Pharmazeutika (Vienna, Austria).

2.2. Chromatographic conditions

For liquid chromatography, we used a high-performance liquid chromatograph with an isocratic pump equipped with a wavelength detector; the detector signals were recorded with an HP-Chemstation and integrator, using an automatic sampling system. The separation was carried out on a 150 mm × 4.6 mm i.d. reversed-phase column (Zorbax SB C18) 3.5 μm. The mobile phase consisted of 40 mm phosphoric acid buffer pH 3.0 (adjusted with 1 M KOH), and acetonitrile (32:68 (v/v)), the column temperature was 50 °C, and the flow rate was 1.4 ml/min. The detector wavelength was set at 215 nm (all components by Merck–Hitachi, Stuttgart, Germany).

2.3. Patients' samples

Kidney, heart, and liver transplant patients received MMF and co-medications such as cyclosporine A, tacrolimus, and prednisolone for immune suppression. Samples of EDTA plasma were obtained from patients and centrifuged immediately at 4500 × *g* at 4 °C for 7 min. Thereafter, the samples were prepared for HPLC analysis.

2.4. Standard solution

Standard stock solutions were prepared in methanol and consisted of MPA (0.5 mg/ml), MPAG (0.68 mg/ml) and AcMPAG (0.1 mg/ml). These solutions were added to drug-free human plasma to prepare six non-zero concentrations in the range of 0.25–15 μg/ml MPA (0.25, 0.5, 2.0, 5.0, 10.0, 15.0 μg/ml), 3.41–218 μg/ml MPAG (3.41, 13.64, 27.28, 54.56, 109.12, 218.24 μg/ml), and 0.2–15 μg/ml ACMPAG (0.20, 0.5, 1.0, 5.0, 10.0, 15.0 μg/ml), respectively, and six quality control concentrations of MPA combined with its metabolites. The internal standard was obtained by dissolving 15 mg of Epilan D in the 1 l precipitation agent, i.e. 0.1 M phosphoric acid in acetonitrile, 1:1000 (v/v) (protein precipitation).

2.5. Sample preparation

Fifty microlitres EDTA plasma were mixed with 100 μl protein-precipitating reagent (with internal standard added) in an Eppendorf tube. The tubes were briefly agitated in a vortex machine and then centrifuged at 4700 × *g* for 5 min. One hundred microlitres of the clear supernatant were transferred to an Eppendorf tube and diluted with 100 μl twice-distilled water; an aliquot of 20 μl was autosampled for HPLC analysis.

2.6. Evaluation

The MPA, MPAG and AcMPAG values were determined automatically with the HP-Chemstation. Linear regression was calculated with the ratio of the peaks height for MPA, MPAG, AcMPG and the internal standard.

3. Results

3.1. Chromatograms and analysis

To investigate possible chromatographic interference by drugs administered to transplant recipients, more than 500 samples of patients who had undergone heart, kidney, and liver transplantation and received MMF and additional immunosuppressive drugs were analyzed. Fig. 2(A) shows representative chromatograms including blank plasma with internal standard; (B) plasma spiked with MPA (1.0 μg/ml), MPAG (7.5 μg/ml) and AcMPAG (1.5 μg/ml); and (C) a representative plasma sample of a kidney transplant patient who also received cyclosporin A and showed concentrations of MPAG of 30.60 μg/ml, AcMPAG of 2.45 μg/ml and MPA of 1.25 μg/ml. No peaks interfering with either the MPAG, AcMPAG, MPA peak or the internal standard peak were observed. The retention times were 2.0 min for MPAG, 3.5 min for AcMPAG, 11.1 min for MPA, and 4.1 min for the internal standard.

3.2. Linearity

Calibration curves in human plasma were linear over a concentration range of 0.25–20 μg/ml MPA, 3.41–218 MPAG μg/ml, and 0.2–15 μg/ml ACMPAG. The correlation coefficient was greater than 0.997 in all calibration curves ($n = 6$) (Table 1). Regression analysis gave the following equations: for MPA: $y = 0.0927x - 0.1543$, $r = 0.997$, for MPAG: $y = 1.276x - 0.1684$, $r = 0.998$, and for AcMPAG: $y = 0.0222x - 0.1009$, $r = 0.999$; in which y is the relative peak height and x is the concentration (μg/ml).

3.3. Accuracy

Accuracy was determined by evaluating analytical recovery of known amounts of standard solutions added to plasma (Tables 2 and 3).

To determine *recovery*, known concentrations of MPA,MPAG and AcMPAG were added to the plasma samples and the concentrations were determined in five replicates. The mean recovery for MPA across the range

Table 1
Parameters of analytical performance for quantification of MPA, MPAG, and AcMPAG in spiked and pooled human plasma samples ($n = 5$)

Sample analytes	Linearity (μg/ml)	Sensitivity (μg/ml)	Correlation coefficient (r)
MPA	0.25–15	0.1	0.99747
MPAG	3.41–218	2.0	0.99775
AcMPAG	0.20–15	0.1	0.99966

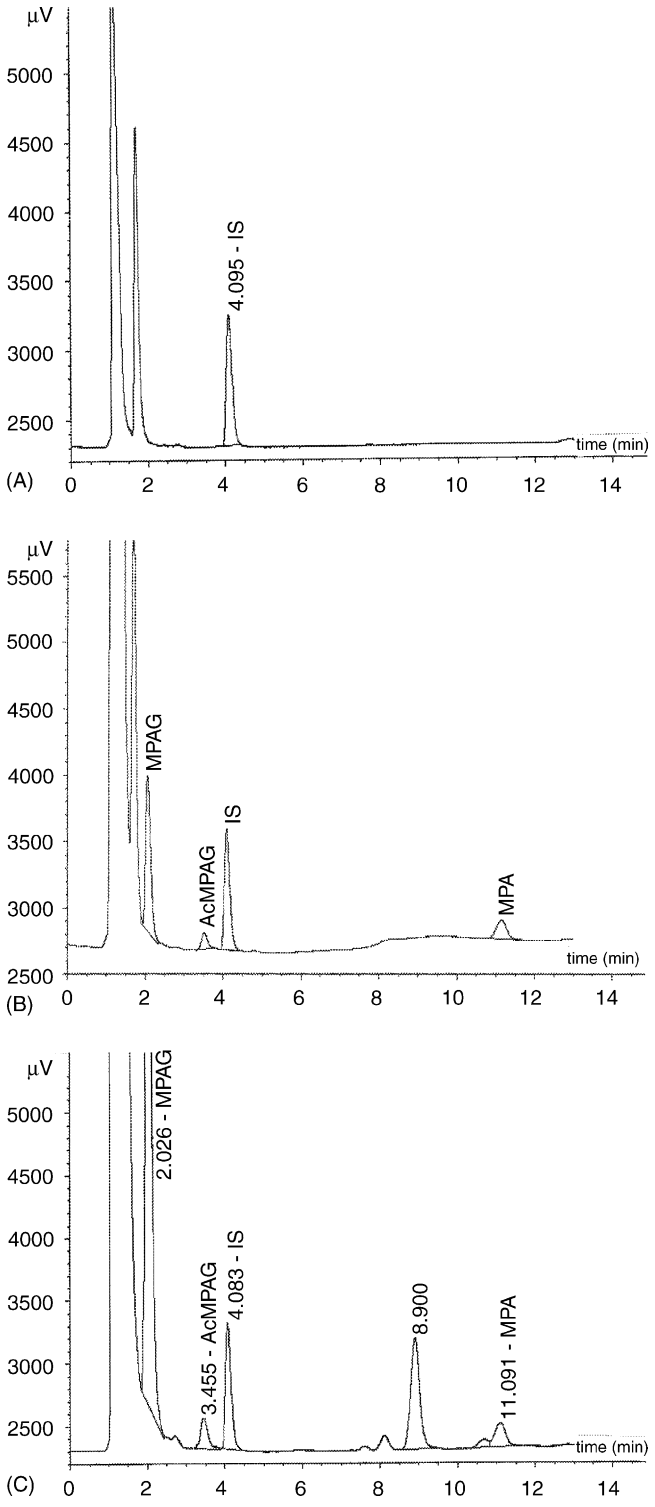

Fig. 2. (A) HPLC chromatograms of MPAG, AcMPAG and MPA in human plasma. Blank plasma with internal standard. (B) Plasma spiked with MPA (1.0 μg/ml), MPAG (7.50 μg/ml) and AcMPAG (1.5 μg/ml). (C) Plasma sample from a kidney transplant patient (cyclosporin A as co-medication): MPAG (30.60 μg/ml), AcMPAG (2.45 μg/ml) and MPA (1.25 μg/ml).

Table 2
Recovery of the HPLC method for determination of MPA, MPAG and AcMPAG from human plasma ($n = 10$)

	Theoretical value (μg/ml)	Measured value (mean + S.D.) (μg/ml)	Mean recovery (%)
MPA	0.75	0.75 ± 0.04	100.0
	1.50	1.44 ± 0.05	96.0
	10.00	9.47 ± 0.56	94.7
	18.50	18.44 ± 1.10	99.7
MPAG	4.50	4.63 ± 0.44	102.9
	16.40	16.64 ± 1.04	101.5
	80.40	79.29 ± 1.39	98.6
	112.60	110.81 ± 6.91	98.4
AcMPAG	0.30	0.29 ± 0.02	96.7
	0.90	0.82 ± 0.04	91.1
	1.25	1.22 ± 0.05	97.6
	2.80	2.78 ± 0.14	99.2

of the assay from 0.75 to 18.50 μg/ml was 97.6% (range 94.7–100%), for MPAG the range of the assay from 4.5 to 112.6 μg/ml was 100.4% (range 98.4–102.9%) and for AcMPAG the range of the assay from 0.30 to 2.80 μg/ml was 96.2% (range 91.1–99.2%), respectively (Table 2).

3.4. Precision

Intra- and inter-assay precision was assayed with control plasma spiked with five different concentrations of the analytes. The intra- and inter-assay precision was expressed as the coefficient of variation (CV (%)). The intra-assay (within-day) precision of MPA concentrations (0.75, 1, 5, 10, and 18.5 μg/ml) was 5.33–5.96%, and for inter-assay (between-day) 1.83–5.88%, respectively. For MPAG (4.5, 16.4, 80.4, and 112.6 μg/ml) these values were 6.23–9.50 intra-assay and 1.87–4.91.0% inter-assay. Values for

Table 3
Parameters of analytical performance of the HPLC method for MPA and its metabolites ($n = 10$)

Sample analytes	Analyte added (μg/ml)	Within-day (mean ± S.D.)		Between-day (mean ± S.D.)	
		Analyte found	CV (%)	Analyte found	CV (%)
MPA	0.75	0.75 ± 0.04	5.33	0.68 ± 0.04	5.88
	1.50	1.44 ± 0.05	3.45	1.57 ± 0.06	3.82
	10.0	9.47 ± 0.56	5.91	9.54 ± 0.18	1.89
	18.50	18.44 ± 1.10	5.96	19.10 ± 0.35	1.83
MPAG	4.5	4.63 ± 0.44	9.50	4.28 ± 0.08	1.87
	16.4	16.64 ± 1.04	6.25	15.82 ± 0.41	2.59
	80.4	79.29 ± 1.39	1.73	81.82 ± 1.48	1.81
	112.6	110.81 ± 6.91	6.23	114.11 ± 5.61	4.91
AcMPAG	0.30	0.29 ± 0.02	6.89	0.30 ± 0.03	10.0
	0.90	0.82 ± 0.04	4.88	0.96 ± 0.03	3.12
	1.25	1.22 ± 0.05	4.10	1.33 ± 0.03	2.26
	2.80	2.78 ± 0.14	5.03	2.76 ± 0.04	1.45

Table 4
Results of analyses of commercial therapeutic drug monitoring control samples compared with values provided by manufacturer for MPA (n = 10)

Sample analyte	Nominal value[a] (μg/ml)	Analyte found[b] (mean ± S.D.)	CV (%)
Control low	1.0	1.07 ± 0.05	4.31
Control medium	7.5	7.74 ± 0.31	4.01
Control high	12.0	12.11 ± 0.39	3.22

[a] Values provided by the manufacturers.
[b] Results obtained in our laboratory.

AcMPAG (0.30, 0.90, 1.25, and 2.80 μg/ml) were 5.03–6.89% intra-assay and 1.45–10.0% inter-assay. For the inter-assay CV the same concentration were analyzed over a period of 10 days, for the intra-assay CV 10 analyses were done on the same day. The results are summarized in Table 3.

To further check the reliability of our method, we used commercially available control samples of MPA (level I (1.0 ng/ml), level II (7.5 ng/ml) and level III (12 ng/ml) from "Dade Behring, Vienna, Austria") as external quality control. Table 4 shows the results of analyses of these commercial controls compared with values provided by the manufacturer for MPA (n = 10). The total coefficient of variation for MPA was 3.22–4.31%.

3.5. Limit of quantification and detection limit

The lower limit of quantification was 0.25 μg/ml for MPA, 3.41 μg/ml for MPAG and 0.2 μg/l for AcMPAG, as assessed by adding different concentrations of standard solutions to pooled plasma samples and 20 μl sample injection into the HPLC column. The detection limits, defined as the concentration of drug giving a signal-to-noise ratio >3:1, were 0.1, 2, 0 and 0.1 μg/ml for MPA, MPAG, and AcMPG, respectively.

3.6. Stability of samples

The stability of MMF metabolites in plasma samples was evaluated by comparing triplicate assays in freshly spiked samples, prepared daily at four different concentrations, and in samples spiked with the same concentrations and stored under different conditions. No significant loss was observed after storage at room temperature for 24 h or at 4 °C for 10 days.

4. Discussion

Immunosuppressive drugs are toxic and cause numerous side effects, which can be more pronounced when their metabolites accumulate. Similar to other immunosuppressive drugs, MMF therapy is associated with several adverse effects [3]. As MMF metabolites are excreted through the kidneys, accumulation can lead to concentrations of MPAG in plasma 100 times greater than those of the drug itself, i.e. MPA. Hence, dosage optimization in individual transplant patients depends on monitoring of plasma levels.

Measurement of MPA and its metabolites in transplant patients requires a reliable method featuring sensitivity, ease of sample preparation, and simple instrumentation and chromatographic conditions, without sacrificing precision and reproducibility. Most published methods for the quantification of MPA, the MPA-glucuronide (MPAG) metabolite and AcMPA in human plasma are time consuming and require the use of a variety of organic solvents for extraction, rendering them unwieldy for routine use with large numbers of samples.

The method presented here combines a simple and rapid procedure with easy sample preparation. A major advantage is that plasma samples are prepared by protein precipitation without extraction (5 min centrifugation). Results are available in a short time. The process is further facilitated by the use of an autosampler and an integrator (Hewlett-Packard HP-Chemstation).

The intra- (within-day) and inter-assay (between-day) coefficients of variation ranged from 1.73 to 9.50% and from 1.45 to 10.0%, respectively. The absolute (measured) and relative (analytical) recovery for MPA, MPAG, and AcMPAG varied from 96 to 100%, respectively. These values indicate that this method provides acceptable precision and accuracy.

Commercially available therapeutic drug control samples (levels I, II and III therapeutic drug monitoring (MPA) control samples) were used for external quality control assessment and confirmed the reliability of our method (Table 4). Neither external drug control samples nor patient samples interfered in the assay. There was no problem with the analysis of plasma samples from patient plasma (Fig. 2).

The method is easy on the HPLC equipment. The temperature is maintained at 50 °C, assuring optimum column performance, as well as better maintenance of the retention time. Exposure to impurities is kept low through protein precipitation, dilution with distilled water, and small sample volume needed for injection (20 μl), allowing approximately 700 analyses per column.

A further advantage is that this method uses an internal standard which can be purchased commercially without any restrictions. This method is useful for routine monitoring or pharmacokinetic studies of this drug in the given concentrations in serum or plasma.

5. Conclusions

An optimized method for determination of MPA, MPAG and AcMPAG in plasma is presented. Our results indicate that the determination of drug metabolites by reversed-phase isocratic high-performance liquid chromatography is a fast and accurate method suitable for simultaneous therapeutic monitoring in clinical routine. This simple and

uncomplicated, yet very sensitive method for monitoring MPA and its phenolic and acyl glucuronide metabolites can on the one hand prevent accumulation and crossing of toxic thresholds, and on the other, permits timely dosage adjustments as required for successful treatment. It is an efficient and cost-effective way of measuring MPA and its metabolites in a single assay.

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