Study on a New Precolumn Derivatization Method in the Determination of Metformin Hydrochloride

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Abstract

Metformin hydrochloride is successfully determined by reversed-phase high-performance liquid chromatography with a new precolumn derivatization method using 9,10-anthraquinone-2-sulfonyl chloride as the derivatization agent. Several derivatization systems are tried to optimize the derivatization conditions, and a new post-derivatization treatment method is established. The derivatization product is analyzed on a Lichrosper C_{18} column (6.0 mm × 150 mm, 5 µm) at 256 nm with methanol–water (70:30, v/v) as the mobile phase. The calibration curves of the derivatives for the UV detector (0.01–4 mg/L) are linear with respect to peak area. The detection limit (peak area) for the metformin hydrochloride is 0.01 mg/L for a signal-to-noise ratio of 3:1. In human plasma, the detection limit is 0.02 mg/L. This assay is rapid, sensitive, and highly reproducible.

Introduction

Metformin hydrochloride (METF) or 1,1-dimethylbiguanide was used clinically as an oral hypoglycaemic agent for patients with Type 2 diabetes (1). Several methods have been published that determine the concentration of metformin in preparation (2,3) or in plasma (4–6), such as reverse-phase, ion-pair liquid chromatography (LC) (2,4,7), LC–mass spectrometry (MS)–MS (6,8), or cation exchanging with normal phase (5,8). However, these methods have required complicated instrumentations and some complex and time consuming procedures like solid-phase extraction (3,7). For example, take the normal-phase method into consideration: it required an expensive column, which had a relative short service life. However, LC–MS is not ideal for the instrumentation and is not always available in a common lab. When using ion-pair chromatography, the viscosity of the mobile phase increased greatly, therefore, the column would possibly block up, which is difficult to clean.

Besides these methods, a derivatization method was another choice by which METF could be analyzed on an ordinary octadecyl silica column without using sodium dodecanesulphonate. Despite the difference between each derivatization method, they could be basically divided into two species according to their derivatization mechanism. One method was to form *s*-triazine derivatives. The derivatization agents that appeared early were trifuoroacetic acid (9) and monochlorodifluoroacetic anhydride (10). Also, the procedures needed to keep waterless, so it involved being extracted twice with a large volume of solvent (9) or vacuum drying (10) to remove water, which made the procedure much more complex. Later, Ross (11) improved the *s*-triazine derivatation method with a kind of two-phase derivatization system, which consisted of acetonitrile and NaOH solution staurated with NaCl, and *p*-nitrobenzoly chloride was used as the derivatization agent. This development simplified the derivatization procedure a great deal, but at the same time 2 h and a lot of derivatization agent must be used. The other method was to form 2,3,5-substituted imidazole, for which the derivatization agents could be phenanthrenequinone, phenanthrenequinonesulphomate, or benzoin (12–14). The advantage of the imidazole derivatation method was that the derivatization agents reacted specifically with guanide to produce fluorescent product, and the derivatization agents itself had no fluorescence. Thus, the analytical selectivity was improved. There were two modes in the application of these agents, postcolumn and precolumn derivatization. The former had high sensitivity but required troublesome pretreatment, and the latter was simple at the cost of the sensitivity (12). What's more, no matter which mode was used, the derivatization reaction could only be rapid at high temperature; room temperature would prolong this reaction by several hours.

In recent years, there were few reports focused on improving the derivatization method of METF, which attracted our interest, and thus the purpose of this study was to find a better method for the derivatization of METF.

Anthraquinone-2-sulfonyl chloride (ASC) was a new derivatization agent synthesized by our group (15) and had been successfully applied in the determination of polar amine (15), which had some similar properties to METF. When using this agent, a two-phase derivatization system that was not popular in derivatization analysis was adopted. Because hydrochloric acid (one of the reaction products) could be neutralized by

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sodium hydroxide in the water, the reaction equilibrium could rapidly move to the end at room temperature. This derivatization agent was expected to exhibit its special character in METF derivatization.

In this work, we wanted to evaluate the possibility of derivatizing guanide with ASC prior to LC, with METF selected as the model compound in an attempt to develop a rapid, simple, and sensitive derivatization method for the analysis of METF by high-performance liquid chromatography (HPLC) with UV detection. Later, this method was applied for the determination METF in human plasma taken from healthy volunteers after oral administration METF.

Experimental

Reagents

The following solvents and reagents were used without specific purification: dichloromethane, cyclohexane, sodium chloride, sodium hydroxide (Nanjing Chemical Co., Nanjing, China) (analytical grade). Methanol (HPLC-grade, Hanbang Co., CHN, Huaiying, China) and water were filtered and degassed under vacuum prior to use.

ASC was made in our laboratory according to the reported method (15). METF was supplied by the department of Pharmaceutical Development (Hua Sheng Pharmaceutical, Chang Zhou, China).

Standard solutions

ASC solution was made by dissolving 15 mg of ASC in 10 mL of dichloromethane. Sodium hydroxide solution (12 mol/L) was prepared by dissolving 4.8 g sodium hydroxide in 10 mL of water. The stock solution of METF was prepared by dissolving 0.004 g METF in 100 mL of water. The stock solution was then diluted to yield the appropriate working solutions. All the solutions were stored at 4°C.

Linearity samples were prepared by diluting the METF stock solution with water to obtain METF solutions at the concentration of 4, 2, 1, 0.4, 0.2, 0.1, 0.04, 0.02, and 0.01 µg/mL before operating as mentioned in the Precolumn derivatization procedure section. Precision samples were prepared, five portions at each concentration (0.04, 0.4, and 4 µg/mL) of METF, following the same scheme.

Chromatography

Chromatographic separation and determination of the derivatives was achieved on a Shimadzu LC-10Atvp HPLC (Columbia, MD) equipped with a Shimadzu SPD-10Avp UV detector. The mobile phase consisted of methanol–water (70:30, v/v). Following filtration and degassing, the mobile phase was pumped at a flow rate of 1.0 mL/min through a 150- × 6-mm column containing 5-µm particles of octadecyl-bonded silica (Merck). The detection wavelength was set at 256 nm, and the column temperature was 20°C. Mass spectra were recorded on a Finnigan surveyor-TSD (Thermo-Finnigan, San Jose, CA) quantum tandem MS.

Derivatization

Preparation of the standard METF derivatives

In a 50-mL three-necked flask equipped with a thermometer and efficient stirrer, 0.34 g of METF (2 mmol) was dissolved in 7 mL of water containing 1.5 g of NaOH. Five milliliters of CH_2Cl_2 was added to the solution, and it was then cooled to 15°C by stirring. The ASC solution (0.74 g of ASC in 15 mL of CH_2Cl_2) was dropped into the flask within 30 min. The mixture was stirred for another 1 h and cooled with ice to keep the temperature under 10°C. The resulting precipitates were filtrated and washed with water until the washings were free of alkali. The rude product was recrystallized from acetone–water (8:2, v/v), giving a pale yellow piece.

Precolumn derivatization procedure

The derivatization reaction of METF was carried out in a 10-mL glass-stopped vial. First, 0.4 mL METF solution was mixed with 0.1 mL NaOH solution (12 mol/L) and approximately 0.2 g NaCl (to form a solution saturated with NaCl). Then, 1.0 mL ASC solution was (1.5 ng/mL) to the vial. The reaction mixture was shaken for 5 min at room temperature using a vortex mixter and centrifugated at 4000 rpm for 3 min, and 0.8 mL of the organic layer was transferred to another vial. The solvent was removed under the N_2 flow. The residue was dissolved by 1 mL of dichloromethane–cyclohexane (1:6, v/v), which was consequently extracted with 0.2 mL of methanol–1mM H_3PO_4 (3:2, v/v) for approximately 15 s. Twenty microliters of the lower layer was injected into chromatographic column.

Human plasma samples

A 0.4-mL plasma sample and 0.1-mL NaOH solution (12 mol/L) were transferred into a 10-mL glass-stopped vial. The following procedure was the same as in the precolumn derivatization procedure section.

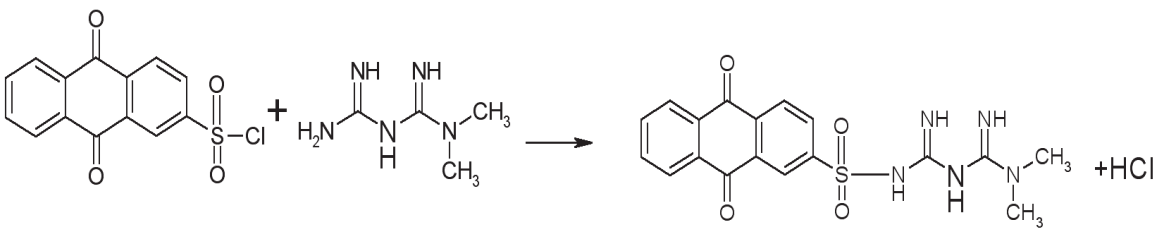

Figure 1. The reaction mechanism of ASC with METF.

Results

Identification of the standard derivatization product

Figure 1 shows the reaction mechanism between ASC and METF. The product was anthraquinone-2-sulfanilyl guanidine (ASG) and hydrochloric acid.

Figure 2 shows the mass spectra of the product ASG determined under electrospray ionization in positive mode. The accurate molecular weight of the ASG was 400.11, which was consistent with the $[M+H]^+$ and the molecular constitution of ASG calculated by the computer. The daughter ions of the 400.11 were 383.04 and 207.01 (Figure 3), and the relationship between them is demonstrated in Figure 4.

Derivatization method validation

The present derivatization method was assessed by several aspects, including linearity, precision, stability, and limit of detection.

Compared with the chromatograph of the blank sample derived from water (shown in Figure 5A), a clear chromatographic peak with a retention time at 5.5 min appeared in the sample chromatograph derived from the METF solution (shown in Figure 5B), which corresponded to ASG, and the shape of the peak was satisfactory. Data of the precision is presented in Table I, and the relative standard deviations (RSD) were all below 10%. The limit of detection could reach 0.01 µg/mL for a signal-to-noise ratio of 3:1 (shown in Figure 5C).

The method developed exhibited good linearity between the ASG response (As) and the corresponding concentration of METF (C) over the range of 0.01 to 4 µg/mL. The linear equation was:

$$As = 809790 \cdot C - 8759 \qquad \text{Eq. 1}$$

where $r = 0.999$. The result of the precision experiments are presented in Table I, and the RSDs are all below 10%, and the analytical recoveries were between 95% and 1114% (shown in Table II).

The final extraction solution was stable for 1 h when analyzed by HPLC, but it was not stable when stored longer than 3 h. Thus, when the whole precolumn derivatization procedure was finished, the sample should be analyzed as soon as possible.

Application to clinical pharmacokinetic study

To check the clinical applicability of the method, it was applied to a pharmacokinetic study in four healthy volunteers after a single oral dose of METF. The method was linear over the conceration range of 0.02–4.00 µg/mL. The limit of detection was determined to be 20 ng/mL. The linear equation was:

$$As = 337688 \cdot C - 3121.9 \qquad \text{Eq. 2}$$

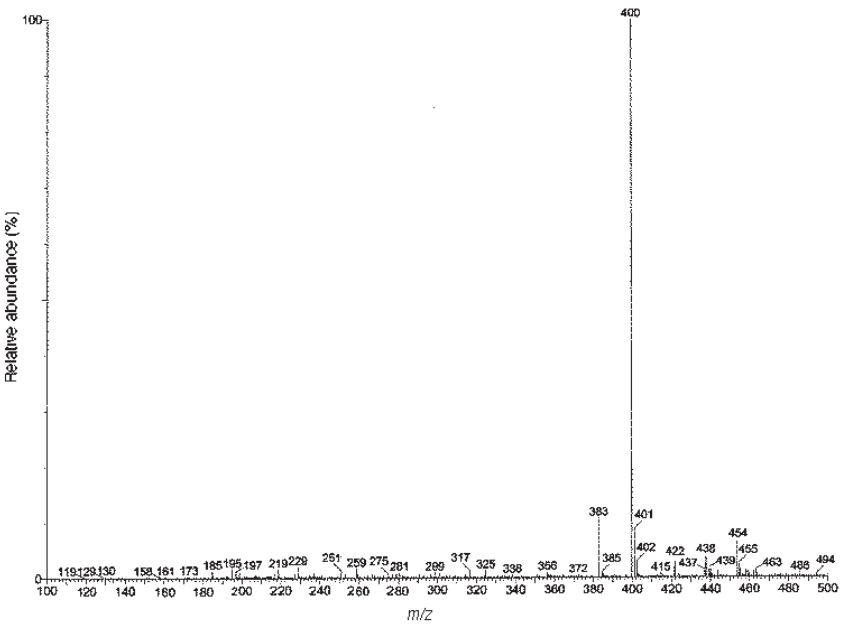

Figure 2. The mass spectra of derivatization product ASG.

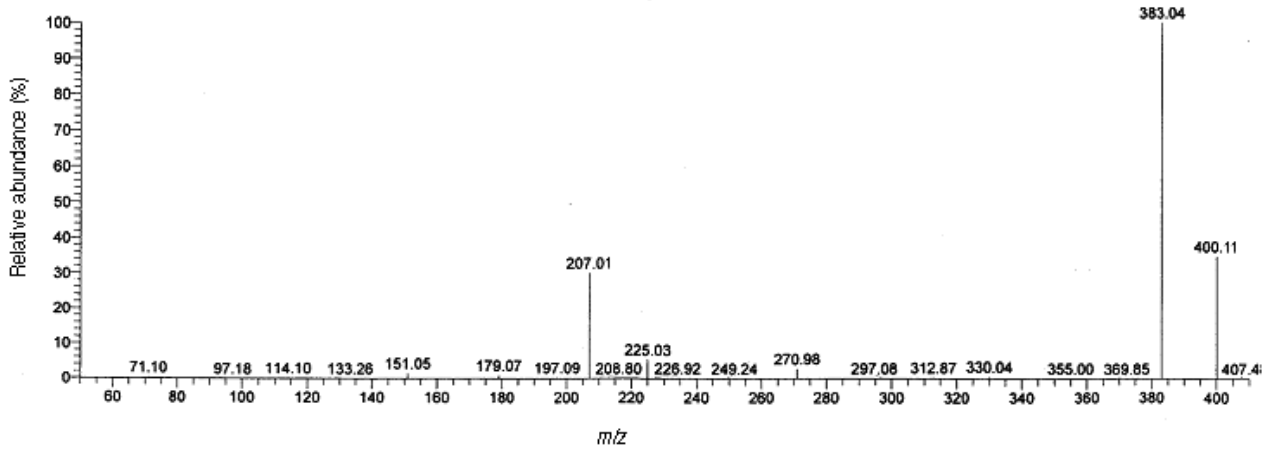

Figure 3. Mass spectra of the ASG daughter ion.

where r = 0.9998. The intra- and interday accuracies were all less than 9%; analytical recoveries were between 96% and 106%. The concentration-time profile is shown in Figure 7, and the pharmacokinetic parameters are shown in Table III.

Discussion

Optimization of the derivatization conditions

Selectivity of the derivatization system

Two derivatization systems were studied and compared: (*i*) CH_3CN–NaOH solution saturated with an NaCl system that had been used by Ross (10) and (*ii*) CH_2Cl_2–NaOH solution that had been used by Fang (15).

From the results, the latter system was found to exhibit some advantages over the former. First, the amount of ASC required in the latter system was much less than the former. ASC hydrolyzed fast in the former system, so it needed much more ASC to maintain a certain concentration in the CH_3CN during the derivatization process. Second, the former had two derivatives, though ASG was dominant, the other was still significant in the chromatogram. The third was the most important, as the derivatization efficiency of the latter was nearly 2.5 times that of the former. In addition, some research was done in one-phase derivatization; acetone was chosen to prepare an ASC solution because of its good solubility of ASC, which was mixed with the METF solution and basified with NaOH for derivatization. However, the result of the one-phase derivatization system was even worse than that of the former system (it needed more ASC and had much lower derivatization efficiency) and created more second derivative. It seemed that with the intersolubility between the organic solution and water decreasing, the ASC needed in the derivatization became less, the specificity of the derivative became better, and the derivatization efficiency increased.

In order to find out the most appropriate organic solvent that was fit for the derivatization, we had tried toluene, ethyl ether, ethyl acetate, and chloroform. The result showed that $CHCl_3$ and CH_2Cl_2 as solvents for the derivatization reaction provided the most intense peak for the ASG, ethyl acetate came out to be the second, ethyl ether was much less than ethyl acetate, and there was almost no derivatization product when toluene was used. This phenomenon could be explained by the Hughes-Ingold principle in organic synthesis: if a transient state product had more charge density than the reactant, then the reaction kinetics would increase with the solvent polarity (Figure 6). According to the magnitude of the polarity of solvents (30), we found the order was basically consistent with the reaction activity, which was toluene (33.9) < ethyl ether (34.6) < ethyl acetate (38.1) < chloroform (39.1).

Investigation of other factors in derivatization

Other factors including the reaction time, concentration, and volume of the ASC solution used, as well as the concentration of NaOH solution, were investigated.

Reaction time. In the reaction time range from 1 to 10 min, 5 and 10 min provided almost maximum and constant derivatization efficiency. Thus, 5 min for reaction time was adopted in the recommended procedure.

The concentration of the ASC solution. The influence of the concentration of ASC in the reaction yield was examined over the range 0.2–10 mg/mL. The analytical response increased with increasing ASC concentration up to 1.5 mg/mL. A further increment did not significantly improve the analytical signal. Thus, 1.5 mg/mL was the concentration selected for the experiment.

The volume of the ASC solution. The volume of the ASC solution was of greater importance, as either more or less would decrease the derivatization efficiency. The best result was obtained when the volume ratio between the water layer and organic layer was 1:2.

The concentration of NaOH solution. METF was a strong

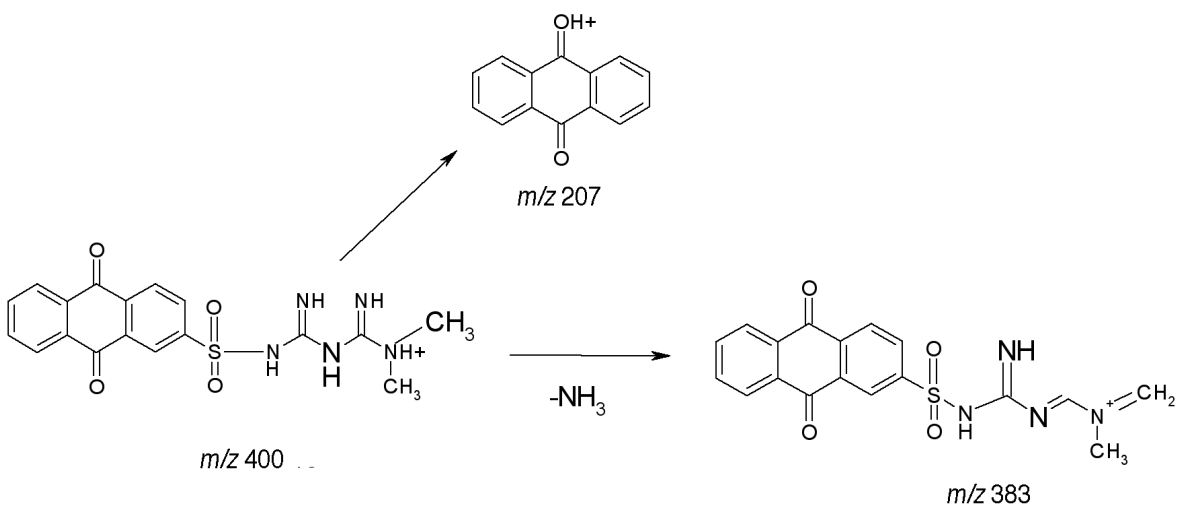

Figure 4. The relationship between the mother and daughter ion of ASG.

base (pK_a 12.9), which forced the use an NaOH solution at a very high concentration to maintain it in its dissociative form in order to keep its nucleophilic character. Finally, when the concentration of the OH^- in the METF solution was found to be bigger than 2 mol/L, the difference caused by the alkali could not be accounted for.

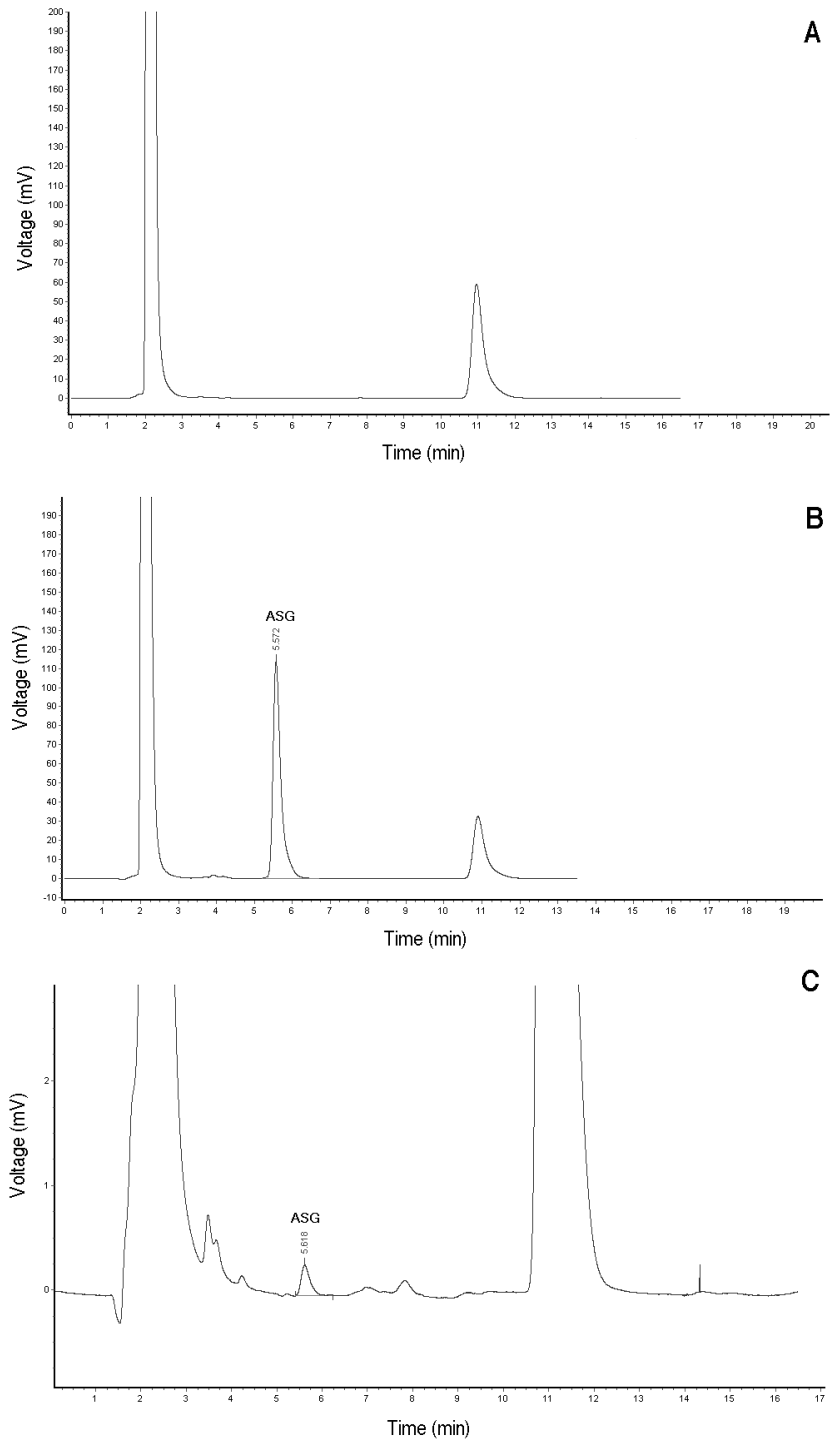

Figure 5. Derivaization chromatograms: blank derivatization with water (A); derivatization with 4 µg/mL METF solution (B); derivatization with 0.01 µg/mL METF solution (C) (the peak with retention time at about 2 min corresponds to 9,10-anthraquinone-2-sulphonic acid and the peak with retention time at about 11 min corresponds to the methyl ester product of ASC).

Influence of the salt

Because of the strong polarity, METF could not be extracted completely by the organic solvent, even when basified with a sodium hydroxide at a high concentration. However, reactions in one phase were always better than on the interface of two phases because of the more efficient touch. Theoretically, a better result would be obtained if more METF existed in the organic solvent. Considering the salting out effect, several salts were tried, including NaCl, KCl, and Na_2SO_4. The salt did increase the derivatization yield by approximately 10%, and the difference among the three kinds of salt was not significant, so the NaCl was selected.

Optimization of post-derivatization treatment

During the course of two-phase derivatization, the product ASG was present in the CH_2Cl_2 layer. Therefore, when the derivatization was finished, the remaining problem was how to treat the organic solvent to make it fit for the chromatographic system. First, the organic layer was removed, and the CH_2Cl_2 was eliminated under the N_2 flow. Then, the residue was dissolved with the mobile phase. However, there was still too much ASC unreacted in the residue that could not be conveniently dissolved.

The reason for the bulk remains of ASC was rooted in the inefficient hydrolyzation of the derivatization system. Thus, the first method attempted was aimed at hydrolyzing ASC after derivatization. However, ASG was not very stable in solutions containing water, so the common hydrolyzation with acid or base did not satisfy the method. Enlightened by the fast hydrolyzation character of ASC in the CH_3CN–NaOH solution saturated with NaCl system, some CH_3CN solutions were added to the derivatization system, and the reaction then continued. The ASG was stable during the process, and ASC was hydrolyzed, but this process required at least 20 min.

Because of the great polarity difference between ASG and ASC, a back-extraction method was attempted to help separate the two after the derivatization reaction. Among the good solvents of ASC, benzene (a solvent of low polarity) was attempted to dissolve the residue. It was then back-extracted with water or acid water. To our disappointment, the efficiency of back extraction was very low. Great improve-

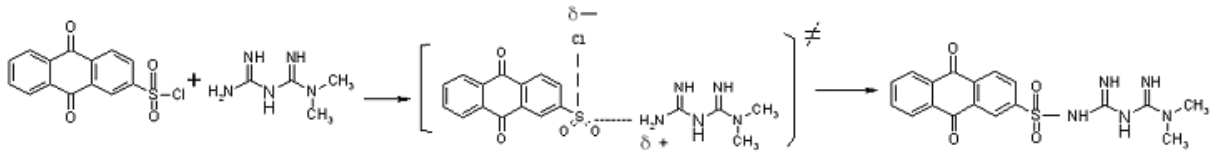

Figure 6. The course of the derivatization reaction.

ment was achieved when a mixture of methanol and water was used instead of water or acid water for back extraction. The idea came from another use of the extraction and dispenses principle advanced by Folch (16), which was often used in the extraction of the lipid. Another improvement was obtained when a less polar solvent, a mixture of CH_2Cl_2 and cyclohexane, was used to dissolve the residue. In the following experiments, the methanol content and the acidity in the back extraction solution as well as the proportion of CH_2Cl_2 and cyclohexane in the organic solvent were optimized. Finally, the extraction procedure was carried out as follows: the residue was dissolved to 1 mL of dichloromethane–cyclohexane (1:6) and the acquired solution was back-extracted with 0.2 mL of methanol–1mM H_3PO_4 (3:2). The back-extraction recovery of ASG could be 95%. In addition, the duration time for back extraction was also an important factor because when ASC met with methanol, the methyl ester product of ASC rapidly appeared. The longer the extraction process lasted, the more methyl ester product was generated. A back-extraction time over the range of 15 to 120 s was tested; the result showed extraction time in this range had little impact on the extraction efficiency of ASG. Therefore, 15 s was chosen as the time for back extraction.

Table I. The Precision of the METF Derivatization Method

Added concentration (µg/mL)	0.04	0.4	4.0
Intraday RSD	8.6	5.0	2.9
Interday RSD	9.4	5.6	3.7

Table II. The Analytical Recoveries of the METF Derivatization Method

Added concentration (µg/mL)	Recovery (%)					Mean (%)	SD (%)	RSD (%)
0.04	95.13	95.69	108.1	95.07	113.5	101.5	8.69	8.6
0.40	110.0	106.9	98.94	105.8	113.4	106.9	5.38	5.0
4.0	97.63	102.3	102.7	96.75	97.7	99.41	2.83	2.9

Table III. The Pharmacokinetic Parameters of the Healthy Volunteers

Subjects∞	C_{max}* (µg/mL)	T_{max}† (h)	$t_{1/2}$‡ (h)	MRT§ (h)	AUC** 0–24 h (mg/h/L)	AUC 0–∞ (mg/h/L)
A	1.57	1.5	6.57	6.88	7.69	8.15
B	1.89	2.0	4.72	4.93	9.03	9.21
C	1.33	1.5	4.39	5.22	7.11	7.24
D	1.17	2.0	3.76	5.82	8.29	8.40
Mean	1.49	1.75	4.86	5.71	8.03	8.25
± s	0.31	0.29	1.21	0.86	0.82	0.81

* C_{max} = highest drug concentration observed in plasma.
† T_{max} = time at which the highest drug concentration occurs.
‡ $t_{1/2}$ = half-life.
§ MRT = mean time a molecule resides in body.
** AUC = are under plasma drug concentration-time curve.

Comparison between derivatization methods

Compared with the derivatization methods previously reported, the proposed method was completed very rapidly at room temperature, which was the most important characteristic of the method. The sensitivity of the method was relatively high, even higher than post-column fluorescence derivatization (12).

With the post-derivatization treatment method developed, not only was almost all of the unreacted ASC removed, but also ASG was condensed. As far as we know, no back-extraction method like the one presented here has been reported. Thus, there is hope that the method may have great use in other experiments and become popular.

Application

The procedure using human plasma did not use NaCl because the plasma became gelatinous when NaCl was used. All of the pharmacokinetic parameters were generally in agreement with those reported previously (17). This method is suitable for clinical pharmacokinetic study (Figure 7).

Conclusion

A new derivatization method with 9,10- ASC, a new derivatization agent, is rapid, simple, and sensitive.

The research on applying the method to the plasma samples was successful. From the results obtained, there is hope that the method can be successfully applied to the determination of METF in other samples such as urine or tissues.

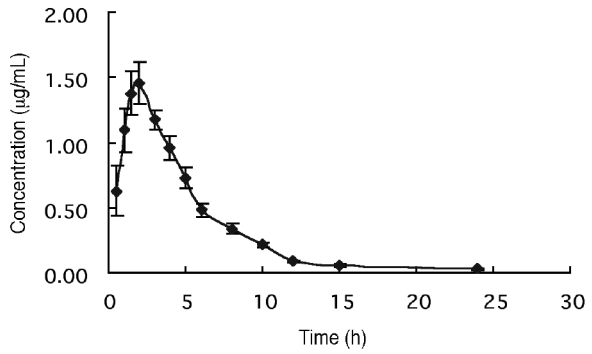

Figure 7. Mean plasma concentrations versus time profile of METF in four subjects.

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