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Short communication

Determination of Guan-Fu Base A, a new anti-arrhythmic, in human plasma by gas chromatography and electron–capture detection

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Abstract

A gas chromatographic–electron capture detection (GC–ECD) method has been developed for determining Guan-Fu Base A (GFA), an experimental anti-arrhythmic, in human plasma. The method was based on one-step liquid–liquid extraction with toluene and chemical derivatization with pentafluoropropionic anhydride followed by GC–ECD. The derivatives of GFA and metoprolol (Met, internal standard) were confirmed by gas chromatography–mass spectrometry (GC–MS) to be dipentafluoropropionyl-GFA and dipentafluoropropionyl-Met. The method was linear over the concentration ranges of 0.1–20.0 and 1.0–30.0 μg/ml with the detection limit of 0.05 μg/ml at $S/N=5$. The intra- and inter-assay precisions were less than 6 and 10%, and accuracy 99.70±3.30 and 97.60±5.99%, respectively. The absolute recoveries were 81.88, 77.35, 80.79 and 83.85% for GFA at concentrations of 0.5, 1.0, 5.0 and 14.0 μg/ml and 88.24% for Met at 3.0 μg/ml, respectively.
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1. Introduction

Guan-Fu Base A (GFA, Fig. 1) is a natural substance first isolated by Liu et al. [1,2] from the root of *Aconitum coreanum* (Lèvl.) Rapaics. It was found effective on both prevention and treatment of experimental arrhythmia of different animal models [3–5]. Further experiments on cardiac conductivity and myocardial contractility in anesthetized rabbits suggested that the inhibitory effects of GFA on heart automaticity and conductivity might be the pharmacological basis of its anti-arrhythmic effect. Tachyarrythmia and re-entry, therefore, could be held up effectively [6]. It is more appealing that the chemical structure of GFA is quite different from any of the anti-arrhythmics we are now using in the clinic.

GFA is a diterpaniod alkaloid with the skeleton of hetisin [7]. It seemed difficult to analyze GFA with high sensitivity either by high-performance liquid chromatography (HPLC) or gas chromatography

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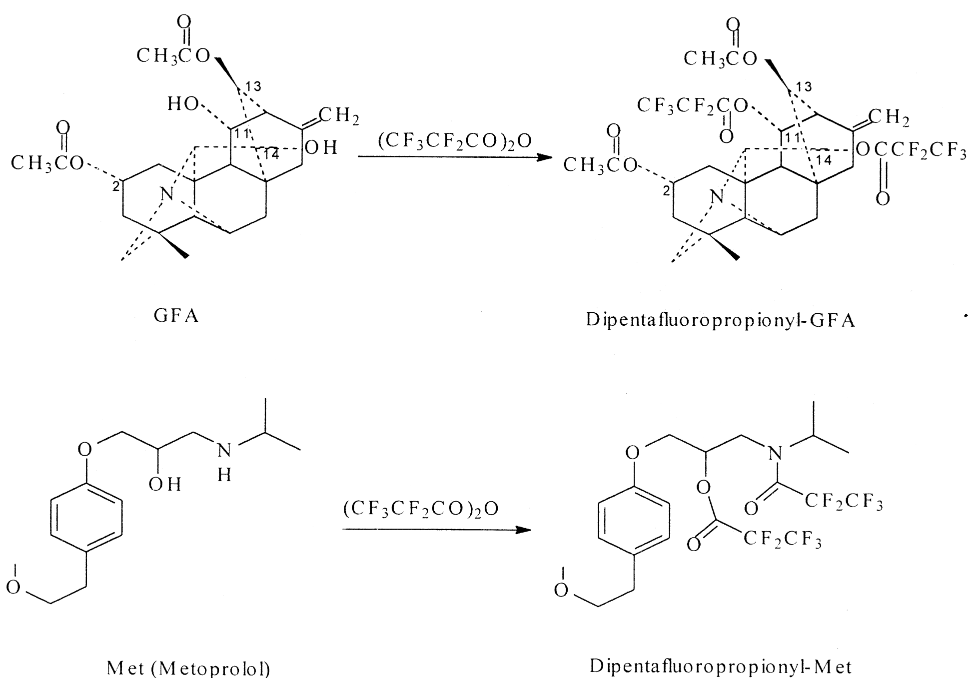

Fig. 1. Chemical structures of Guan-Fu Base A (GFA) and Met (I. S.), and their reaction schemes with PFPA.

(GC) without previous derivatization, because of its weak UV absorption, lack of fluorescence in the molecular structure and poor volatility. It was obviously, however, that a method with high sensitivity was needed before it was first applied to the clinical investigation, since no information about GFA levels in human plasma was available at that time. Derivatization of GFA with a halogenated acylating reagent such as trifluoroacetic anhydride (TFAA) or pentafluoropropionic anhydride (PFPA) followed by gas chromatographic–electron capture detection (GC–ECD) analysis was therefore preferred for obtaining significantly improved sensitivity and volatility as well as the rapidity of the reaction. We reported a GC–ECD method for the analysis of GFA in rabbit plasma used in preclinical pharmacokinetic investigation [8]. In that method, however, the TFAA derivative of GFA was so unstable in injection solvent that it was even impossible to duplicate the injection.

In this report we describe a method appropriate for the analysis of GFA in human plasma. PFPA, instead of TFAA, was used as the derivative reagent. The stability of the derivative was investigated and improved. The method was successfully used in Phase I clinical investigation of GFA conducted by Fuwai Heart Hospital in Beijing.

No other methods for analysis of GFA in human plasma have been reported yet.

2. Experimental

2.1. Chemicals and reagents

GFA and GFA·hydrochloride (GFA·HCl) were supplied by Professor J. Liu. Metoprolol tartrate (Met) was purchased from Sigma (St. Louis. MO, USA). Pentafluoropropionic anhydride (PFPA) was purchased from Tokyo Chemicals (Tokyo, Japan). All the solvent used were analytical grade. Blank human plasma, collected with sodium heparin, was purchased from Nanjing Blood Bank (Nanjing, P.R. China).

2.2. Gas chromatography and gas chromatography–mass spectrometry

Shimadzu GC-9A gas chromatograph (Kyoto, Japan) equipped with ^{63}Ni electron capture detector was used. Peak areas were recorded using Shimadzu CR3A chromatopac data module integrator at a chart speed of 2 mm/min. The stainless column, 2 m×3 mm I.D. (Shimadzu, Japan), was packed with Chromasorb W (DMCS, 80–100 mesh, Applied Science Laboratory Inc., USA) which was covered with 2% OV-1. The pulse electricity was 0.5 nÅ. The temperatures of injector, column and detector were 280, 230 and 280°C, respectively. Nitrogen (99.999%) was used as carrier gas at a flow-rate of 30 ml/min.

The derivatives of GFA and Met (I.S.) were identified using ZAB-HS mass spectrometer (VG Analytical, Manchester, UK). A HP-OV1701 fused-silica cross-linked capillary column 25 m×0.20 mm×0.2 μm (Hewlett-Packard, USA) was used with head pressure at 1 kg/cm^2. The temperatures for injector, column and ion source were 280, 230 and 240°C, respectively. Helium (99.999%) was used as the carrier gas at a flow-rate of 1 ml/min. The electron impact ionization energy was 70 eV and the scan was made from m/z 40–800 for the derivative of GFA and 40–600 for that of Met, respectively.

2.3. Sample preparation and derivatization

A 3.0-μl volume of the internal standard (Met, 1.0 μg/ml) and 1.0 ml of plasma were added to a 10-ml conical centrifuge tube followed by the addition of 3.0 ml of toluene and 0.2 ml of 0.5 mol/l NaOH. The sample was then vortexed for 1 min and centrifuged for 5 min at 1100×g. A sample containing 2.4 ml of the organic layer was transferred to a 3-ml reactor and dried with nitrogen at 50°C. Subsequently, 50 μl of acetyl acetate and 50 μl of pentafluoropropionic anhydride were added to the dried sample, successively. The reactor was then tightly sealed, briefly hand shaken to mix and heated at 50°C for 30 min.

After derivatization, the sample was evaporated with nitrogen (flow-rate: 0.9 l/min) at 50°C for 3 min. It was then reconstituted with 0.5–1.0 ml of toluene. An aliquot of 1.0 μl was injected into GC.

2.4. Method validation

The intra-assay precision and accuracy were determined by analyzing five samples spiked with GFA·HCl at concentrations of 0.5, 5.0 and 14.0 μg/ml. The inter-assay precision and accuracy were determined by analyzing the spiked samples on 4 or 5 consecutive days. The method was used to analyze the unknown spiked samples of different concentrations before it was applied to pharmacokinetic study of Phase I clinical trail.

2.5. GFA·HCl administration and blood sampling

The administration of GFA and blood sampling were performed at Fuwai Heart Hospital. Ten male healthy volunteers, aged between 22 and 24 years, were given a bolus dose of GFA·HCl, 4 mg/kg, intravenously. Blood samples were taken just before dosing and at 5, 10, 20, 30, 60, 150, 240, 360, 540 and 720 min after dosing. The plasma samples were stored at −20°C until analysis. They were numbered in a double blind way by a third person before sent for analysis. The real time-sequence of the samples, therefore, was unknown to the analyst until all of the analysis was finished.

3. Results and discussion

3.1. Gas chromatography and mass spectrometry

Fig. 2 shows typical chromatograms of GFA standard, plasma blank, the blank of a volunteer and the plasma sample of a volunteer at 10 min after intravenous administration of GFA·HCl, 4 mg/kg. Met (I.S.) was also added in volunteers' blank since the real time sequence was unknown to the analyst before the end of the analysis according to the double blind principle. The chromatogram of purchased blank plasma, however, could demonstrate that there were no substances interfering with GFA and Met (I.S.). The retention times of GFA and Met (I.S.) were about 14.4 and 4.0 min, respectively, with the overall chromatographic run time established at 20 min.

The chromatographic characteristic of some other drugs that might be prescribed for patients with heart

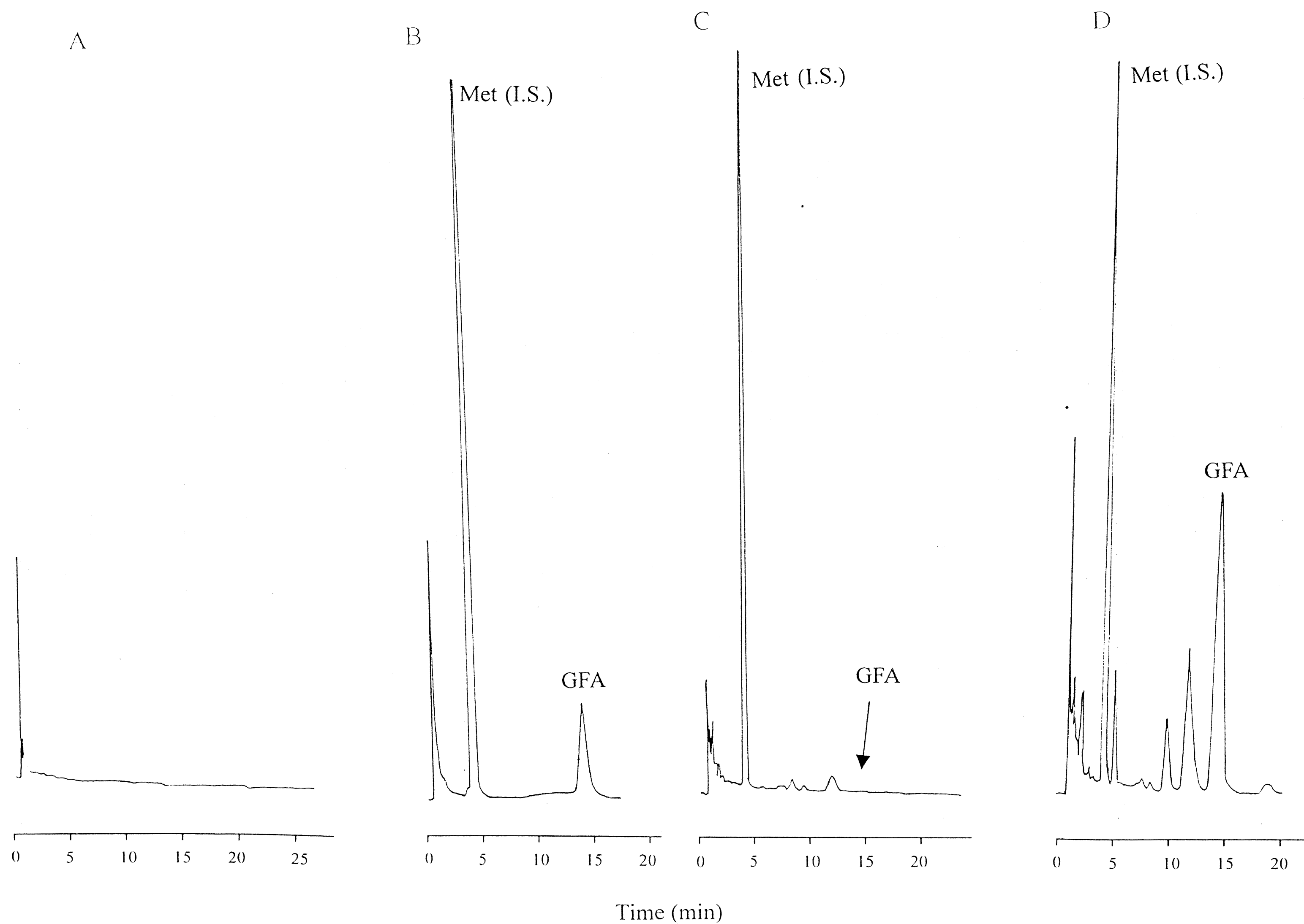

Fig. 2. Chromatograms of (A) blank plasma, (B) plasma spiked with GFA and Met (I.S.), (C) blank plasma sample obtained from a volunteer and spiked with Met, and (D) plasma sample obtained at 10 min after an i.v. bolus of GFA·HCl, 4 mg/kg. Stainless column (2 m×3 mm I.D) packed with 2% OV-1 covered Chromasorb W (DMCS, 80–100 mesh); carrier gas: nitrogen (99.999%) at 30 ml/min; temperatures of injector, column and detector: 280, 230 and 280°C, respectively; ECD pulse electricity: 0.5 nÅ.

disease were also investigated. Among those, verapamil, amiodaron, lidocaine, propafenone, mexiletine and digoxin would not interfere, while proprenolol (R_t: 5.29 min), atenolol (5.15 min), quinidine (13.37 min) and diazepan (15.67 min) potentially would. Therefore separation with capillary column (Fig. 3) is under experiment in order to suit further clinical investigation.

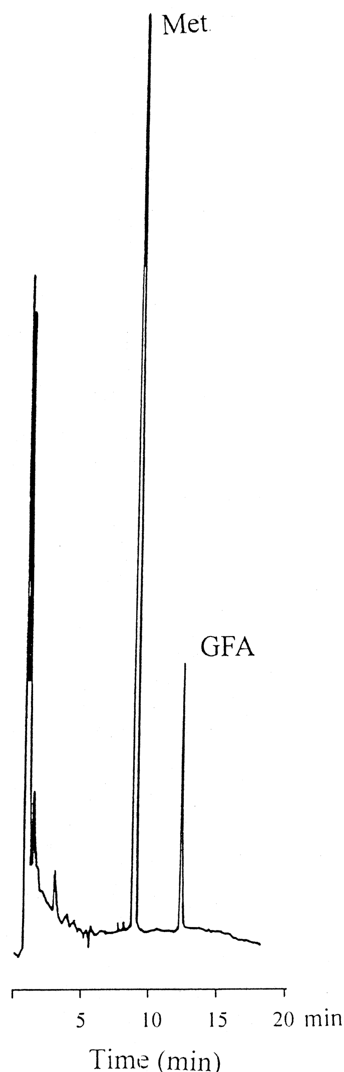

Fig. 3. Chromatogram of GFA and Met with a capillary column. The retention time of GFA and Met were about 8.9 and 12.7 min, respectively. Column: CP-Sil 5 CB 15 m×0.25 mm×0.25 μm (Chrompack, The Netherlands); head pressure of the carrier nitrogen (99.999%): 30 kPa at 1 ml/min; temperature: injector and detector: 280°C, column: 90°C (2 min), 20°C/min to 230°C (10 min). Injection: split at 1:30.

3.2. The structure elucidation of the derivatives

In the mass spectrum of GFA derivative could be found the molecular ion *m/z* 721 (relative abundance: 27.37%) and other main fragment ions: *m/z* 662$[M-CH_3OCO]^+$(70.45%), 574$[M-CF_3CF_2OC]^+$ (18.64%), 558$[M-CF_3CF_2OCO]^+$(5.82%), and the base peak *m/z* 43$[CH_3OC]^+$. These ions clearly indicated that 11,14-hydroxyl groups on GFA had reacted with PFPA to produce dipentafluoropropionyl-GFA (DPFP-GFA) (Fig. 1).

Similarly, the hydroxyl and amino groups on Met reacted with PFPA to produce dipentafluoropropionyl-Met (DPFP-Met) (Fig. 1). The molecular ion was *m/z* 559 (relative abundance: 4.02%) and other main fragment ions were *m/z* 408$[M-CH_3OCH_2CH_2C_6H_4O]^+$(65.94%), 45$[M-CH_3OCH_2]^+$(73.57%) and the base peak *m/z* 366$[408-CH_3CH_2CH_2]^+$ which was produced by McLafferty Rearrangement from *m/z* 408.

3.3. The stability of the derivatives

DPFP-Met was stable in injection solvent even at ambient temperature, while DPFP-GFA was still not stable enough. The instability of the GFA derivatives in reconstitute solvent might be related to the molecular structure. GFA had the skeleton of hetisin, of which the rigidity and the steric effect proved the pentafluoropropionyl group or the trifluoroacetyl group from rotating freely and made them easy to be replaced by the trace amount of the proton existed in the solvents. The presumption was confirmed when dried and freshly redistilled diethylether was used as the solvent. When in such a solvent DPFP-GFA showed good stability. It was, however, very difficult to keep the solvent dry. Therefore a study was conducted at −20°C using toluene as the solvent, because of its hydrophobic property and higher boiling point. The stability was then significantly improved. Fig. 4 shows that DPFP-GFA was stable at least for 28 h when kept in a sealed vial in toluene at −20°C.

3.4. Method validation

The linear range was calculated based on the peak area ratio of GFA to Met (I.S.) versus the nominal

Table 1
Intra-assay and inter-assay precision and accuracy for the determination of GFA in plasma

	Concentration added (μg/ml)	Concentration found (μg/ml)	n	RSD (%)	Accuracy (%)
Intra-assay	14.0	14.49	5	3.44	103.5
	5.0	4.88	5	5.96	97.6
	0.5	0.49	5	3.39	98.0
Inter-assay	14.0	14.01	5	5.95	100.1
	5.0	4.54	5	7.20	90.8
	0.5	0.51	4	9.97	102.0

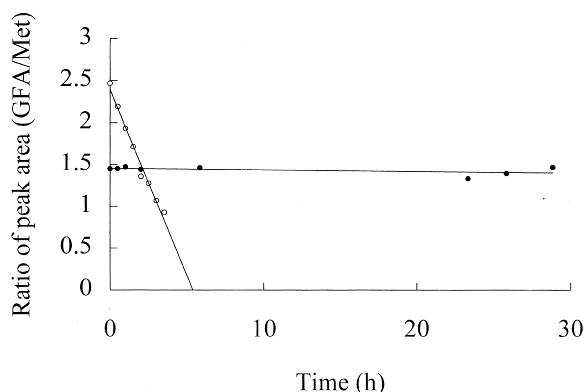

Fig. 4. Stability of DPFP-GFA and DPFP-Met in toluene at ambient temperature (○) and at −20°C (●).

concentration with the least-square regression. For the ranges of 0.1–20.0 and 1.0–30.0 μg/ml, obtained $Y=0.04068+0.2345C$ ($r=0.999$, $n=8$) and $Y=-0.1106+0.2121C$ ($r=0.999$, $n=8$), respectively (Y: ratio of GFA to Met, C: concentration of GFA·HCl). The limit of detection, as defined at $S/N=5$ for spiked plasma standard, was 0.05 μg/ml. The absolute recoveries were 81.88, 77.35, 80.79 and 83.85% for GFA at concentrations of 0.5, 1.0, 5.0 and 14.0 μg/ml and 88.24% for Met at 3.0 μg/ml, respectively. The intra- and inter-assay precision of this method shown in Table 1 were less than 6 and 10%, respectively. The over all intra- and inter-assay accuracies were 99.70±3.30 and 97.60±5.99%, respectively. The predicted concentrations for un-

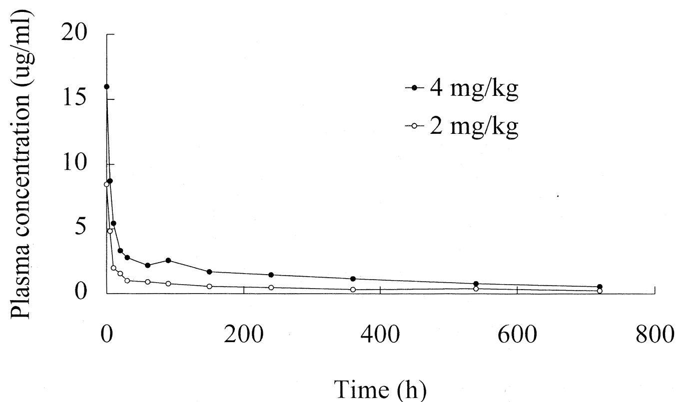

Fig. 5. Plasma concentration versus time profiles of a volunteer receiving an i.v. bolus of GFA·HCl, 4 mg/kg (●) and weeks later the half dose, 2 mg/kg (○).

known spiked samples were within 9% of the nominal values.

3.5. Application in clinical study

The method described was successfully applied to the analysis of plasma samples from 10 healthy volunteers receiving an i.v. bolus of GFA·HCl, 4 mg/kg and later from two of the volunteers receiving only half of the dose, i.e. 2 mg/kg. All of the pharmacokinetic curves could be fitted to a triexponential equation using 3P87 software package [9], assuming a three-compartment model for the distribution and elimination processes. Fig. 5 shows GFA·HCl concentration–time profiles of a volunteer receiving an i.v. bolus of GFA·HCl, 4 mg/kg and weeks later the half dose, 2 mg/kg.

This study is the first to determine GFA concentrations in human plasma for the investigation of its pharmacokinetic model and parameters. Consequently, comparison could not be made with other studies. In addition, the analytical method was also successfully applied in the study of electrophysiology and hemodynamics of GFA in clinical trial.

4. Conclusion

This report describes the development of a GC–ECD method for the determination of a new experimental anti-arrhythmic GFA in human plasma following derivatizing GFA and Met (I.S.) with PFPA in mild conditions. The structures of the derivatives, DPFP-GFA and DPFP-Met (I.S.), were identified by GC–MS. They could be kept stable in toluene for at least 28 h if stored at −20°C. The method was validated with satisfying precision, accuracy, linear range and detection limit and has been successfully used in Phase I clinical trial.

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