QUANTITATIVE ANALYSIS OF THE COMPONENTS OF THE COMBINED METABOLITES OF DEOXYPEGANINE AND DEOXYVASICINONE

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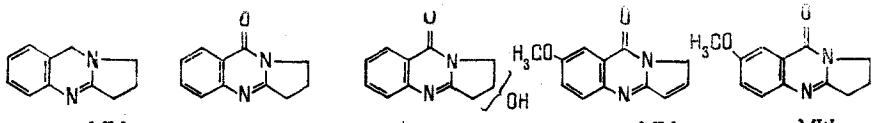
The quantitative composition of the combined metabolites isolated from the rat organism after the administration of the anticholinesterase preparation deoxypeganine and its analog deoxyvasicinone has been determined by the mass-spectrometric method of integrating the ion current. The results of the determination have been compared with those obtained by UV spectroscopy.

In the investigation of the biotransformation of biological active substances, the quantitative estimation of the metabolites is very important. In searches for a sufficiently reliable method of determining individual components of the combined metabolites isolated from rat urine after the administration of the anticholinesterase preparation deoxypeganine (I) and its deoxyvasicinone (II) [1], we have dwelt upon the mass-spectrometric method as the most sensitive and selective.

The methods widely used today for determining the components of biological mixtures, such as mass fragmentography [2], SIM (selected ion monitoring) [3], and MIM (multiple ion monitoring) [4] presuppose, as a rule, the preliminary chromatographic separation of the mixture, followed by the recording of individual peaks or of a section of the mass spectrum under low-resolution conditions. Since our aim was to perform the direct analysis of extracts of biological material, any method using low resolution would introduce an error into the quantitative determination of the metabolites because of the overlapping of the analytical peaks with isobaric peaks of biogenic impurities.

We have used IIT (integrated ion current technique) method [5], which consists in the cyclic recording of a small (within the range of 1 amu.) section of the spectrum under high-resolution conditions with subsequent integration of the contour obtained. Here the heights of the peaks of the ions of only one elementary composition are measured, and therefore the influence of foreign impurities is practically excluded. We have developed an analytical form of calculation applicable to the IIT method including the introduction of corrections for a change in the sensitivity of the mass spectrometer.

As reported previously [6], deoxyvasicinone (II) is transformed in the rat organism in two directions: by hydroxylation of the alicyclic ring and the formation of isomers of vasicinone (III), and by methoxylation of the aromatic ring (metabolite (IV)). We have established that the metabolism of deoxypeganine (I) passes through the stage of the formation of (II) and then to products (III) and (IV).



A scheme of the metabolism and a proof of the structures of the individual metabolites will be published.

For the quantitative determination of the individual components of the combined metabolites by the IIT method, we performed a calibration of the instrument with respect to the molecular ions of the initial substances (I) and (II), and also with respect to vasicinone

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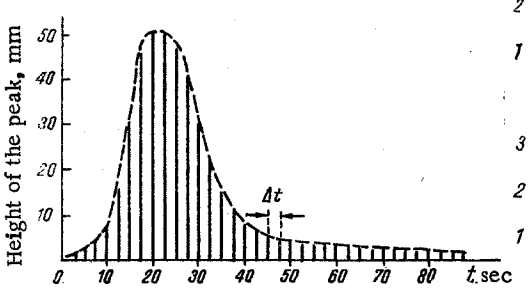

Fig. 1

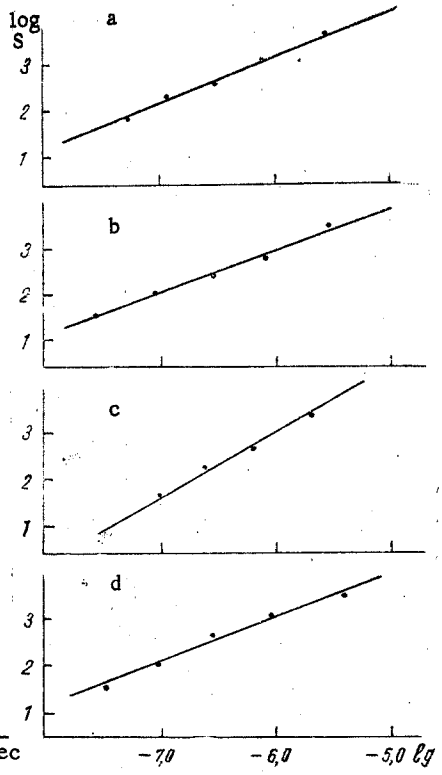

Fig. 2

Fig. 1. Contour of the ion current of M^+ 186 (II) for a sample weighing $19.2 \cdot 10^{-8}$ g (10^{-9} mole).

Fig. 2. Calibration graphs: a) deoxypeganine (I); b) deoxyvasicinone (II); c) vasicinone (III); d) 6-methoxydeoxyvasicinone (V).

(III). For the determination of (IV), as the calibration substance we used its close analog — 6-methoxydeoxyvasicinone (V).

The integration of the ion current amounts to measuring the area under the contour of the peak (Fig. 1). The calibration graphs for the individual substances were constructed as plots of the logarithm of the measured area S against the logarithm of the weight of the sample q introduced into the mass spectrometer [7]. Within the limits necessary for these experiments of the weight of the sample, of from $2 \cdot 10^{-7}$ to $2 \cdot 10^{-10}$ g, the relationship between the logarithms proved to be linear (Fig. 2).

The equation of the straight line (1) is the analytical expression of this relationship:

$$\log S = k(b - \log q), \tag{1}$$

where k and b are coefficients varying somewhat from sample to sample (Table 1).

With the aim of checking the possible mutual influence of the main components of the combined metabolites, we have analyzed model mixtures of compounds (I-III, and V). No deviations from the calibration curve of the individual substances were detected.

The sensitivities of the instrument for substances (I-III) in relation to its sensitivity for compound (V), taken as unity, were calculated from formula (2) [7]

$$V_i = \frac{S[M_i^+]}{S[M_V^+]} \cdot \frac{q_V}{q_i}, \tag{2}$$

where $S[M_i^+]$ is the area under the contour and q_i is the weight of the substance in moles.

It must be mentioned that the value of the relative sensitivity V_i for each component varies with a change in the weight of the sample because of some difference in the coefficients k and b. The detectable minimum amount of substance for the performance of an

TABLE 1. Values of the Coefficients in Eq. (1), the Relative Sensitivities V_i, and the Sensitivity Thresholds q_{min} for Samples (I-III, V)

Sample	m/z (M^+)	k	b	V_i ($q=10^{-9}$ mole)	$q_{min} \times 10^{-12}$ mole)
I	172	—0,996	—10,23	0,989	3,4
II	186	—0,931	—10,37	0,896	2,7
III	202	—1,050	—10,08	1,220	3 5
V	216	—0,976	—10,26	1,000	2,6

TABLE 2. Absolute and Percentage Contents of the Free Metabolites of Deoxyvasicinone (II) and Deoxypeganine (I) in Rat Urine Determined by the Integrated Ion Current Technique (IIT) and by UV Spectroscopy

	IIT			
Sample	(I) — free metabolites (0.0165 g administered)		(II) — free metabolites (0.050 administered)	
	metabolites found, g	% of the dose	metabolites found, g	% of the dose
I	$17,4\times10^{-4}$ ±5%	10,50	—	—
II	$1,3\times10^{-4}$ ±8%	0,79	$3,6\times10^{-4}$ ±5%	0,72
III	$3,9\times10^{-5}$ ±8%	0,24	$4,0\times10^{-4}$ ±6%	0,79
IV	8×10^{-7}	0,005	5×10^{-7}	0,001
Σ	$19,0\times10^{-4}$	11,53	$7,6\times10^{-4}$	1,51
	UV method			
Σ	$37,0\times10^{-4}$		$8,0\times10^{-4}$	

analysis is approximately the same for all substances (see Table 1). The results of the measurement of the logarithm of the area for one and the same weight of substance are reproduced with an accuracy of 0.3%.

Simultaneously with the determination of the individual components of the combined metabolites from (I) and (II), we estimated the total amount of metabolites in the material from the UV spectral curves, using averaged extinction coefficients of the characteristic maxima and mean molecular weights (Table 2). As can be seen from Table 2, the combined metabolites of deoxypeganine include mainly the initial substance, but half the free metabolites of deoxypeganine consist of isomers of vasicinone.

The orders of the total amount of metabolites determined by the two methods agree, but the numerical values for the case of the metabolites of (I) differ substantially (19.0 and 37.0). Judging from the composition of the mixture of metabolites of deoxypeganine determined mass-spectrometrically, the high result obtained by the UV method is not due to the superposition of the maxima characteristic for (II) and (III) on the maximum of the deoxypeganine itself. It is most probably caused, in the case of the total material from (I), by an increase in the influence of the background from biogenic impurities because of the substantially lower extinction coefficient of the 280 nm maximum (I) in comparison with the 268 nm maximum (II and III).

The GLC analysis of the primary extracts of the metabolites is difficult not only in view of the large amount of impurities, but also because of the complexity of the detection of the metabolites themselves. Thus, preparation (I) is labile, which leads to a pronounced broadening of the chromatographic peak. To determine the isomers of vasicinone (III) it is necessary to silylate the sample, which also complicates quantitative analysis. The method that we use is free from these deficiencies. Furthermore, a small change in the procedure, namely concentrating the combined material and increasing the resolving capacity of the instrument to 25,000 has permitted us to determine quantitatively the amount of metabolite

(IV) in the combined materials from (I) and (II), and this amounts to only 0.01% of the preparation administered in the case of (II), which is 1500 times less than the combined amounts of metabolite (II) and (III) (see Table 2). It must be mentioned that in crude extracts the amount of biogenic impurities was about 50% by weight, which was not reflected in the results of the analysis.

EXPERIMENTAL

Calibration of the Instrument. Weighed samples (∿3 mg) of substances (I)-(III) were dissolved in 96% ethanol in calibrated 10-ml measuring flasks. From these solutions a series of dilutions were prepared: 1:3; 1:10; 1:30; 1:100; and 1:300. Aliquots (6 µl) of the solutions were placed in a heating tube and with the aid of an autonomous forevacuum apparatus the solvent was pumped off. The ion currents of the molecular ions (I-III and V) with m/z 172, 186, 202, and 216 were recorded on the MKh 1310 instrument under the following conditions: SVP 5 system for the direct introduction of the sample; ionizing voltage 50 V, collector current 40 µA; voltage of the amplifier 5 kV; temperature of the ionization chamber 100°C; resolving capacity 10,000; rate of scanning 12.3 sec per mass decade; rate of movement of the recorder strip 2.5 mm/sec. The areas under the contour were calculated from the formula

$$S=\sum h_i \cdot \Delta t, \tag{3}$$

where h_i is the height of the i-th peak, mm, and Δt is the distance between the individual peaks (see Fig. 1).

A graph was plotted of the dependence of the logarithm of the area on the logarithm of the weight. The coefficients k and b for Eq. (1) were obtained from the formulas

$$k_i=\frac{\log S_{i+1}-\log S_i}{\log q_i-\log q_{i+1}}; \tag{4}$$

$$k=\frac{\sum^{n} k_i}{n}; \tag{5}$$

$$b_i=\frac{\log S_i - k\log q_i}{k}; \tag{6}$$

$$b=\frac{\sum^{n} b_i}{n} \tag{7}$$

where log S_i and log q_i are the logarithms of the areas and weights of a single determination; and k and be are the mean values of k_i and b_i for all the determinations.

To take into account the change in the sensitivity of the instrument with time, a correction was introduced into Eq. (1) for the magnitude b (Δb) from the formulas

$$b'=b+\Delta b; \tag{8}$$

$$\Delta b=\frac{\Delta \log S}{k} \tag{9}$$

where $\Delta \log S$ is the change in the logarithm of the area with the change in the sensitivity of the instrument (for a given weight of substance).

Quantitative Analysis of the Metabolites. The combined metabolites were obtained as described previously [8]. A chloroform extract of the biological material was brought to a definite volume, and a series of dilutions (1:10; 1:30; 1:100) were prepared: Aliquots, after the solvent had been distilled off from the tubes, were introduced into the instrument successively with aliquots of standard solutions so that the areas under the curves of the ion current did not differ very considerably. A series of parallel experiments performed to take into account the possible changes in the sensitivity of the instrument. The amounts of metabolites were calculated from the following formula derived on the basis of Eq. (1):

$$\log q_m=b'-\frac{\log S_m}{\log S_{st}}\cdot\left(b'-\log q_{st}\right), \tag{10}$$

where b' is the corrected value of the coefficient b; log S_m and log S_{st} are the logarithms of the areas under the ion-current curves for solutions of the combined metabolites and of the standard; and log q_m and log q_{st} are the logarithms of the corresponding weights.

The results of not less than three determinations were averaged, and the amount of metabolite in the sample was calculated.

To calculate the total amount of metabolites in the samples by the UV-spectroscopic method, the following equation was used:

$$\sum_{UV} = \frac{D \cdot L \cdot MW_{av}}{\varepsilon_{av}} \cdot \frac{V_1}{V_2}, \quad (11)$$

where D is the optical density; L is the dilution, liters; MW_{av} is the mean molecular weight (for sample (I), 172 and for (II) 194); ε_{av} is the extinction coefficient (for sample (I), 2630 (280 nm), and for (II) 7915 (268 nm)); V_1 is the volume of the chloroform extract, liters; and V_2 is the volume of the aliquot.

A Hitachi EPS-3T UV spectrometer was used with methanol as the solvent and a cell with a layer thickness of 1 cm.

SUMMARY

The quantitative compositions of the combined free metabolites isolated from the urine of experimental animals after the administration of deoxypeganine (I) and deoxyvasicinone (II) have been determined by the mass-spectrometric integrated ion current technique.

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