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Letter to the Editor

Supplementary data for improved gas chromatographic method of determining diclofenac in plasma — Behavior of the methyl ester and the indolone derivative of diclofenac in gas—liquid chromatography with electron-capture detection

Sir,

Geiger et al. [1] reported a method for determining diclofenac, an anti-inflammatory and antirheumatic agent. Their method consists of derivatizing the compound into its indolone and analyzing the derivative by gas—liquid chromatography. We have presented a different method for determination of diclofenac [2], which utilizes derivatization of diclofenac into its methyl and ethyl esters, and have shown that the method has a three-fold higher sensitivity than the indolone method.

We have now made a precise comparison between these two methods in terms of the rate of derivatization, the efficiency of extraction of the derivative into organic solvents, and the stability of the derivatives during the analytical procedures.

METHODS

Synthesis of diclofenac derivatives

The methyl ester and the indolone derivatives of diclofenac were synthesized as follows.

For the methyl ester formation, diclofenac (200 mg) was mixed with 10 ml of methanol containing 0.5% sulfuric acid and the mixture was heated at 60°C for 1 h. For the indolone compound formation, diclofenac (200 mg) was added to 10 ml of trifluoroethanol (TFE) containing 0.5% sulfuric acid, and heated at 75°C for 75 min. After heating, each reaction mixture was added to 30 ml of water and the reaction products were extracted with *n*-hexane (3 × 50 ml for each compound). The *n*-hexane layers were dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure. The residues were crystallized from methanol—water (2:1, v/v), and recrystallized from methanol. The ester and the indolone derivative proved to be analytically pure (white prisms and pale yellow prisms, respectively). Analytical data are summarized in Table I.

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TABLE I

PROPERTIES OF THE METHYL ESTER AND THE INDOLONE DERIVATIVE OF DICLOFENAC

		Methyl ester	Indolone derivative
m.p.*		107—108°C	127—128°C
R_F value**		0.78	0.33
Elemental analysis			
	C found	57.93%	60.51%
	calc.	58.09%	60.46%
	H found	4.06%	3.19%
	calc.	4.22%	3.26%
	N found	4.34%	4.96%
	calc.	4.52%	5.04%
m/e (M^+)		310	278

*The melting point of the methyl ester is reported as 101—102°C [3, 4] and 103—104°C [5], but that of the indolone derivative is not given [6].
**Thin-layer chromatography on silica gel was conducted using the solvent *n*-hexane—chloroform (4:1) and each spot was visualized by UV absorption.

Derivatization of diclofenac and gas—liquid chromatography of derivatives

Derivatization of diclofenac was conducted in the manner described in the previous paper [2] for the methylation, and according to the method of Geiger et al. [1] for the indolone formation. Briefly, a methanol solution (10 μl) of diclofenac (1 μg) was placed in an ampoule, and methanol was evaporated at about 40°C under a gentle stream of nitrogen. The dried residue was dissolved in 0.15 ml of TFE containing 0.5% sulfuric acid (method A), 0.15 ml of TFE containing 0.5% sulfuric acid and 0.05 ml of methanol (method B), or 0.15 ml of methanol containing 0.5% sulfuric acid (method C). After the ampoule was sealed, the mixture was reacted in a water bath at 75°C for 75 min for method A, or at 60°C for 1 h for methods B and C. After reaction, the ampoule was opened, and 0.4 ml of 25% potassium hydrogen carbonate solution and 2 ml of *n*-hexane were added. The mixture was shaken on a Vortex mixer, and a 1-μl aliquot was injected into the gas chromatograph, which was operated as previously described [2] except that the column oven temperature was maintained at 250°C instead of 260°C.

Rate of conversion to the derivatives and the extraction efficiency

The rate of conversion to the derivatives and the extraction efficiency by the above three methods were examined. Three different systems of experiments were conducted simultaneously. First, diclofenac (1 μg or 3.14 nmol) was derivatized by the above three methods and analyzed as described above. Second, a methanol solution (10 μl) of the authentic indolone derivative (1 μg or 3.60 nmol) was added to a mixture of TFE—0.5% sulfuric acid (0.15 ml) and 25% aqueous potassium hydrogen carbonate (0.4 ml), and a methanol solution (10 μl) of the authentic methyl ester (1 μg or 3.23 nmol) was added to a mixture of TFE—0.5% sulfuric acid (0.15 ml), methanol (0.05 ml) and 25%

aqueous potassium hydrogen carbonate (0.4 ml), or a mixture of methanol–0.5% sulfuric acid (0.15 ml) and 25% aqueous potassium hydrogen carbonate (0.4 ml). The derivatives in the mixtures were extracted with 2 ml of *n*-hexane, and 1-μl aliquots of the *n*-hexane layer were subjected to gas–liquid chromatography. Third, *n*-hexane solutions containing 500 ppb of the authentic indolone derivative and the authentic methyl ester were prepared, and 1-μl aliquots were injected into the gas chromatograph.

The rate of conversion to the derivatives can be calculated by dividing the respective values obtained from the first experiment by the values from the second, which were multiplied by 3.14/3.60 for the indolone and 3.14/3.23 for the methyl ester.

The extraction efficiency can be calculated by dividing the values from the second experiment by the values from the third.

Stability of the derivatives in an alkaline medium

A methanol solution (10 μl) of the authentic methyl ester (1 μg) was added to a mixture of methanol–0.5% sulfuric acid (0.15 ml) and water (0.4 ml), a mixture of methanol–0.5% sulfuric acid (0.15 ml) and 25% aqueous potassium hydrogen carbonate (0.4 ml), a mixture of methanol–0.5% sulfuric acid (0.15 ml) and 25% aqueous potassium carbonate (0.4 ml), or a mixture of methanol–0.5% sulfuric acid (0.15 ml) and aqueous potassium hydroxide (0.4 ml). A set of the mixtures was prepared, and they were allowed to stand at 20°C. At desired periods, the mixtures were extracted with 2 ml of *n*-hexane, and 1 μl of the *n*-hexane layer was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Electron-capture detector response to the authentic derivatives

n-Hexane solutions containing 50, 100 and 200 ppb of the authentic methyl ester and the authentic indolone derivative were prepared, and 1-μl aliquots were injected into the gas chromatograph. Results obtained are shown in Table II, and indicate that the methyl ester gives 1.95 times (by peak height), or 2.25 times (by peak area), more sensitive responses than the indolone derivative. When the sensitivity is compared on a molar basis, the methyl ester is 2.2 times (by peak height), or 2.4 times (by peak area), more sensitive than the indolone derivative.

Rate of conversion to the derivatives and the extraction efficiency

Using the authentic derivatives, we examined the rate of conversion to the derivatives and the extraction efficiency under the standard derivatization conditions. The results are summarized in Table III, and indicate that diclofenac was quantitatively methylated by method C and in a 95% yield by method B, while the conversion to the indolone derivative occurred in a 76% yield by method A. On the other hand, the extraction efficiency with *n*-hexane was almost 100% by methods A and C, but was about 94% by method B.

By dividing the difference in the electron-capture detector response (2.2 by peak height) by the rate of conversion to the indolone (0.76), it is clear that

TABLE II

ELECTRON-CAPTURE DETECTOR RESPONSE TO THE AUTHENTIC METHYL ESTER AND THE AUTHENTIC INDOLONE DERIVATIVE

All values are expressed as mean ± S.D. (n = 5).

Compound	Concentration (ppb)	Peak area (cm^2)	Peak height (cm)
Methyl ester	50		18.5 ± 0.07* (20.6)***
	100		6.4 ± 0.03** (7.1)***
	200	2.0 ± 0.01** (2.23)***	14.0 ± 0.06** (15.6)
Indolone derivative	50		9.5 ± 0.2*
	100		3.3 ± 0.1**
	200	0.89 ± 0.02**	7.1 ± 0.2**

*The detector was operated with a pulse-rate of 2.5 kHz and the electrometer setting was kept at range 10^2 and attenuation 4.
**The detector was operated with a pulse-rate of 10 kHz and the electrometer setting was kept at range 10^2 and attenuation 8.
***The values were calculated on a molar basis; namely, the values marked * or ** were multiplied by 310/278.

TABLE III

THE RATE OF CONVERSION OF DICLOFENAC TO THE METHYL ESTER OR THE INDOLONE DERIVATIVE AND THE EXTRACTION EFFICIENCY OF THE DERIVATIVES

All values are expressed as mean ± S.D. (n = 5). The detector was operated with a pulse-rate of 10 kHz, and the electrometer setting was kept at range 10^2 and attenuation 16.

Method*	Peak height (cm)	Peak height (cm) of authentic samples (500 ppb)		Rate of conversion (%)	Rate of extraction (%)
		With extraction	Without extraction		
A	5.81 ± 0.46**	7.65 ± 0.09***	7.68 ± 0.10	76.0	99.6
B	13.8 ± 0.3**	14.5 ± 0.2***		95.2	94.2
C	14.8 ± 0.3**	15.3 ± 0.2***	15.4 ± 0.1	99.3	99.4

*Derivatization methods A, B and C are defined in the text (A, TFE—H_2SO_4; B, TFE—methanol—H_2SO_4; C, methanol—H_2SO_4).
**Each 1 μg of diclofenac was derivatized by method A, B or C, and analyzed as described in Methods. All values were corrected for molar concentration of the authentic samples.
***A methanol solution (10 μl) of the authentic methyl ester (1 μg) or the indolone derivative (1 μg) was treated according to methods B and C for the methyl ester or method A for the indolone, except that the heating at 60°C for 1 h or at 75°C for 75 min was omitted. Details are given in Methods.

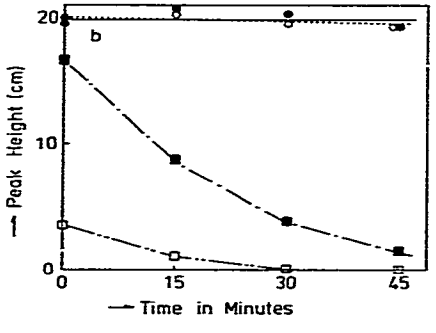
method C should be about three times more sensitive than method A. This figure completely agrees with the results demonstrated in the previous paper [2].

Stability of the derivatives in an alkaline medium

We incidentally noticed that the peak height of the methyl ester obtained by method C decreased with the passage of time when the ester was allowed to stand prior to extraction in 0.4 ml of 25% aqueous potassium hydrogen carbonate prepared a month previously. We were interested in this phenomenon, and examined the stability of the authentic methyl ester in an alkaline medium. The result is shown in Fig. 1, which indicates that the methyl ester apparently decomposed under alkaline conditions of potassium carbonate or potassium hydroxide, but not under alkaline conditions of potassium hydrogen carbonate freshly prepared. The indolone derivative formed by method A, however, was stable in an alkaline medium of potassium hydrogen carbonate whether the potassium hydrogen carbonate reagent was prepared a month previously or was freshly prepared (data not shown). Therefore, it is recommended that 0.4 ml of water instead of 25% potassium hydrogen carbonate in the previous report should be added to the reaction mixture in method B or C.

Measurements of diclofenac below 100 ppb

In the previous paper [2] measurements of diclofenac below 100 ppb were not reported. However, measurements below 100 ppb are required for pharma-



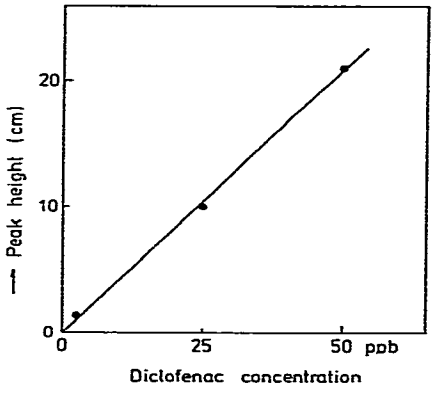


Fig. 1. Time course of decomposition of the authentic methyl ester under various alkaline conditions as described in Methods. (•) $KHCO_3$, (▪) K_2CO_3, (□) KOH, (○) water. The detector was operated with a pulse-rate of 10 kHz, and the electrometer setting was kept at range 10^2 and attenuation 8.

Fig. 2. Standard curve of diclofenac after derivatization with methanol—H_2SO_4. The standard curve was prepared as described in the previous paper [2] using 2.5, 25 and 50 ng of diclofenac, except that the derivative was extracted with 1 ml of *n*-hexane instead of 2 ml in the previous method [2] after the addition of 0.4 ml of water instead of 0.4 ml of aqueous 25% $KHCO_3$. A 1-μl aliquot of the *n*-hexane layer was injected into the gas chromatograph. The detector was operated with a pulse-rate of 2.5 kHz, and the electrometer setting was kept at range 10^2 and attenuation 4.

cokinetic studies after a usual 25-mg oral dose in man. Therefore, measurements below 100 ppb were attempted by method C. For this purpose, the detector was operated with a pulse-rate of 2.5 kHz instead of 10 kHz. The former gives about a three-fold increase in sensitivity over the latter. A typical standard curve is shown in Fig. 2. This shows that diclofenac below 1 ng in 1 ml of plasma can be measured by this method.

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