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244. Hisashi Murata and Tadao Wada*1 : A Fluorometric Method for the Estimation of Benhepazone.

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Benhepazone is activated maximally at 395 mμ and emits a fluorescence which shows a maximum at 410 mμ. Furthermore, Benhepazone was found to be quantitatively extracted into chloroform in alkaline conditions from a mixture consisting of Benhepazone and related compounds. On the basis of these findings, a fluorometric method has been adopted for the measurement of Benhepazone in tissues, urine, and feces. A procedure for assaying Benhepazone consists of its extraction with chloroform followed by the measurement of its fluorescence intensity at 410 mμ and it has been ascertained that neither metabolic products of Benbepazone nor native substances in biological samples interfered by the determination of Benhepazone in the course of this procedure.

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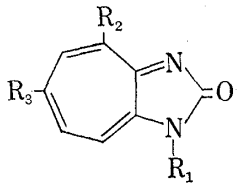
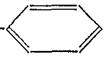
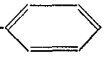
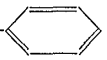
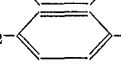
In the course of screening analgesic agents among a number of seven-membered ring compounds, Benhepazone (1-benzylcycloheptimidazol-2(1*H*)-one) has been found to have potent analgesic and antiphlogistic activities.[1] To clarify whether these activities are due to Benhepazone itself or to its metabolic products, it is necessary to investigate the metabolic degradation of Benhepazone. Accordingly, an attempt was made to follow its metabolic fate in animals. In the present paper a specific and sensitive method for the estimation of Benhepazone is described.

Benhepazone in various tissues, urine, and feces was easily determined by a procedure involving its extraction with chloroform and the measurement of the fluorescence at 410 mμ resulting from its activation at 395 mμ. This method was found to be adequate for the measurement of Benhepazone in biological specimens, because neither its metabolites nor normal constituents of the mammalian organism interfered with the fluorescence assay.

Fig. 1. Chemical Structure of Benhepazone and Its Related Compounds



Compounds	R_1	R_2	R_3
Benhepazone	CH_2- 	H	H
A1	CH_2- 	OH	H
A2	H	H	H
A3	H	OH	H
A4	H	H	OH
A5	CH_2- 	H	OH
A6	CH_2-  -OH	H	H

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1) H. Minakami, H. Takagi, S. Kobayashi : Life Sciences, **3**, 305 (1964).

Experimental

Materials——Benhepazone[2] and its related compounds[2~4] were synthesized in our laboratories. The structures of these compounds are presented in Fig. 1.

Standard Procedure for the Fluorometric Determination of Benhepazone in Biological Materials——Biological samples were prepared from various tissues, urine, and feces by the procedure which will be described in a forthcoming paper.[5]

To an aliquot of biological samples containing 10 μg. or less of Benhepazone, 1 ml. of 5*N* NaOH and 10 ml. of $CHCl_3$ were added, the mixture was shaken mechanically for 5 minutes, and centrifuged. After the $CHCl_3$ phase was dried over 1 gm. of sodium sulfate, Benhepazone was estimated by a fluorometry. The intensity of the fluorescence of Benhepazone was measured at 410 mμ by the Kotaki AKA fluorometer using an excitation wave length at 395 mμ, comparing with the standards carried through the entire procedure.

Results and Discussion

Fluorescence Properties of Benhepazone

The maximum of the fluorescence intensity of Benhepazone, which is activated maximally at 395 mμ, is observed at 410 mμ (Fig. 2). The intensity of its fluorescence is directly proportional to the concentration over the entire range shown in Fig. 3.

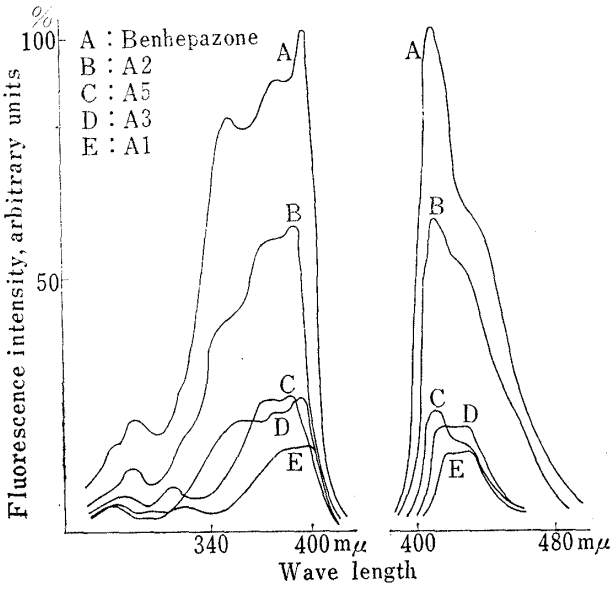

Fig. 2. Activation and Fluorescence Spectra of Benhepazone and Its Related Compounds

Benhepazone or its related compounds were dissolved in chloroform to a concentration of 0.01 m*M*. The activation (left) and fluorescence (right) spectra of these compounds were measured with Hitachi spectrofluorometer MPF-2.

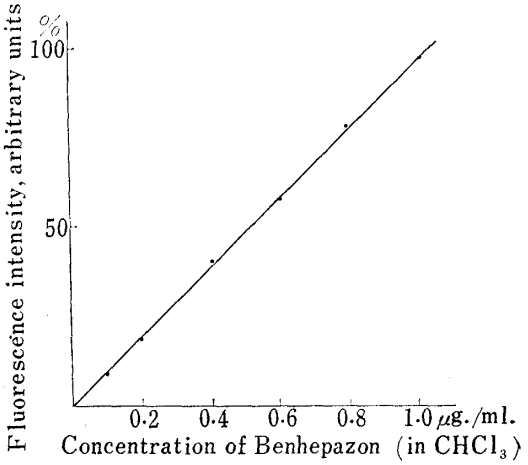

Fig. 3. Standard Curve of Benhepazone

Extraction of Benhepazone from Its Aqueous Solution

The solubility of Benhepazone in various solvents was examined (Table I). Benhepazone is easily soluble in chloroform, slightly in ethanol, acetone, and 0.1*N* hydrochloric acid, and scarcely in ether and 0.1*N* sodium hydroxide. Therefore, it seemed that Benhepazone might be easily extracted into chloroform from its aqueous alkaline solution.

2) H. Nakao, N. Soma, Y. Sato, G. Sunagawa : This Bulletin, **13**, 473 (1965).
3) T. Nozoe, T. Mukai, I. Murata : J. Am. Chem. Soc., **76**, 3352 (1954).
4) T. Nozoe, T. Mukai, T. Asao : Bull. Chem. Soc. Japan, **35**, 1188 (1963).
5) H. Murata, M. Mori : This Bulletin, **15**, 1988 (1967).

As indicated in Table II, the quantity of Benhepazone extracted with chloroform was measured by changing pH of its aqueous solution and these results indicated that Benhepazone would be quantitatively extracted by chloroform at pH 3 or higher pH.

Table I. Solubility of Benhepazone in Various Solvents at 20°C

Solvents	Solubility (g./dl.)	Solvents	Solubility (g./dl.)
Toluene	0.18	Chloroform	16.5
Dioxane	0.65	Ethylacetate	0.25
Diethylether	0.042	0.1*N* HCl	1.31
Ethanol	1.26	0.1*N* NaOH	0.089
Acetone	1.07	Water	0.098

Table II. Effect of pH on the Extraction of Benhepazone from Its Aqueous Solution

pH of Benhepazone aqueous solution	Relative fluorescence intensity (%)	pH of Benhepazone aqueous solution	Relative fluorescence intensity (%)
0.14	9.7	7.3	96.4
1.0	43.3	9.3	96.0
3.0	96.3	12.0	96.5
5.0	96.3		

Ten ml. of chloroform was added to 10 ml. of an aqueous solution containing 10 μg. of Benhepazone, of which pH was adjusted with HCl or NaOH as indicated, shaken and centrifuged. The fluorescence intensity of the chloroform phase was measured by the procedure for the Benhepazone assay described in Experimental.

Interference in Benhepazone Assay

The activation and fluorescence spectra of compounds related to Benhepazone were investigated, including A1, A2, A3 and A4 already isolated and identified as the metabolites of Benhepazone.[6] Among these compounds, A4 and A6 exhibited no fluorescence in their chloroform solution, while other compounds did. As shown in Fig. 2, the activation and fluorescence spectra of these compounds were found to be similar to those of Benhepazone, but they showed no interference in the fluorometric determination of Benhepazone, since these compounds would not be extracted by chloroform under the same conditions for the estimation of Benhepazone, as shown in Table III. This result showed that Benhepazone alone could be determined even when the assay was applied to a mixture of Benhepazone and its derivatives. Therefore, mixtures of Benhepazone and its derivatives were treated by the same procedure for the determination of Benhepazone, and fluorescence of the chloroform phase was measured at 410 mμ. As indicated in Table IV, the same fluorescence intensity as that of Benhepazone was obtained in every mixture of Benhepazone and its derivatives.

Usually, the blank value was very small and was equivalent to 0~0.04 μg. of Benhepazone for every g. or ml. of biological samples such as blood, liver, urine, and feces. This blank had practically no effect on the estimation of Benhepazone. As shown in Table V, Benhepazone added to an aliquot of urine of rats was almost completely recovered.

From these results the present fluorometric assay of Benhepazone is considered to be a valuable method for the estimation of Benhepazone in biological specimens.

6) H. Murata, A. Yasumura: Seikagaku, **37**, 461 (1965).

Table III. Extraction of Benhepazone or Its Related Compounds into Chloroform

Added to the mixture	Relative fluorescence intensity (%)				
	Benhepazone	A1	A2	A3	A5
Water	96.0	97.0	2.0	1.0	60.0
HCl	63.0	99.0	0.0	0.0	1.0
NaOH	98.0	1.0	0.0	0.0	0.0

To 1 ml. of 0.1 m*M* Benhepazone or related compounds were added 10 ml. of chloroform and 5 ml. of water, 0.2*N* HCl or 0.2*N* NaOH. After shaking and centrifuging the mixture, the fluorescence intensity of the chloroform solution was measured respectively at the maximum wave length of fluorescence resulting from activation at maximum wave length of individual compounds in Hitachi spectrophotofluorometer MPF-2. The relative fluorescence intensity was obtained by comparing respectively with fluorescence intensity of 0.01 m*M* chloroform solution of individual compounds, which was set to 100%.

Table IV. Extraction of Benhepazone from the Mixtures of Benhepazone and Its Derivatives

Compounds added to the mixture ($\times 10^{-2}$ μmoles)		Recovery (%)	Compounds added to the mixture ($\times 10^{-2}$ μmoles)		Recovery (%)
None	0	100	A4	2.0	100.0
				4.0	100.4
A1	2.0	100.7		8.0	99.7
	4.0	100.0			
	8.0	101.0	A5	2.0	100.7
				4.0	99.3
A2	2.0	100.0		8.0	100.0
	4.0	99.3			
	8.0	100.4	A6	2.0	98.6
				4.0	100.7
A3	2.0	101.0		8.0	100.0
	4.0	100.0			
	8.0	101.0			

Various concentrations of the derivatives of Benhepazone were added to the mixture consisting of 0.5 ml. of 0.01 m*M* Benhepazone, 5 ml. of 1*N* NaOH and 10 ml. of chloroform, shaken and centrifuged. The fluorescence intensity of the chloroform phase was measured with Kotaki AKA fluorometer and compared with that of chloroform phase without the derivatives of Benhepazone. "Recovery" was given by setting the fluorescence intensity in the latter case to 100%.

Table V. Estimation of Benhepazone added to Urine of Rats

Added (μg.)	Found (μg.)	Recovery (%)
3.00	3.10	103.3
6.00	5.90	98.3
9.00	9.15	101.6

Various concentrations of Benhepazone indicated were added to 1 ml. of urine of rats. The estimation was followed by the procedure for the Benhepazone assay described in Experimental.

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