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MICRO-DETERMINATION OF TOLMETIN IN PLASMA BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A highly sensitive, specific and precise gas chromatographic method for the determination of the anti-inflammatory agent tolmetin in micro-samples of blood plasma is described. The pentafluorobenzyl ester derivative of tolmetin, which can be detected down to picogram levels using electron-capture detection, has been prepared. The lowest concentration of tolmetin which can be measured accurately and precisely (coefficient of variation $< 15\%$) in a 25-μl plasma sample is 0.1 μg/ml. Previously reported assays require a sample size 20–80 times larger and have a detection limit of 0.5 μg/ml.

The pentafluorobenzyl ester derivatives of both tolmetin and the internal standard have been identified from their chemical ionization mass spectra. The present method was found to be in good agreement with another published gas chromatographic assay when both methods were employed to analyze the same set of plasma samples from four juvenile rheumatoid arthritic patients.

INTRODUCTION

Tolmetin [1-methyl-5-(4-methylbenzoyl)-1H-pyrrole-2-acetic acid] (I) is a non-steroidal anti-inflammatory agent for the treatment of rheumatoid arthritis. It is commercially available as the sodium salt (Tolectin® tolmetin sodium; McNeil Laboratories, Fort Washington, Pa., U.S.A.). The absorption and excretion of this drug in man have been described in previous publications[1–3]. The major metabolite of tolmetin in man[4] is 5-(4-carboxybenzoyl)-1-methyl-1H-pyrrole-2-acetic acid (II).

A gas–liquid chromatographic (GLC) method for the determination of I in plasma and both I and II in urine was reported[5]. A comparison between GLC and spectrometric analyses of I (ref. 6) and a high-pressure liquid chromatographic determination of I and II in plasma[7] were also published.

These published methods require at least 0.5 ml of plasma for each determination. However, when dealing with juvenile rheumatoid arthritic patients as well as with small mammals such as the mouse and the rat, smaller sample sizes (10–100 μl) are preferred, and yet submicrogram per ml levels of sensitivity have to be maintained. Therefore, a highly sensitive assay had to be developed.

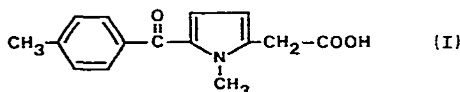
This paper describes the pentafluorobenzylation of tolmetin by extractive alkylation. Extractive alkylation affords a method of isolating polar compounds with simultaneous derivatization. The derivatization step is carried out by adding the organic phase containing the derivatizing agent to the aqueous phase containing the underivatized compound. The usual plasma extract concentration step of evaporating the organic phase to dryness before derivatization is omitted. Therefore the overall extraction and derivatization procedure becomes relatively simple. An average of 32 plasma samples can be analyzed routinely during an 8-h working day.

EXPERIMENTAL

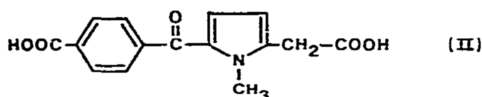
Reagents

Sulphuric acid, potassium carbonate and isoamyl alcohol were of analytical grade (Mallinckrodt, St. Louis, Mo., U.S.A.). Ethyl acetate was of nanograde quality (Mallinckrodt). Benzene was of pesticide grade (Fisher Scientific, Pittsburgh, Pa., U.S.A.). Heptane was of glass distilled grade (Pollard & Co., Wilmington, Del., U.S.A.). 2,3,4,5,6-Pentafluorobenzylbromide (PFBB) was from Pierce Chemical Co. (Rockford, Ill., U.S.A.).

A dichloro analogue of tolmetin (III) was used as the internal standard. Tolmetin (I) was obtained as the sodium salt dihydrate. II and III were obtained as free acids.



(I)



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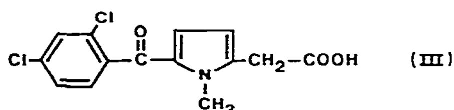

(III)

Plasma standard solutions

Plasma standards containing 0.1–45.0 μg of tolmetin per ml of plasma were prepared by adding 0.5 ml of tolmetin sodium salt solution containing the appropriate amount (2–90 μg equivalent) of the tolmetin free acid per ml of distilled water to drug-free plasma to a total volume of 10 ml.

Glass equipment

Fifteen-ml centrifuge tubes (volume: 15 ml; Kimble Glass) with PTFE-lined screw caps were used for both extraction and derivatization. Prior to use, all glassware was soaked in chromic acid for 1 h, rinsed thoroughly with distilled water and heat treated for 3 h at 270°. PTFE-lined screw caps were soaked in *n*-heptane for 1 h and dried at 60° prior to use.

Extraction and derivatization procedure

To 100-μl samples of plasma containing tolmetin as standards or unknowns in a 15-ml centrifuge tube were added 1 ml of 2 *N* H_2SO_4 and 5 ml of 1.5% isoamyl alcohol in *n*-heptane containing 0.5 μg of internal standard (III). The capped centrifuge tube was then shaken for 15 min on a table-top shaker (Eberbach Corp.) at 120 oscillations per minute and centrifuged at 681 *g* for 10 min. A 4-ml volume of the supernatant organic layer was transferred to another centrifuge tube containing 1 ml of 1 *M* K_2CO_3. The mixture was shaken for 15 min and centrifuged at 681 *g* for 10 min. The supernatant organic layer was aspirated and discarded. One ml of 0.5% PFBB in ethyl acetate was added to the remaining aqueous phase. The centrifuge tube was capped and heated in an oven at 70° overnight. After cooling to room temperature, 100 μl of the supernatant organic phase was pipetted into another centrifuge tube and evaporated to dryness under a stream of dry nitrogen at room temperature. An amount of 300 μl of benzene was added to the residue and mixed on a Vortex mixer for 5 sec. A 3-μl aliquot of the benzene solution was then injected directly into the gas chromatograph.

Mass spectrometry

For the positive identification of the pentafluorobenzyl (PFB) ester derivatives of tolmetin (PFB-I) and the internal standard (PFB-III), a Finnigan 3300 quadrupole mass spectrometer was employed in conjunction with a Finnigan Model 9500 gas chromatograph and a Finnigan Model 6000 data system. The gas chromatograph–mass spectrometer system was operated in the chemical ionization mode using methane as the reagent gas. A 91.4 × 0.2 cm I.D. silanized glass column packed with 1% OV-17 on Gas-Chrom Q (80–100 mesh) was used with a helium gas flow-rate of 20 ml/min. During analysis, the interface and the transfer-line were maintained at 250°. The column temperature was 235° and the injection port temperature was at 280°. The chemical ionization source was operated without external heating. The source pressure was maintained at 1 torr. The ion source settings were: ion energy 4 V; ion repeller 40 V; ion lens —40 V and electron energy 100 eV. The electron beam emission was adjusted to 0.5 mA. The voltage across the electron multiplier was —1,500 V.

Gas chromatography

A Perkin-Elmer Model 900 gas chromatograph equipped with a ^{63}Ni electron-capture detector (ECD) was used. The column was a 61.0 × 0.4 cm I.D. silanized glass column packed with 3% XE-60 on Gas-Chrom Q (80–100 mesh). The column was conditioned at 250° overnight with an argon–methane (95:5, v/v) flow-rate of 30 ml/min.

Chromatographic conditions were: column oven 230°, injection port 260°, detector 300°. The carrier gas (argon–methane, 95:5, v/v) flow-rate was 60 ml/min. The retention times for the PFB ester derivatives of tolmetin (PFB-I) and of the internal standard (PFB-III) were 3.8 min and 6.7 min, respectively (Fig. 1).

Quantitation

A standard curve for tolmetin in plasma was prepared by analyzing standard plasma solutions according to the procedure described above. Ratios of peak heights (drug/internal standard) were plotted against concentrations of tolmetin (Fig. 2).

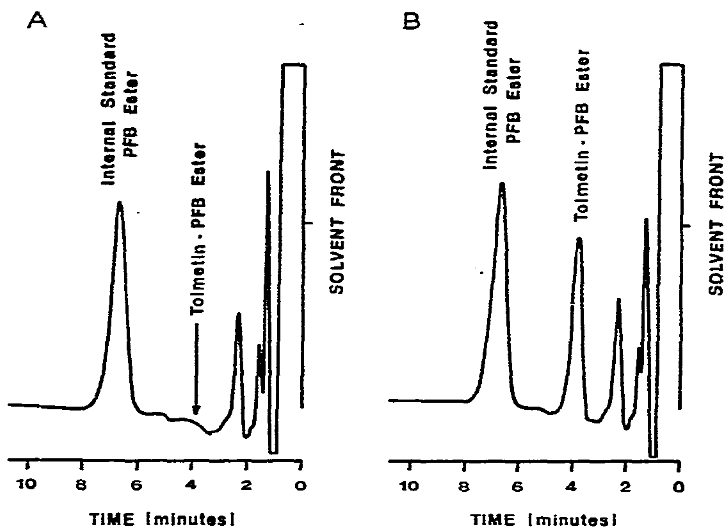

Fig. 1. Gas chromatograms from (A) 100 μl of blank plasma containing 500 ng of internal standard and (B) 100 μl of plasma containing 700 ng of tolmetin and 500 ng of internal standard.

Linear regression analysis was applied to the data. The standard curve was found to be linear in the concentration range of 0.1–45.0 μg of tolmetin per ml of plasma. Therefore peak height ratios were used in the analysis of unknown samples.

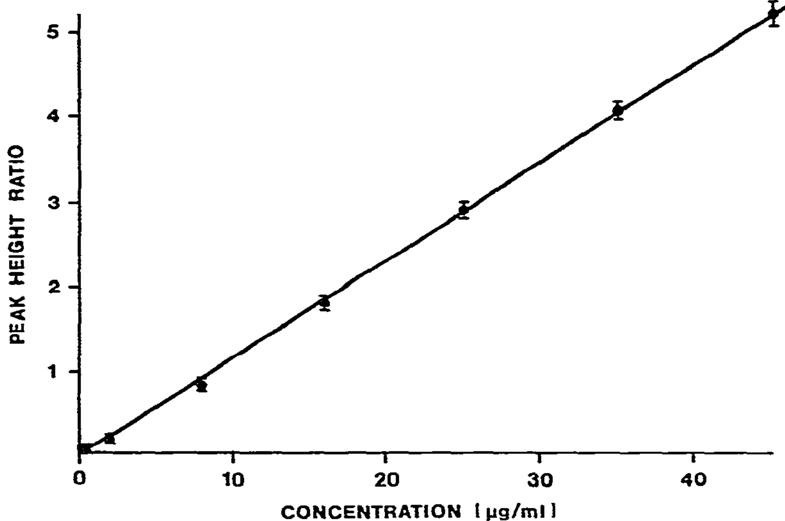

Fig. 2. Standard curve for tolmetin in plasma (500 ng of internal standard III per sample). Points and vertical bars represent the mean ± standard deviation of six separate determinations at each concentration. The straight line was obtained by least squares analysis for best fit (correlation coefficient = 0.999).

RESULTS AND DISCUSSION

Reaction conditions

Pentafluorobenzylation of organic acids and phenols has previously been investigated[8,9]. Derivatization occurs when the acid or phenol is heated with PFBB in strongly basic acetone or alcohol for some hours. More recently, extractive alkylation technique has successfully been employed[10,11] for derivatization. In extractive alkylation, organic acids in a basic aqueous medium are extracted by means of a positively-charged counter ion (*e.g.* tetrabutylammonium ion) into an organic phase containing the PFBB. In a poorly solvating organic phase, the anion of the organic acid becomes a highly reactive nucleophile in the displacement of bromide ion from the reagent. However, if the partition ratio between the organic and aqueous phases is very low, as in the case of short-chain fatty acids, the extraction of the organic acids into the organic phase will be inefficient and the reaction will be very slow and incomplete[12]. Since the partition coefficient of an organic acid is a function of the organic phase and of the pH of the aqueous phase, reaction conditions for an individual organic acid can be optimized by manipulation of these two parameters.

With tolmetin (I) and internal standard (III), pentafluorobenzylation in the presence of tetrabutylammonium ion was rapid and complete using ethyl acetate as the organic phase and 1 *M* K_2CO_3 as the aqueous phase. At pH values greater than 12, however, two different derivatives of tolmetin were obtained. It was also found that in the absence of the counter ion, the derivatization was completed for both I and III within 4 h at 70°. The time course of extractive alkylation of I and III under these reaction conditions is shown in Fig. 3.

Interestingly the major metabolite of tolmetin (II) was not derivatized in the absence of the counter ion due to the low partition coefficient. By leaving out the

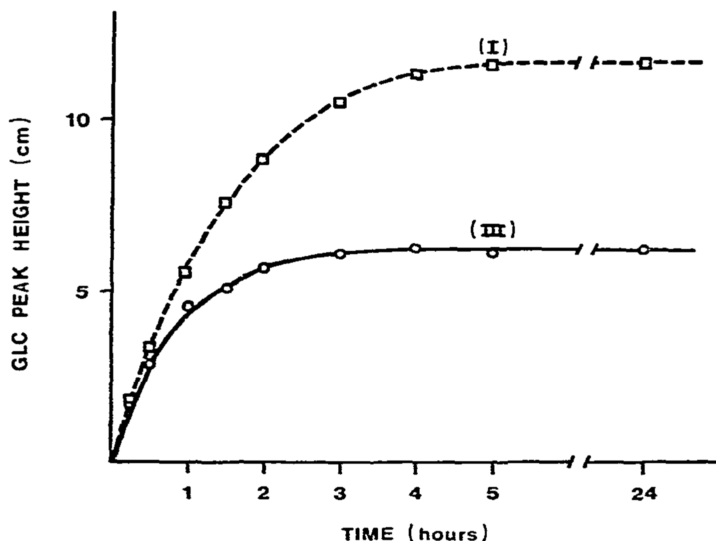

Fig. 3. Time course of the extractive pentafluorobenzylation of tolmetin (I) and internal standard (III). Organic phase, 0.5% PFBB in ethyl acetate. Aqueous phase, 1 *M* K_2CO_3 containing 0.7 μg of tolmetin (I) and 0.3 μg of internal standard (III). Temperature, 70°.

counter ion, the derivatization reaction became selective. This resulted in much cleaner chromatograms. Because of the relatively long (4 h) reaction time, the reaction mixture was kept overnight in the oven.

Reaction mixtures were subjected to gas chromatography–mass spectrometry analysis. Only one derivative was found for both tolmetin (I) and the internal standard (III). The chemical ionization spectrum for the PFB derivative of tolmetin (PFB-I) is shown in Fig. 4. Major *m/e* peaks observed were: 438 $(M+1)^+$, 466 $(M+29)^+$, 478 $(M+41)^+$, 119 $(CH_3\text{–}C_6H_4\text{–}CO)^+$, 181 $(M+1 - \text{tolmetin})^+$, 212 $(M - \text{PFB–COO})^+$ and 346 $(M - CH_3\text{–}C_6H_5)^+$. These ions are consistent with the addition of one PFB group to the carboxy group in tolmetin (MW = 437).

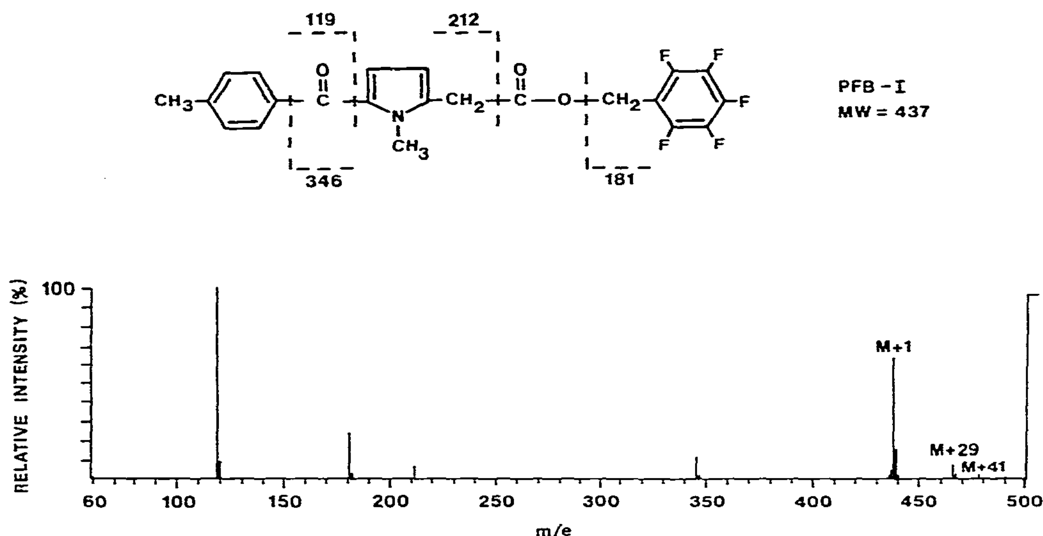

Fig. 4. Chemical ionization mass spectrum and proposed fragmentation pattern of the pentafluorobenzyl derivative of tolmetin (PFB-I).

Sensitivity

The PFB ester derivative of tolmetin (PFB-I) is highly electron-capture sensitive. An amount of 10 pg of this derivative when injected into the gas chromatograph under the stated conditions will give a peak with a signal-to-noise ratio of 10.

The lowest level of tolmetin that has been determined quantitatively in plasma using a sample size of 25–100 μl is 0.1 μg/ml which is adequate for all practical purposes. However, it is obvious that concentrations lower than 0.1 μg/ml can be determined since only 0.1 % of the derivatives in each sample was injected into the gas chromatograph in the present procedure. The percentage of sample utilized can be readily increased by using a smaller amount of benzene for reconstitution or by injecting a larger amount of the benzene solution.

Interference

No interference peaks due to endogenous materials have ever been observed using the present procedure. This procedure, to date, has been employed successfully

to analyze over 500 tolmetin plasma samples. The major circulating metabolite (II) will not be derivatized under the experimental conditions and thus is eliminated in the derivatization step.

Stability

Freshly prepared plasma standard solutions were compared with plasma standard solutions at the same drug concentrations kept frozen at $-10°$ for two months. The variations in the GLC peak height ratios at each drug level between 0.1 μg/ml and 45 μg/ml were insignificant.

Benzene solutions of derivatized plasma extract containing PFB-I and PFB-III were examined by repeated injections of aliquots into the gas chromatograph. It was found that both PFB ester derivatives were stable in the presence of plasma extract for at least four days at room temperature. The stability of PFB esters has previously been demonstrated[8,13].

Response curve

The linearity of the ECD response was demonstrated by injecting samples of different concentrations of the PFB ester derivatives of both I and II into the gas chromatograph. Peak height values were plotted against the absolute amounts of the derivatives injected to obtain response curves. The response curve for the tolmetin derivative was linear between 0.01 ng and 5.00 ng and the response curve for the internal standard derivative was linear between 0.05 ng and 5.00 ng. The detector response for both derivatives was found to be non-linear above the 5-ng level.

Recovery

GLC response curves which correlate peak height with the amount of PFB-I and PFB-III per sample offer the possibility of determining total yields after extraction and derivatization for tolmetin (I) and internal standard (III) in this procedure. For 0.2 μg of I and 0.5 μg of III seeded in 100 μl of plasma, the total recoveries after extraction and derivatization were 74% (coefficient of variation (C.V.) = 3.2%) for I and 75% (C.V. = 3.8%) for III (eight determinations).

Standard curve

A standard curve prepared by analyzing 100 μl of the plasma standard solutions is shown in Fig. 2. Excellent correlation was observed between the peak height ratios and the tolmetin plasma concentrations. Linear regression analysis gave a correlation coefficient of 0.999 with a Student's t of 154.

The reproducibility of the assay was very good as is shown in Table I. For six independent determinations at each concentration over the course of three weeks, the C.V. values were less than 10% in the concentration range of 0.1–45 μg/ml of tolmetin.

An identical standard curve was obtained by analyzing 25 μl of the plasma standard solutions. The reproducibility in this case was still very good. The C.V. values for six determinations at each level in the same concentration range were less than 15%.

For 100-μl plasma samples, the standard curve was non-linear above 45 μg/ml.

TABLE I

MEAN PEAK HEIGHT RATIO OF PFB-I TO PFB-III, STANDARD DEVIATION AND COEFFICIENT OF VARIATION OF WORKING STANDARD CURVES PREPARED BY ANALYZING SEEDED PLASMA SAMPLES OVER THE COURSE OF THREE WEEKS

0.5 μg of III per sample.

Tolmetin plasma concn. (μg/ml)	*Mean peak height ratio*	*Number of determinations*	*Standard deviation*	*Coefficient of variation (%)*
0.1	0.012	6	0.001	8.3
0.5	0.057	6	0.005	8.8
2.0	0.187	6	0.010	5.3
8.0	0.848	6	0.047	5.5
16.0	1.812	6	0.068	3.8
25.0	2.893	6	0.076	2.6
35.0	4.055	6	0.118	2.9
45.0	5.207	6	0.176	3.4

Therefore samples with concentrations above 45 μg/ml should be diluted with blank plasma before analysis.

Comparison of two GLC methods

The procedure described here for the determination of tolmetin levels in plasma was compared to a previously reported GLC method[6]. Plasma samples from four juvenile rheumatoid arthritic patients who had been undergoing tolmetin therapy for one to two years were analyzed by means of the two methods. The results were in good agreement (see Table II).

TABLE II

COMPARISON OF TWO GLC METHODS FOR THE DETERMINATION OF TOLMETIN CONCENTRATIONS IN PLASMA SAMPLES FROM JUVENILE RHEUMATOID ARTHRITIC PATIENTS

Method 1 was described by Cressman *et al.*[6]: double extraction of tolmetin and internal standard is followed by methylation with ethereal diazomethane solution and chromatography of the methyl derivatives using flame ionization detection. The method requires 1 ml of plasma and has a lower detection limit for tolmetin of 0.5 μg/ml. Method 2 is the procedure described in this paper.

Sample	*Concentration (μg/ml) determined by Method 1*	*Concentration (μg/ml) determined by Method 2*
1	0	0.46
2	2.3	1.73
3	7.8	8.01
4	0	0
5	0	0.10
6	0	0
7	3.8	3.94
8	5.0	3.93
9	19.4	19.20
10	0	0.20
11	42.0	43.20
12	0	0.49

ACKNOWLEDGEMENTS

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